STUDIES ON THE INTRACELLULAR MEMBRANES OF MAMMALIAN EXOCRINE PANCREAS

Dissertation for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
RAYMOND J. MacDONALD
1974

This is to certify that the

thesis entitled

STUDIES ON THE INTRACELLULAR MEMBRANES OF MAMMALIAN EXOCRINE PANCREAS

presented by

Raymond J. MacDonald

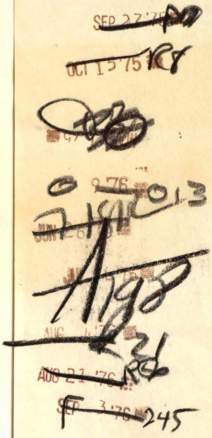
has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Department of Biochemistry

Major professor

Date May 16, 1974

O-7639



ABSTRACT

STUDIES ON THE INTRACELLULAR MEMBRANES OF
NORMAL AND SECRETING PANCREAS

by

Raymond J. Jacoboski

The exocrine pancreas is representative of several tissues that temporarily store secretion products within membrane-bound granules. Distinct membrane functions including membrane-membrane interactions are implicit during the processes of secretory protein synthesis, intracellular transport, concentration [illegible] granules, and exocytosis. The aims of this research were to characterize the intracellular membranes of the secretion [illegible] and to relate their structural and functional properties to the processes of secretion.

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ABSTRACT

STUDIES ON THE INTRACELLULAR MEMBRANES OF MAMMALIAN EXOCRINE PANCREAS

By

Raymond J. MacDonald

The exocrine pancreas is representative of several tissues that temporarily store secretion products within membrane-bound granules. Distinct membrane functions including membrane-membrane interactions are implicit during the processes of secretory protein synthesis, intracellular transport, concentration within storage granules, and exocytosis. The aims of this research were to characterize the intracellular membranes of the mammalian exocrine pancreas and to relate their structural and functional properties to the mechanism of secretion.

Membranes of microsomes, mitochondria and zymogen granules from adult rat pancreas were purified by sequential extraction of the subcellular organelles with 0.2 M $NaHCO_3$ and 0.25 M NaBr. Enzymatic analyses of the final granule membrane preparation indicated that less than 2% of the protein represented granule contents and mitochondrial membrane. The granule membrane constituted only approximately 0.5% of the total granule protein.

Purified intracellular membranes were fractionated by polyacrylamide gel electrophoresis in 1% sodium dodecyl sulfate and stained for protein with Coomassie blue and for carbohydrate by the periodic acid-Schiff procedure. Nine polypeptides ranging in molecular weight from 10,000 to 130,000 were consistently enriched during purification of the zymogen granule membrane and have been designated membrane components. The predominant granule membrane polypeptide accounted for approximately 38% of the Coomassie blue staining intensity and had an apparent molecular weight of 74,000. This polypeptide was also the major component which stained by the periodic acid-Schiff procedure and which contained a majority of the membrane sialic acid. A membrane component of very low molecular weight stained both by Coomassie blue and periodic acid-Schiff was identified as lipid. The characteristic zymogen granule membrane profile was also observed for membranes from dog, beef, pig and rabbit zymogen granules.

The polypeptide profiles of smooth and rough microsomal membranes were similar; their complexity contrasted with the characteristic simplicity of the granule membrane profile. The microsomal membranes contained approximately 35 discernible species, only 5 to 10 of these contained carbohydrate. The glycopolypeptide composition of smooth microsomal membranes resembled granule membranes. The 74,000 molecular weight glycopolypeptide was enriched in smooth microsomal membranes, but not in rough microsomal or mitochondrial membranes. The postmicrosomal supernatant and the granule contents, two major soluble subcellular fractions, contained only minor glycopolypeptide components. These results indicate that pancreatic glycoproteins are

preferentially associated with membranes, and that the granule membrane contains a small number of unique glycopolypeptides. It is postulated that the granule membrane glycopolypeptides are important either for the segregation of polypeptides of the granule membrane or for membrane-membrane interactions, most importantly with the luminal plasmalemma during secretion.

Mg^{2+}-dependent adenosine triphosphatase activity has been observed to be firmly bound to rat zymogen granule membrane. Kinetic analysis of the triphosphatase activity implies that two enzymes with distinctly different Km's are present. Possible roles of two granule membrane adenosine triphosphatases in the packaging and release of the exocrine cell products are discussed.

Since cyclic AMP has been implicated as an intermediate in the secretion stimulus, it was of interest to investigate the possible involvement of cyclic AMP-dependent phosphorylation of the structures immediately involved in the secretion process. Alterations of the granule membrane surface, such as phosphorylation of specific sites by a protein kinase, could alter the rate of granule discharge. A single protein was phosphorylated when granule membrane was incubated with (γ^{32}P)ATP. The activity required a divalent metal cation and was nearly equally active with Ca^{2+} or Mg^{2+}. The phosphorylation was not stimulated by cyclic nucleotides. The lack of cyclic AMP stimulation may indicate that the association of the protein kinase with the granule membrane is induced by cyclic AMP, facilitating phosphorylation of a specific membrane-bound substrate.

STUDIES ON THE INTRACELLULAR MEMBRANES OF MAMMALIAN EXOCRINE PANCREAS

By

Raymond J. MacDonald

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1974

to galvin hoyt swift

Beware of the man who works hard to learn something, learns it, and finds himself no wiser than before, Bokonon tells us. He is full of murderous resentment of people who are ignorant without having come by their ignorance the hard way.

K. Vonnegut

ACKNOWLEDGMENTS

I would like to express my deep and sincere appreciation to Dr. Robert A. Ronzio for his continual help and guidance throughout my graduate studies. His concern for the professional development of this graduate student was particularly encouraging. I would like to thank the members of my doctoral committee, Drs. Charles Sweeley, Clarence Suelter, and especially Steve Aust and Walter Esselman, for their helpful discussions during the course of my research. I would also like to note special satisfaction, perhaps hidden to them, in frequent interactions with Drs. Fritz Rottman, Loren Bieber and John Boezi, in addition to numerous colleagues and members of the laboratory.

I would like to express my appreciation to Joe Harlan for his invaluable assistance in the development of the preparative polyacrylamide slab gel electrophoresis, and to Diana Ersfeld for performing numerous amino acid analyses.

I especially thank my uncle, Steve F. Wronski, whose generosity and subtle faith permitted my academic endeavors.

Financial assistance from the Department of Biochemistry and the National Institutes of Health is appreciated.

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ATPase	undefined adenosine triphosphatase
C2	zymogen granule membrane component 2
Ca^{2+}-Mg^{2+}-ATPase	Ca^{2+}- or Mg^{2+}-stimulated adenosine triphosphatase
CbG	Coomassie blue G
CbR	Coomassie blue R
cyclic AMP	adenosine 3'5' cyclic monophosphate
(γ^{32}P)ATP	adenosine triphosphate containing phosphorus-32 in the terminal phosphate
Mg^{2+}-ATPase	Mg^{2+}-dependent adenosine triphosphatase
PAS	periodic acid-Schiff
SBTI	soybean trypsin inhibitor
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
TEMED	N,N,N'N'-tetramethylethylenediamine
ZGM-1	zymogen granule membrane fraction 1
ZGM-2	zymogen granule membrane fraction 2
ZGM-3	zymogen granule membrane fraction 3
ZGS-3	NaBr extracted fraction from ZGM-2

LITERATURE REVIEW

Secretion

Secretion of specific cell products is requisite for the maintenance of multitissue organisms and involves such diverse phenomena as endocrine mediated homeostasis and exocrine directed digestion. The most evident mechanism of secretion involves concentration and storage of the cell products in electron opaque secretory granules within the cytoplasm. In these cases the release reaction of the packaged material appears to be a general, basic phenomenon related to endo- and exocytosis. This section is limited to a review of the processes involved in the formation of secretory granules and the extracellular release of their contents.

Table 1 presents a brief survey of secretory cells which concentrate and store cell products in membrane-bound cytoplasmic granules prior to release. The table encompasses endocrine cell secretion of polypeptide hormones and monoamines, and characteristic exocrine cells which secrete hydrolytic enzymes. Except for chick oviduct, the secretory granules of the sources indicated have been isolated and their contents have been verified as the tissue secretory material by enzymatic and chemical analyses. In oviduct, egg white proteins have been localized within the cytoplasmic granules by

Table 1. Survey of secretory cells which store and secrete cell products via secretory granules.

Tissue-cell type	Secretion products	References
Pancreas		
α-cells	glucagon	Mathews (1970)
β-cells	insulin	Howell et al. (1969)
Adenohypophysis (anterior pituitary)		
somatotroph	growth hormone	Hymer and McShan (1963)
mammatroph	prolactin	"
thyrotroph	thyroid stimulating hormone	"
gonatotroph	follicle stimulating hormone and luteinizing hormone	"
corticotroph	adrenocorticotrophic hormone	"
Neurohypophysis (posterior pituitary) neurosecretory neurons	oxytocin and vasopressin	Poisner and Douglas (1968)
Adrenal medulla chromaffin cells	epinephrine and norepinephrine	Poisner and Trifaro (1967)
Platelets	5-hydroxytryptamine	Holmsen et al. (1969)
Mast cells	histamine	Stormorken (1969)
Adrenergic neurons	norepinephrine	Hokfelt (1968)
Cholinergic neurons	acetylcholine	Whittaker (1965)
Parotid acinar cell	hydrolytic enzymes, eg., amylase, DNase	Schramm and Danon (1961) Amersterdam et al. (1971)
Pancreas acinar cell	hydrolytic enzymes and proenzymes, eg., amylase, chymotrypsinogen	Greene et al. (1963) Hokin (1955)
Chick oviduct tubular gland cells	egg white proteins, eg., ovalbumin, lysozyme	Palmiter (1972)
Polymorphonuclear leucocytes	lysosomal hydrolases, eg., β-glucuronidase, RNase	Woodin and Wieneke (1970)

fluorescent antibody techniques (Palmiter, 1972). Fusion of the granule membrane with the cell surface membrane to facilitate release has been observed for all cell types listed (Poste and Allison, 1974). In addition, for all those secretory systems adequately investigated, the release of granule contents after exposure to a secretion stimulus has been shown to require active energy metabolism and Ca^{2+}, to be inhibited by excess Ca^{2+} or Mg^{2+}, and to involve the displacement of intracellular membrane-bound Ca^{2+}.

The uniform occurrence of cytoplasmic storage granules, the demonstration of storage granule exocytosis, and the striking similarity of the biochemical requirements for secretion, strongly indicate a common mechanism. The sequence of steps outlined below and expanded in the sections which follow appear fundamental to this process.

1) Protein secretory products are synthesized on ribosomes attached to the endoplasmic reticulum, then sequestered inside the reticulum cisternae.

2) The products are transported to and concentrated into secretory granules, each bounded by a smooth-surfaced unit membrane.

3) Movement of the granule displaces it from the site of formation near the Golgi complex to the cell surface.

4) Extrusion of the granule contents by exocytosis (reverse pinocytosis) is initiated by fusion of the granule membrane with the surface membrane.

The exocrine pancreas, representative of tissues utilizing this secretion mechanism, will be discussed to illustrate further details.

Morphology of the Pancreatic Exocrine Cell

The appearance of a pancreatic acinar cell is that of a cellular factory structured for protein synthesis and secretion. The subcellular organelles are strictly polarized within the cytoplasm. Occupying approximately 60% of the cell volume, the rough endoplasmic reticulum is crowded into the basal pole of the cell. Its flattened cisternae, oriented parallel to the circumference of the nucleus, enclose a functionally and physically distinct cellular compartment. Ribosomes, although frequently seen in the cytoplasm, are largely associated with reticulum-bound polysomes arranged in whorls, rosettes or linear arrays. The nucleus occupies a region of the cell extending from the basal section to the middle of the cell, surrounded on all but one face by the rough endoplasmic reticulum. The Golgi complex generally occupies the region free of rough endoplasmic reticulum adjacent to the nucleus, with the mature face of the Golgi apparatus extending toward the lumen. Numerous small smooth-surfaced vesicles are associated with the periphery of the Golgi stacks. Clustered in the apical portion of the cell toward the luminal plasma membrane are numerous electron dense zymogen granules containing the hydrolytic digestive enzymes and proenzymes destined for secretion into the duct system of the gland. Centrally located in the Golgi complex are a small number of immature zymogen granules characterized by a scalloped profile and internal material of variable density. Mitochondria are prevalent in the middle and apical cytoplasm. Secondary lysozomes are occasionally observed.

The acinar lumen is formed by laterally joining each exocrine cell to its neighbor. Contact is maintained between surface membranes by tight junctional complexes which extend completely around each cell, effectively segregating the duct lumen from the remainder of the tissue. The structure implies differentiation of the luminal plasma membrane from the rest of the surface membrane and precludes mixing of membrane components by translocation along the plane of the membrane.

The morphology of the pancreatic acinar cell applies, with minor modifications, to the other secretory cell types listed earlier (Table 1).

Microscopy studies by Jamieson and Palade (1971) noted loss of zymogen granules and concomitant increase in the amount of luminal plasma membrane after stimulation of secretion by carbamylcholine. Morphological evidence of the secretion mechanism is limited to the static electron microscopic observations of structures which appear to be granule ghosts, judged by their distinct spherical shape, fused with the luminal plasmalemma. In such instances the granule contents are partially or completely lost to the duct space.

Greene et al. (1963) and Keller and Cohen (1961) have demonstrated that the protein complement of isolated zymogen granules is identical to that of pancreatic juice collected from the gland. Since this evidence is representative of the average granule population, it remains to be shown whether each granule contains all, one, or a limited number of the digestive enzymes. Most other secretory tissues delegate the synthesis and export of each cell product to an

individual cell type (for instance, the endocrine pancreas and the adenohypophysis, Table 1).

Intracellular Transport of Secretory Protein

Extensive evidence has accumulated in support of the proposal that exportable proteins are synthesized on polysomes attached to the endoplasmic reticulum, while the synthesis of cellular non-exportable proteins occurs on polysomes free in the cytoplasm (Hicks et al., 1969; Redman, 1969; Takagi and Ogata, 1971). During or after synthesis, the secretory proteins are transported into the cisternal space of the rough endoplasmic reticulum. Isolated rough microsomes with attached ribosomes active in amino acid incorporation are capable of discharging their nascent polypeptide chains vectorally into the microsomal space after normal chain termination or premature termination by addition of puromycin (Redman and Sabatini, 1966; Redman, 1967). The direct transfer across the membrane and the resistance of the nascent polypeptides of membrane-bound ribosomes to proteolytic attack have led to a simple model to describe the mechanism involved (Sabatini and Globel, 1970). Sabatini and Blobel have proposed that the nascent polypeptide grows within a channel in the large subunit which is attached to the membrane; upon continued elongation the polypeptide penetrates the endoplasmic reticulum through a membrane pore.

The original electron microscopic radioautography studies of guinea pig exocrine pancreas by Caro and Palade (1964) indicated transfer of exportable protein through the endoplasmic reticulum cisternae, transient association of the proteins with the peripheral

elements of the Golgi complex, and accumulation of the proteins within zymogen granules. Subsequent refinements were developed by Jamieson and Palade (1967a,b) for a more precise analysis of the intracellular movement of secretory proteins. Pancreatic slices were labeled with (^{3}H) leucine for three minutes, then the incorporation of radioactivity was stopped by the addition of a large excess of unlabeled leucine, and the incubation continued. The intracellular transport of newly synthesized exportable protein was monitored by electron microscopic radioautography and isolation of the subcellular elements involved. The increased resolution resulting from the short, discrete pulse indicated that movement of labeled protein occurred through the cisternal space of the rough endoplasmic reticulum to the transitional elements of the endoplasmic reticulum. The radioactivity accumulated in these transitional elements, which are characterized by being partly covered with ribosomes, partly free and positioned adjacent to the Golgi complex, at about 10 minutes post-pulse. Shortly thereafter the label was associated with small smooth-surfaced vesicles located at the periphery of the Golgi cisternae. At about 20 minutes post-pulse, much of the radioactivity was associated with the condensing vacuoles; the number of radioautography grains associated with the vacuoles increased as protein accumulated within, and the vacuoles became more electron opaque. Continuation of this process led to most of the label in mature zymogen granules. After 60 to 80 minutes radioactivity can be observed in the duct lumen as a result of granule movement to and fusion with the luminal plasmalemma.

In an elegant series of radioautography experiments Jamieson and Palade (1968b, 1971) delineated the energy requirements for intracellular transport of newly synthesized secretory proteins. The studies were made possible by first demonstrating that cycloheximide, at a concentration which blocks protein synthesis by more than 95%, only minimally affects transport (Jamieson and Palade, 1968a). Therefore, inhibition of transport by repressing ATP synthesis cannot be related to a requirement of continued protein synthesis.

In the subsequent experiments cycloheximide was present to maintain a constant level of transport, and antimycin A and sodium fluoride were added to block the synthesis of ATP by oxidative phosphorylation and glycolysis. The tissue ATP level dropped to 50% within 8 minutes after the addition of the metabolic inhibitors, and was at 5% of the initial level after 60 minutes. Several definitive transport steps were analyzed by pulse labeling, followed by a chase incubation to allow the label to accumulate in the compartment preceding the transfer step under consideration. Cycloheximide, antimycin A and sodium fluoride were then added and the transfer of radioactivity to the next compartment relative to a control incubation was assessed by radioautography.

Briefly summarized, the results were as follows. The migration of secretory proteins from their site of synthesis to the transitional elements of the endoplasmic reticulum was not affected by the metabolic inhibitors, while the transfer of radioactive proteins through the peripheral elements of the Golgi apparatus to condensing vacuoles was inhibited. The next step, the conversion of condensing vacuoles

to mature zymogen granules, was not altered at 20 minutes after exposure to the drugs, but was inhibited 20-40% after 60 minutes exposure. The extracellular release process, assayed by the carbamylcholine induced secretion of radioactively labeled protein, was quickly and completely inhibited by the presence of antimycin A and sodium fluoride.

Therefore, of the three prominent discrete steps in the transport process (transfer of secretory material from transitional elements to condensing vacuoles, conversion of the vacuoles to mature zymogen granules, and exocytosis of the granule contents), the first and last strictly require an energy source, presumably ATP. The energy requirements of condensing vacuole maturation are equivocal. The low level of inhibition might be due to affects not primarily associated with the granule, and thus only become apparent after an extended period. Alternatively, the energy requirement may have a high affinity for ATP or access to a select intracellular ATP pool (Jamieson and Palade, 1971).

Properties of Secretory Granules

Secretory granules display surprising size uniformity when grouped into two tissue classes (Mathews, 1970; Poste and Allison, 1974). The average diameter for most of the hormone containing granules of the tissues listed in Table 1 is 0.15 μ, while granules of exocrine tissues (acinar cells of the parotid and pancreas, oviduct and leukocytes) are approximately 1 μ in diameter.

Little is known of the surface properties of secretory granules. Consistent with the anionic nature of many membrane

components, the granules of the adrenal medulla (Banks, 1966; Mathews, et al., 1972) and the neurohypophysis (Poisner and Douglas, 1968) possess a net negative charge. A significant fraction of the charge has been attributed to external sialic acid residues (Mathews, et al., 1972). The presence of Ca^{2+} neutralizes the surface charge and causes the granules to aggregate.

Numerous secretory granules have been observed to be osmotically insensitive. The basis of this phenomenon can be readily demonstrated for adrenal medulla chromaffin granules, which contain norepinephrine and ATP. If Ca^{2+} is added to a solution of norepinephrine and ATP to yield final molar ratios of 3:1:0.1 (norepinephrine: ATP: Ca^{2+}), an insoluble complex is formed (Pletscher et al., 1970). Pancreatic zymogen granules also have unusual stability properties. The granules are stable in distilled water, 0.3 M sucrose and 0.3 M urea (Hokin, 1955; Jamieson and Palade, 1971; Burwen and Rothman, 1972). Addition of increasing amounts of cations induces lysis. These results imply that secretory vesicles function as a sink for the accumulation of secretory products, and once formed to not require a continuous energy supply to maintain their integrity.

Secretory granules contain few enzymatic activities. Chromaffin granules from adrenal medulla and their isolated membranes possess dopamine β-hydroxylase activity. Since this activity is responsible for the intragranule conversion of dopamine to norepinephrine, its distribution would be expected to be limited to granules involved in catecholamine secretion. Ca^{2+}- or Mg^{2+}-dependent ATPase has been found associated with secretory vesicles from the adrenal

medulla (Trifaro and Warner, 1972; Trifaro and Dworkind, 1971), neurohypophysis (Poisner and Douglas, 1968), polymorphonuclear leukocytes (Wooden and Wieneke, 1963, 1970), platelets (Stormorken, 1969), synapses (Kadoto et al., 1967), and guinea pig exocrine pancreas (Meldolesi et al., 1971c). The ATPase activity appears to be membrane-bound and has been envisaged as the energy requiring step in granule exocytosis (see below). A second ATP-requiring enzymatic activity, that of a protein kinase, is associated with secretory granules of the adenohypophysis (Trifaro and Warner, 1972) and adrenal medulla (LaBrie et al., 1971).

A Model of the Secretory Granule Release Reaction: Exocytosis

A series of obligatory steps may be anticipated as requisites to membrane fusion. The two membranes must become directly opposed, probably to within 10 Å (Mathews, 1970; Poste and Allison, 1969). Membrane destabilization must occur at the nearest adjacent sites. Formation of a new membrane continuum at the fusion point must favor inclusion of the granule membrane, and quick restabilization of the newly formed membrane must follow. One of the original models incorporating these main features was a proposal by Woodin (1968) for the extrusion of secretory granules by polymorphonuclear leukocytes. The model has been further refined for secretory granule release (Poisner and Trifaro, 1967; Mathews, 1970) and general membrane fusion (Poste and Allison, 1969, 1974).

The proposal takes into account the negative surface charge of both the secretory granule and the interior of the plasma membrane.

The granule may overcome the potential energy barrier imposed by the juxtaposition of two similar charges through its kinetic energy derived from Brownian motion. Once the energy barrier has been surmounted, the rise of intracellular Ca^{2+} associated with the secretion stimulus facilitates adhesion of the membranes through salt bridges ($-COO^-...Ca^{2+}...^-OOC-$, or possibly $-OPO_3^-...Ca^{2+}...^-O_3PO-$). The granule membrane associated Ca^{2+}-Mg^{2+}-ATPase is postulated to promote membrane destabilization at the fusion site by hydrolysis of membrane bound ATP. Released orthophosphate is presumed to remove membrane Ca^{2+} by chelation. Fusion occurs at the sites and stabilization recurs by reassociation of Ca^{2+} and ATP.

The release reaction is strictly limited by the concentration of Ca^{2+} available, since fusion is prevented in both the absence of Ca^{2+} and in the presence of excess Ca^{2+}. These observations are readily incorporated in the model: The rise in intracellular Ca^{2+} during stimulation of secretion is required for adhesion of the secretory granule to the plasma membrane. Saturating levels of Ca^{2+}, however, do not permit localized loss of Ca^{2+} generated by the granule ATPase, and loss of membrane stability is prevented.

Control of Exocrine Pancreas Secretion

Hormonal control of the physiological state and function of many metazoan cells is mediated by intracellular concentrations of adenosine 3'5' cyclic monophosphate (cyclic AMP). For example, the catecholamine stimulation of cardiac muscle glycogenolysis and contracting force (Rasmussen et al., 1972), the hormonal-induced increase of lipolysis in adipose tissue (Butcher et al., 1968), and the

glucagon stimulation of liver gluconeogenesis (Krebs, 1972) are mediated by levels of cyclic AMP. Control of cell secretion in many instances is controlled in a similar manner: Glucagon stimulation of insulin release by β-cells of pancreatic islets (Bdolah and Schramm, 1965) and catacholamine induced secretion of amylase by the parotid gland (Schramm and Naim, 1970) have been documented.

The functional relationship between extracellular hormones and cyclic AMP, originally described by Sutherland et al. (1965) as the second messenger hypothesis, may be summarized: An extracellular messenger, generally a hormone, binds to its specific receptor on the cell surface, resulting in the activation of a plasma membrane associated adenylyl cyclase. This activation generates an increase in the intracellular concentration of cyclic AMP, the product of the adenylyl cyclase reaction. Thus the information originally contained in the hormone is translated intracellularly through changes in cyclic AMP levels. The cyclic AMP concentration is determined by the balance between synthesis from ATP by adenylyl cyclase and hydrolysis to 5'AMP by specific phosphodiesterases. As yet, only the cyclase activity has been shown responsive to extracellular signals. Acting as a second messenger, cyclic AMP activates the target cell to perform its specific function. More recently, Kuo and Greengard (1969) and Exton et al. (1971) have postulated that most, if not all, of the affects of increased cyclic AMP levels are mediated by activation of cellular protein kinases.

Sutherland and Robison (1966) developed a set of criteria as a guide for assessing the involvement of cyclic AMP as the specific

messenger in cell activation by hormones. In summary these criteria are 1) an increase in intracellular cyclic AMP in response to hormone; 2) the increase accompanies or precedes the physiologic effect; 3) the hormone is able to activate adenylyl cyclase in cell homogenates; 4) exogeneous cyclic AMP or an analog has the ability to mimic the hormone effects; and 5) phosphodiesterase inhibitors such as the methyl xanthines are able to mimic or potentiate the hormone effects.

Analysis of agents which cause amylase secretion by mouse pancreas incubated in vitro implicates cyclic AMP participation in control of exocrine secretion (Kulka and Sternlicht, 1968). Both pancreozymin, which controls exocrine secretion in situ, and carbamylcholine stimulated amylase release 3-fold over control values. Cyclic AMP, its monobutryl and dibutryl analogs, and the phosphodiesterase inhibitor theophylline also stimulated amylase secretion. Acting as a cyclic AMP antagonist, 3'AMP inhibited the stimulation induced by pancreozymin, carbamylcholine and cyclic AMP. Increasing concentrations of cyclic AMP reversed 3'AMP directed inhibition, indicating that the effect of this antagonist is readily reversible and relatively specific.

Further investigations with rat pancreas indicated that the control mechanism is more complex. Baudin et al. (1971) have shown that while carbamylcholine and pancreozymin stimulate glycolysis, (^{14}C) glucose incorporation into protein, oxygen uptake, and $^{32}PO_4$ incorporation into phospholipid in addition to enzyme secretion, cyclic AMP or its analogs only increase the rate of secretion without

affecting the other parameters. Although carbamyl choline and pancreozymin elicit a 6-fold increase in the rate of secretion, maximal cyclic AMP stimulation is limited to approximately 2-fold. Obviously cyclic AMP has a limited ability to mimic hormone action in this tissue. While methyl xanthines have little or no effect on secretion in rat exocrine pancreas (Heisler et al., 1972; Baudin, et al., 1971), they do potentiate pancreozymin-induced secretion (Baudin et al., 1971). Furthermore, the increase in the rate of secretion by optimal concentrations of carbamylcholine plus dibutryl cyclic AMP is greater than the sum of the two alone (Heisler et al., 1972). Consequently, the increased intracellular level of cyclic AMP induced either indirectly by phosphodiesterase inhibitors or directly by exogeneous dibutryl cyclic AMP appears to stimulate a step in secretion not maximally affected by hormones, and therefore one which appears to become rate limiting only under certain conditions.

The rat exocrine pancreas requires a sufficient supply of Ca^{2+} to maintain secretion (Heisler et al., 1972). The presence of EDTA or repeated carbamylcholine stimulation in Ca^{2+}-free medium inhibited further secretion. Addition of Mn^{2+}, which competes for Ca^{2+} uptake, also inhibited carbamylcholine stimulation. Dibutryl cyclic AMP and theoplylline induction, however, was not strictly dependent upon uptake of extracellular Ca^{2+}, as if cyclic AMP acts to release intracellular sequestered Ca^{2+}. With the information accumulated to date, it is difficult to distinguish between Ca^{2+} functioning as an essential cofactor in secretion, or as an intracellular second messenger responsive to hormone stimulation, as suggested by Rasmussen et al. (1972).

The presence of cyclic AMP in the pancreas (Johnson et al., 1970), its ability to stimulate enzyme release (Kulka and Sternlicht, 1968; Baudin et al., 1971; Ridderstap and Bonting, 1969), the ability of an analog, 3'-AMP, to inhibit induced secretion (Kulka and Sternlicht, 1968), and the induction (Heisler et al., 1972; Kulka and Sternlicht, 1968) or potentiation (Baudin et al., 1971) of release by theophylline are strong arguments for the participation of cyclic AMP in exocrine secretion. The most direct evidence, a measurable increase in intracellular cyclic AMP prior to or concomitant with hormone stimulated enzyme secretion, is negative (Heisler et al., 1972). Consequently, the specific function of cyclic AMP and whether it is a primary intracellular messenger are not yet fully resolved.

The direct action of cyclic AMP in many tissues which respond to hormones is the activation of protein kinases (for reviews, Hittelman and Butcher, 1971; Krebs, 1972). Lambert et al. (1973) have observed that pancreozymin and caerulin stimulation of exocrine pancreas induced a significant increase in protein phosphorylation. The maximal increase occurred with the phosphorylation of zymogen granule membrane. A similar observation is the association of protein kinase activities with secretory granule membranes of anterior pituitary (LaBrie et al., 1971) and adrenal medulla (Trifaro and Warner, 1972). These observations link the common intracellular mediator of hormone action to the control of a secretory granule membrane-bound enzyme activity and afford an intriguing mechanism for controlling the rate of secretion.

Statement of the Problem

The central role of pancreatic intracellular membranes in the biosynthesis, transport, storage and release of the hydrolytic enzymes and proenzymes is evident. Immediately after synthesis the proteins destined for export are segregated into protective membrane-bound compartments, and remain sequestered until extracellular release. However, the membranes function more than as mere containers. Membrane interactions are implicit in intracellular transport and exocytosis. The rate of secretion is almost certainly controlled at the point of fusion of granule membrane with the luminal plasma membrane. In an attempt to gain further insight into the attributes of membranes which function in secretion, an intensive study of the pancreatic exocrine secretory granule (zymogen granule) membrane was undertaken. The rationale was to analyze the structural properties and enzymatic functions unique to the granule membrane in relation to the other intracellular membranes. Characteristics which would be expected to play a role in the secretion process were emphasized.

Recently polyacrylamide gel electrophoresis in SDS has been routinely employed in the analysis of membrane polypeptides. The principal advantages are that membranes can be completely dissolved in SDS buffers, and that membrane polypeptides are dissociated from lipids and can be resolved into distinct minimum molecular weight classes. The hydrophobic interaction of SDS with proteins causes denaturation and forces the polypeptides into a singular conformation (Reynolds and Tanford, 1970b). The uniform conformation and charge density of protein-SDS complexes accounts for the observed

regular relationship between protein molecular weight and mobility during electrophoresis in SDS-polyacrylamide gels. To fully realize the potential of this analytical tool, the electrophoresis procedure must resolve microgram quantities of membrane protein into discrete classes reproducibly and without artifacts. In the study reported herein electrophoretic analysis of the number, distribution and nature of the granule membrane components was coupled with enzymatic analysis of granule membrane function.

MATERIALS AND METHODS

Materials

Electrophoresis Reagents

Proteins for molecular weight standards were obtained from several commercial sources.

acrylamide, technical grade, recrystallized from chloroform before use (Loening, 1967)	Eastman Organic Chemicals, Rochester, N.Y.
N,N'-methylenebisacrylamide, recrystallized from acetone before use (Loening, 1967)	Canalco, Rockville, Ma.
sodium dodecyl sulfate, sequanal grade	Pierce Chemical Co., Rockford, Ill.
ammonium persulfate	Canalco
N,N,N',N'-tetramethylethylenediamine (TEMED)	Bio-Rad Laboratories, Richmond, Ca.
Coomassie brilliant blue R	Sigma Chemical Co., St. Louis, Mo.
Coomassie blue G (xylene brilliant cyanin G)	K and K Laboratories, Plainview, N.Y.
pyronin B	Harleco, Philadelphia, Pa.
basic fuchsin	Harleco
dimethyldichlorosilane	Sigma Chemical Co.
urea, recrystallized from ethanol before use	Mallinckrodt, St. Louis, Mo.

Reagents for Analytical Procedures

cytochrome c, Type V	Sigma Chemical Co.
soluble starch reagent (for amylase assay)	Nutritional Biochemicals Corp., Cleveland, Ohio
bovine serum albumin	Sigma Chemical Co.
N-acetylneuraminic acid	Sigma Chemical Co.
nucleotides	P-L Biochemicals, Milwaukee, Wis.
p-nitrophenylphosphate	Sigma Chemical Co.

Miscellaneous

(^{3}H)sodium borohydride, 120 mCi/mmole	New England Nuclear, Boston, Mass.
(^{32}P)orthophosphate, carrier free	New England Nuclear
soybean trypsin inhibitor	Sigma Chemical Co.
Triton X-100	Rohm and Haas, Philadelphia, Pa.

Tissue Sources

Sprague-Dawley rats were generally obtained from Spartan Research Animals, Haslett. Several hundred rats were the considerate gifts of Dr. Robert Cook, Department of Dairy Science, Michigan State University, and Dr. Jack Gorski, Department of Physiology, University of Illinois, Urbana. Guinea pigs and rabbits were purchased through the Center of Laboratory Animal Research, Michigan State University. Fresh dog pancreases were obtained through the courtesy of Drs. M. D. Bailee, C. C. Chou, and J. Scott, of the Department of Physiology, Michigan State University. Fresh pig and beef pancreases were purchased from local slaughter houses.

Methods

Preparation of Homogenates

All operations were performed at 4°. Excised pancreas was trimmed free of fat, weighed and thoroughly minced with scissors. Large amounts of dog, pig, beef and sheep tissue were frequently processed with a Harvard press (Harvard Apparatus Co., Dover, Mass.) rather than mincing. Ten volumes of homogenization medium containing 0.3 M sucrose and 0.05 to 0.25 mg/ml of soybean trypsin inhibitor (SBTI) were added per gram of minced pancreas and the fatty tissue floating to the surface was removed. Cell disruption was performed by four up and down strokes of a glass-Teflon Potter-Elvehjem homogenizer (clearance of 0.007 inch, Kontes Glass Co.) driven at 620 rpm. Tissue debris was removed by filtering the homogenate through two layers of cheesecloth.

Isolation of Subcellular Fractions (Modifications of the Procedure of Jamieson and Palade, 1967a)

Zymogen granules. The filtered homogenate was centrifuged at 500xg (1600 rpm, HS-4 rotor, I. Sorval Co.) for 10 minutes to remove debris, unlysed cells and nuclei; large pieces of plasma membrane may also collect in this fraction. A zymogen granule fraction was collected by centrifuging the 500xg supernatant in conical centrifuge tubes at 1600xg (2800 rpm, HS-4 rotor) for 25 minutes. The loosely packed brown layer containing mostly mitochondria overlaying the white zymogen granule pellet was removed by gently agitating and rinsing with homogenization medium using a Pasteur pipet. The zymogen granule

pellet was washed once by gently resuspending in homogenization medium and centrifugation at 1600xg for 25 minutes.

Mitochondria. The supernatant and mitochondrial layer above the zymogen granule pellet from the initial 1600xg centrifugation were combined and centrifuged at 8700 xg (8500 rpm, SS-34 rotor, Sorvall) for 15 minutes. The pellet contained a small zymogen granule button overlayed with mitochondria. The mitochondrial layer was resuspended in homogenization medium without disturbing the granules and recentrifuged. Resuspension and centrifugation were repeated approximately three times until zymogen granules were not observed in the pellet.

Microsomes. The 8700xg supernatant was centrifuged at 93,000xg (40,000 rpm, type 40 rotor, Beckman Instruments) for one hour to pellet total microsomes. The material which did not sediment was designated as postmicrosomal supernatant. Smooth and rough microsomes were separated by sucrose density gradient centrifugation of the total microsomal pellet according to Ronzio (1973a). Smooth and rough microsomal fractions from rat (Ronzio, 1973b) and guinea pig (Jamieson and Palade, 1967a; Meldolesi et al., 1971a,b,c) pancreas have been well characterized.

Modifications for isolation of dog and beef zymogen granules. Zymogen granules from dog pancreas were best isolated in two steps. A large fraction of the granules were sedimented by centrifuging the 500xg supernatant for 15 minutes at 1200xg (2500 rpm, HS-4 rotor).

A second fraction was then obtained by centrifuging the supernatant at 1600xg (2800 rpm) for 30 minutes. After removing the mitochondrial layer, both pellets were resuspended and centrifuged. Beef pancreas zymogen granules were prepared by a procedure incorporating similar modifications as described in detail by Greene et al. (1963).

Preparation of Membranes From Subcellular Fractions

Zymogen granule membranes. Washed zymogen granule pellets from 3 to 4 gm of rat pancreas were resuspended in one ml of 0.17 M NaCl containing 0.67 mg/ml SBTI. The protein concentration was generally 12-16 mg/ml. The granules were lysed by adding 3 ml of 0.2 M $NaHCO_3$, pH 8.2, to each ml of suspension. Lysis generally required 1-2 hours at 4° and was monitored as clarification of the solution to an absorbance of less than 0.5 at 660 nm. Membranes were collected by centrifugation for 1 hour at 192,000xg have been designated ZGM-1.

ZGM-1 obtained from 3 or 4 gm of pancreas was resuspended in one ml of 1 M sucrose, requiring approximately 10 strokes of a Dounce glass homogenizer (tight fitting pestle B, Kontes Glass Co.). Generally 5 ml of the membrane suspension was placed in an SW 41 cellulose nitrate tube, overlayed with 0.3 M sucrose, and centrifuged at 192,000xg for 1 hour. Two membrane fractions resulted. Mitochondria, marked by high cytochrome c oxidase activity, accounted for the majority of the pellet. Zymogen granule membrane, banding at the 0.3-1.0 M sucrose interface (Meldolesi et al., 1971a), was designated ZGM-2.

Care was taken to remove all the membrane felt at the interface in less than 1.5 ml of sucrose solution. The suspension was diluted with 0.25 M NaBr in an SW 41 tube, and sonicated at maximum setting for 10 seconds a Biosonik sonic oscillator equipped with a microprobe (Bronwill Scientific). The membrane which sedimented at 192,000xg for 1 hour was designated ZGM-3.

Mitochondrial and microsomal membranes. Mitochondrial and microsomal pellets were resuspended in 0.2 M $NaHCO_3$, pH 8.2, with a Dounce glass homogenizer. The membrane components which sedimented at 192,000xg for 1 hour were resuspended in 0.25 M NaBr, sonicated for 10 seconds (microprobe, maximum setting), and collected by centrifugation at 192,000xg. Membranes isolated in this manner could be directly compared with purified zymogen granule membrane.

Analytical Polyacrylamide Gel Electrophoresis

The gel electrophoresis syste ms surveyed for effective separation of membrane polypeptides are summarized in Table 2. Electrophoresis in 0.1% SDS according to Kiehn and Holland (1970) or Hoober (1970) without modification failed to resolve the major portion of microsomal membrane polypeptides in the molecular weight range below 30,000. The acetic acid-urea system originally described by Takayama et al. (1966) and modified by Zahler et al. (1970) and Ray and Marinetti (1971) did not resolve the entire membrane protein applied, since much of the material remained trapped at the gel surface, presumably as insoluble aggregates. This system was of service, however, since solubilization and electrophoresis without detergent permitted

Table 2. Survey of dissociating polyacrylamide gel electrophoresis systems.

Gel System	Differential Conditions				Results
	Solvent	Buffer	% acrylamide	Duration of run	
I) Kiehn and Holland (1970) Hoober (1970)	alkaline (pH 9) 0.1% SDS with 0.5M[a] urea and 0.1% 2-ME	0.1 M Tris-acetate, pH 9 plus 0.01% EDTA and 0.1% SDS	7	overnight	poor resolution of low molecular weight polypeptides
II) Takayama et al. (1966) Zahler et al. (1970) Ray and Marinetti (1971)	phenol-acetic acid-urea-H_2O-2-ME (2:1:1:1:0.05, w/w/w/v/v)	10% acetic acid	7.5	overnight	alternate basis of separation; significant material trapped at gel surface
III) Fairbanks and Avruch (1972)	acid (pH 2.4) 1% SDS, 1% 2-ME	0.1 M sodium phosphate, pH 2.4, with 1% SDS	9	4 hours	significant material trapped at gel surface; acid pH stabilizes putative acyl-phosphate intermediates and may affect mobility of glycoproteins
IV) Laemmli (1970)	neutral (pH 6.8) 1% SDS with 1% 2-ME	discontinuous system: 0.06 M Tris-Cl, pH 6.8/ 0.15 M Tris-Cl, pH 8.8	4/9[b]	6 hours	stacking procedure aids resolution and use of large sample volumes
V) Fairbanks (1969) Fairbanks et al. (1970)	alkaline (pH 8) 1% SDS with 1% 2-ME	0.04 M Tris-acetate, pH 7.4, 0.02 M EDTA and 1% SDS	9	4 hours	thorough dissolution of membranes; excellent resolution of low molecular weight polypeptides; added protection against proteolysis
Va) Fairbanks et al. (1970) (modified slightly)	alkaline (pH 8) 1% SDS with 1% 2-ME	0.04 M Tris-acetate, pH 7.4, 0.02 M EDTA and 1% SDS	12	8 hours	increased monomer concentration has been reported to alter mobility of glycoproteins (Segrest et al., 1971)
VI) Maizel (1971)	neutral (pH 6.7) 1% SDS, 1% 2-ME	0.1 M sodium phosphate, 0.1% SDS	5-17 gradient	24 hours	preparative slab gel system employed for isolation of membrane component

[a] 2-ME: 2-mercaptoethanol
[b] 4% stacking gel, 9% running gel

an independent evaluation of the size and distribution of membrane components. Acid-SDS polyacrylamide gels were prepared and electrophoresis was performed at pH 2.4 as described by Fairbanks and Avruch (1972). Discontinuous-SDS polyacrylamide gel electrophoresis was performed as described by Laemmli (1970) without modification.

The method of most utility was electrophoresis in 0.04 M Tris-acetate containing 1% SDS, essentially as described by Fairbanks et al. (1970). Membrane pellets were dissolved by sonication in sample solvent (0.01 M Tris-Cl, pH 8.0, containing 1% SDS and 5 mM EDTA). Samples in solutions of high ionic strength were dialyzed against sample solvent overnight. Prior to electrophoresis 2-mercaptoethanol was added to 2% (v/v), and the solution was heated at 60° for 30 minutes or at 100° for 5 minutes. After heating, one volume of sample was mixed with one-third volume of sample solvent containing 20% sucrose and 40 µg/ml pyronin B. Nine percent acrylamide gels (acrylamide to bisacrylamide ratio, 36:1), dimensions 0.5 X 11 cm, were polymerized in chromic acid-cleaned glass tubes coated with dimethyldichlorosilane. A flat gel surface required for high resolution was insured by carefully monitoring the polymerization process. Gels were prerun at 5 volts/cm. Samples were applied in a minimal volume, optimally 7 to 20 µl, to promote sharp protein bands. A potential gradient of 10 volts/cm was employed, and the current did not exceed 6 mamperes per gel. To diminish curvature of polypeptide bands a temperature of 13° was maintained during electrophoresis. A constant length of each electropherogram was obtained by allowing the tracking dye, pyronin B, to migrate 8.8 cm from the origin, requiring

an average of 4.5 hours. Mobilities are expressed relative to the tracking dye, which was marked prior to staining with India ink.

Preparative Polyacrylamide Slab Gel Electrophoresis

The slab gel apparatus employed has been described by Reid and Bielski (1968). Several modifications and detailed procedures have been described by Studier (1973). Preliminary experiments demonstrated that acrylamide gradient slab gels utilizing 0.1 M sodium phosphate buffer, pH 6.65, with 0.1% SDS (Maizel, 1971) gave optimal resolution and separation of individual membrane components. 0.6 cm plexiglass spacers placed between glass plates at the sides and bottom formed the slab gel compartment approximately 11 x 15 x 0.6 cm, which required 110 ml of acrylamide solution.

The acrylamide gradient was prepared using a Beckman Density Gradient Former. Two sets of 50 ml syringes containing the following two solutions yielded a linear 5 to 17% acrylamide gradient:

Light solution, total volume 75 ml: 3.75 ml concentrated acrylamide-bisacrylamide mixture (40 gm acrylamide plus 1.1 gm bisacrylamide per 100 ml water solution), 7.5 ml 10-fold concentrated stock phosphate buffer (81.0 gm of Na_2HPO_4 and 59.3 gm $NaH_2PO_4 \cdot H_2O$ in a final volume of 1 liter), 0.375 ml of 20% SDS (w/v), 37.5 µl TEMED, and 63.0 ml of water. 0.375 ml of a 10% ammonium persulfate solution (w/v) was added immediately before use.

Heavy solution, total volume 75 ml: 37.5 ml of concentrated acrylamide-bisacrylamide mixture, 7.5 ml 10-fold concentrated phosphate buffer, 0.375 ml of 20% SDS, 37.5 µl TEMED, 22.5 ml

glycerol, 6.75 ml water and 0.15 ml of 10% ammonium persulfate solution, just prior to use.

After pouring the gradient, a plexiglass spacer 0.6 cm thick and 11 cm long was inserted into the form from the top to a depth of about 2 cm, displacing some acrylamide solution. Polymerization became apparent 20 minutes after mixing the two solutions. Removal of the top spacer after polymerization and prior to electrophoresis revealed a trough of the same dimensions 1-2 cm deep. The gradient slab gel was prerun at 35 volts for 1 hour.

One to five ml of sample solution containing up to 6.5 mg of membrane protein in 0.01 M sodium phosphate buffer, pH 6.65 with 1% SDS, 1% 2-mercaptoethanol, 5% sucrose and 5 µg/ml pyronin B were applied after heating at 100° for 5 minutes. Buffer reservoirs contained 0.1 M sodium phosphate, pH 6.65, with 0.1% SDS. Overheating during electrophoresis was avoided by maintaining constant current at 70-75 mamperes. Under these conditions the potential difference was approximately 35 volts, and electrophoresis was at room temperature for 18 to 30 hours, depending upon the separation desired.

After electrophoresis was complete, the protein band of interest was located by quick staining three 3 mm thick vertical slices removed from the middle, left and right sections of the gel. The slices were marked for identification and stained with Coomassie blue G for 1-2 hours as described below. The observed migration distances of the top and bottom of the band visualized by staining required corrections for changes in the gel length caused by the staining procedure in order to calculate the corresponding positions

in the unstained slab gel sections. The regions containing the band were excised for elution of the protein from the polyacrylamide matrix.

Preparative Electroelution of Polyacrylamide Gel Slices

A special apparatus was constructed for large-scale electroelution of polyacrylamide gel pieces obtained from slab gels. The apparatus consisted of two buffer reservoirs, each with a two liter capacity, containing platinum wire electrodes. The upper reservoir, supported directly above the lower reservoir by a ring stand, had a 7.5 cm length of 1.9 cm I.D. glass tubing projecting through the bottom. The lower end of the glass tube was sealed with Saran Wrap and filled with 10 ml of 5% acrylamide solution (with 0.14% bis-acrylamide, 0.05% ammonium persulfate, 0.05% TEMED, 0.1 M sodium phosphate, pH 6.65, and 0.1% SDS) containing the gel pieces to be eluted. After polymerization the Saran Wrap was replaced with nylon screen (73 μ mesh; Tobler, Ernst and Traber, Inc.) secured with a rubber band. A 6 inch length of 7/8 inch diameter dialysis tubing containing 40 to 50 ml of electrophoresis buffer was fitted over the bottom of the glass tube containing the gel plug and nylon screen. The dialysis tubing was firmly secured with two lengths of copper wire fastened around protective tygon rings. Air bubbles collecting within the system were removed. Electroelution was generally performed at 40 volts for 18 hours, at which time the dialysis bag was replaced and electroelution continued for an additional 18 hours. The eluate was dialyzed for 18 hours against two changes of 2 liters

of distilled water containing 3 gm washed Dowex AG-1X and 5 ml of toluene. The dialysate was lyophilized and subsequently stored at -20°.

Polyacrylamide Gel Staining

Protein staining. Two methods were employed to detect protein bands within polyacrylamide gels. The technique most frequently employed utilized Coomassie blue R (CbR) and required removal of SDS for optimal results. After electrophoresis gels were soaked in 30 ml screw cap culture tubes for a minimum of two days with four changes of 10% TCA. Gels of higher cross-linkage and larger diameters required a longer extraction period. TCA extracted gels were stained overnight in 0.4% CbR in 10% TCA-33% methanol (Johnson et al., 1971), then destained for 8 to 10 hours in 10% TCA-33% methanol with an apparatus designed after the Hoefer diffusion destainer (Hoefer Scientific Instruments, San Francisco). Removal of background stain was then completed in 10% TCA in 30 ml screw cap culture tubes at 37° overnight with shaking.

A second protein staining technique was employed for polyacrylamide slab gels. The procedure for the preparation of quick staining solution from treatment of Coomassie blue R described by Malik and Berrie (1972) was applied to Coomassie blue G (R. Blakesly, personal communication) as follows: 0.2 gm of CbG was dissolved in 100 ml of distilled water, then diluted with 100 ml of 2 N H_2SO_4. After vigorous stirring, the insoluble material was removed by filtration. The brown filtrate was titrated to dark blue by slow addition of 10 N NaOH (5 to 10 ml). 26.4 gm of TCA was added and

the solution was used immediately. Gels were not soaked in 10% TCA, but were rinsed in distilled water to remove excess SDS. To locate protein bands for the electroelution procedure, gel slices were incubated for about 1 hour in the stain solution. Excess stain was then removed from the slices by two 10 minute washes with 0.2 N H_2SO_4, and the color intensified with several water rinses. This procedure was adequate for locating bands quickly. The length of time exposed to stain was increased to 6 hours to properly stain all detectable bands. Water rinses over a period of several days intensified the stain color.

Carbohydrate staining by the periodic acid-Schiff procedure (PAS). The procedure described by Fairbanks et al. (1970) for detecting carbohydrate within polyacrylamide gels was employed with minor modifications. Complete removal of SDS by exhaustive soaking in 10% TCA (minimum of 8 changes over 4 days) was imperative to prevent artifactual staining of non-glycoprotein species (Glossman and Neville, 1971). All operations were performed in 30 ml screw cap culture tubes with shaking. The incubation times were extended to 0.5% periodic acid, 2 hours; 0.5% sodium arsenite with 5% acetic acid, 1 hour; 0.1% sodium arsenite with 5% acetic acid, 1/2 hour repeated twice; 5% acetic acid, 1/2 hour; Schiff reagent, overnight in the dark; 0.1% sodium metabisulfite in 0.01 N HCl, several changes of one hour duration until the rinse solution failed to turn pink upon the addition of formaldehyde. PAS stained gels were either scanned at 560 nm with a Gilford linear transport or photographed with Kodak Extapan film through a yellow filter.

Determination of Radioactivity in Polyacrylamide Gels

Polyacrylamide gels containing tritium were fractionated with a Savant Autogeldivider (Savant Instrument, Inc.) (Maizel, 1966). Gels containing phosphorus-32 were sectioned into 2 mm transverse fractions using a stainless steel support and cutting guide. Each fraction from ^{3}H and ^{32}P containing gels was placed in a scintillation vial, and 0.1 N NaOH containing 1% SDS was added to bring the fraction volume to approximately 1.4 ml. After incubating for a minimum of 16 hours at 37°, the solution was neutralized by adding an equivalent amount of 1 N HCl. After 10 ml of a Triton X-100 based scintillation fluid (Mostafa et al., 1970) were added to each scintillation vial, the vials were capped, shaken, and monitored for radioactivity in a liquid scintillation spectrometer.

Scintillation Counting

Radioactivity was routinely measured in a Packard Tri-Carb utilizing the Triton X-100/toluene scintillation fluid reported by Mostafa et al. (1970). Tritium was optimally counted at 55% gain with window settings at 50-1000; efficiency was approximately 20%. Phosphorus-32 was monitored at 2% gain with window settings at 50-infinity; appropriate corrections were made for ^{32}P decay during the course of experiments.

In Vitro Radioactive Labeling With Formaldehyde and $(^{3}H)NaBH_4$

Reductive alkylation similar to the method described by Rice and Means (1971) was employed to incorporate radiolabel into standard

proteins and zymogen granule membrane components. Preliminary labeling experiments with bovine serum albumin in the presence of SDS demonstrated the feasibility of the modified procedure. All operations were performed in the hood at room temperature. 0.2 mg of bovine serum albumin in 0.1 M sodium borate, pH 10, containing 1% SDS was treated first with 10 µl of 0.04 M formaldehyde, then after one minute with 4 sequential 2 µl additions of (^{3}H)$NaBH_4$ (0.14 M, 120 mCi/mmole). 10 µl of unlabeled sodium borohydride was added to the mixture after two minutes. Unreacted sodium borohydride was hydrolyzed by adding 5 µl of glacial acetic acid. Low molecular weight components of the reaction mixture were removed by chromatography on a Bio-Gel P-4 column equilibrated with 0.1 M triethylammonium bicarbonate, pH 8, containing 0.1% SDS. The void volume fractions were pooled and lyophilized. The specific radioactivity of ^{3}H-labeled bovine serum albumin varied between 7 x 10^3 and 15 x 10^3 cpm/µg. More than 95% of the protein-bound radioactivity co-electrophoresed with bovine serum albumin in 1% SDS, 9% acrylamide gels. Labeling of purified zymogen granule membrane was performed by the same procedure.

Enzyme Assays

Amylase was assayed by a micromodification (Sanders, 1970) of the method of Smith and Rowe (1949), which measures the disappearance of starch-iodine chromophore at 540nm. The technique was extremely sensitive; samples with high activity were assayed immediately after dilution with 0.05 M Tris-Cl, pH 7.2, containing 0.02 M NaCl and 0.1 mM $CaCl_2$. Units of activity are expressed as the change in absorbance at 540nm per minute.

Cytochrome c oxidase was assayed as described by Wharton and Tzagoloff (1967). Units of activity are expressed as μmoles of cytochrome c oxidized per minute.

Hydrolysis of ATP was assayed by measuring both the release of total orthophosphate from unlabeled ATP and the release of ^{32}P-orthophosphate from ($\gamma^{32}P$)ATP. For both methods 50 μl reaction mixtures contained 3-5 mM disodium ATP, 3-5 mM $MgCl_2$ and 50 mM imidazole-Cl, pH 7.1. After incubating 30 minutes at 37°, assays were terminated by adding 150 μl of cold 10% TCA and centrifuging briefly at 0°. For the nonradioactive assay (assay A), orthophosphate in the supernatant was measured spectrophotometrically as described by Lindberg and Ernster (1956). To assay the specific hydrolysis of the terminal phosphate of ATP (assay B), the standard Mg^{2+}-ATPase assay was supplemented with approximately 100,000 cpm of ($\gamma^{32}P$)ATP. After incubation a 100 μl aliquot of the acid quenched assay mixture was added to a 13 x 100 mm test tube containing 0.4 ml of 1.5% ammonium molybdate in 0.5 N H_2SO_4 at 4°. 0.5 ml of cold isobutanol-benzene (1:1, v/v) was added to each, and the samples were mixed thoroughly by vortexing for precisely 30 seconds. After standing for 20 minutes to equilibrate to room temperature, a 250 μl aliquot of the top layer of isobutanol-benzene was placed in a scintillation vial containing 10 ml of scintillation fluid to determine the ^{32}P content. When the assay was performed as described, the observed ^{32}P-orthophosphate release was directly proportional to incubation time and enzyme concentration for all subcellular fractions assayed.

Protein kinase activity was assayed at 23° in a reaction volume of 100 μl contained in a 400 μl polyethylene microfuge tube

(Beckman Instruments). The standard mixture, unless otherwise indicated, contained 20 mM imidazole-Cl, pH 7.1, 75 μM (γ^{32}P)ATP (7.5 nmoles, approximately 4 x 10^7 cpm), 1 mM $MgCl_2$, and 2 mg/ml of membrane protein. The assay was initiated by the addition of (γ^{32}P)ATP, and terminated after five seconds by the addition of 300 μl of 0.3 N perchloric acid containing 5 mM H_3PO_4 and 2.5 mM ATP (Avruch and Fairbanks, 1972). After standing 15 minutes at 4°, the labeled membrane was sedimented by centrifugation for 10 minutes at 100,000xg (40,000 rpm, type 50 rotor; Beckman) and the supernatant discarded. The pellet was thoroughly resuspended in another 300 μl of perchloric acid solution by sonication. The capped tubes were taped to the bottom of a large plastic beaker, and covered with ice water to a depth of about 1 cm. The tip of a Biosonik sonic oscillator probe (maximum setting) was brought into contact with the wall of each tube for 10 seconds. The membrane was again collected by centrifugation at 100,000xg. Resuspension in the perchloric acid solution and centrifugation were repeated four times. A final wash with distilled water removed excess perchloric acid. The washed pellets were stored at -80°. The pellets were prepared for electrophoresis by adding to each tube 100 μl of 0.3 M sucrose and 7.5 μl of 20% SDS, mixing thoroughly, then adding 7.5 μl of sodium phosphate, pH 2.4, 3 μl of 2-mercaptoethanol, and 10 μl of 40 μg/ml pyronin B. After a 10 minute incubation at 37° the solutions were layered on 0.6 x 11 cm, 9% acrylamide gels. The gels were prepared and electrophoresis was performed in 1% SDS at pH 2.4 as described elsewhere (Avruch and Fairbanks, 1972; Fairbanks and Avruch, 1972). The distribution of

^{32}P in the gels was determined as described earlier. The data have not been corrected for loss of radioactive protein during the perchloric acid washes. Protein kinase activity is defined as the transfer of ^{32}P from ($\gamma^{32}P$)ATP to membrane components within the polypeptide region of SDS-electropherograms.

Preparation of ($\gamma^{32}P$)ATP

($\gamma^{32}P$)ATP was prepared by both the methods of Penefsky (1967) and Richardson (1971) with comparable results. Characteristics of a typical preparation were specific radioactivity, 4 mCi/mmole; A_{250}:A_{260}, 0.81; and A_{280}:A_{260}, 0.20. When analyzed by DEAE column chromatography (Whatman microgranular DE 52), 97% of the ^{32}P eluted coincident with carrier ATP; the remaining 3% eluted distinct from added AMP and ADP standards.

Analytical Procedures

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. Samples containing interfering substances such as sucrose and 2-mercaptoethanol were precipitated at 4° by the addition of an equal volume of 10% trichloroacetic acid. The precipitates were collected by centrifugation for 5 minutes (Microfuge, Beckman), washed by resuspension in 10% trichloroacetic acid and recentrifugation at 4°. Particulate protein samples were dissolved in 0.1 N NaOH with 1% SDS prior to analysis. Sialic acid content of membrane samples and polyacrylamide gel fractions was analyzed by the thiobarbituric acid procedure of Warren (1959), using N-acetylneuraminic acid as the standard. The

sodium dodecyl sulfate content of protein samples was determined by extraction of the dodecyl sulfate-methylene blue complex into chloroform and measuring the absorbance of the chloroform layer at 655nm (Reynolds and Tanford, 1970a).

RESULTS

Isolation and Characterization of Rat Zymogen Granule Membrane

Isolation

The isolation of zymogen granule membrane first requires the preparation of an enriched and intact zymogen granule fraction. Purification from this subcellular fraction is readily monitored. Contaminants group into two easily identifiable classes, granule contents and mitochondria, which can be monitored by enzymatic analyses and characteristic electrophoretic mobilities of specific polypeptides of each class. The purification scheme is biased to remove each group sequentially.

Alkaline lability of zymogen granules was first noted by Hokin (1955). Meldolesi et al. (1971a) exploited this observation to partially purify the granule membrane from guinea pig pancreas. The first two steps of Meldolesi's procedure formed the basis of the isolation technique employed in this study. Isotonicity was maintained to prevent rupture of contaminating mitochondria, and zymogen granules were lysed by the addition of 0.2 M $NaHCO_3$, pH 8.2. Lysis was monitored as a marked decrease of turbidity. After centrifugation, the soluble fraction, representing the granule contents (Hokin, 1955; Meldolesi et al., 1971a), contained 96% of the protein and 98% of the

amylase activity (Table 3). When examined by polyacrylamide gel electrophoresis in 1% SDS, pH 7.4, nine major polypeptide species were observed (Figure 1A). The major component at 3.1 cm accounted for approximately 50% of the total stain intensity and had a molecular weight of 52,000 when compared to proteins of known size in parallel gels. This agrees well with the reported relative amount (Sanders, 1970) and molecular weight (Sanders and Rutter, 1972) of amylase from adult rat pancreas. Procarboxypeptidase B, molecular weight 50,000, can be tentatively identified at 3.3 cm immediately adjacent to amylase by similar criteria (Sanders, 1970). Likewise, the peaks at 5.5 cm and 7.2 cm are assumed to be chymotrypsinogen (molecular weight 25,000) and ribonuclease (molecular weight 14,000), respectively. Amylase, procarboxypeptidase B and chymotrypsinogen correlate with bands 5, 6 and 16 in electropherograms of zymogen granule membrane fractions discussed below.

Table 3 summarizes the purification of zymogen granule membrane away from amylase and cytochrome c oxidase activities. Membranes, ZGM-1, collected from the lysate by centrifugation accounted for 4% of the total granule protein, all of the mitochondrial cytochrome c oxidase activity, and a significant fraction of the granule contents, monitored as amylase activity. The buoyant density difference between the granule ghosts and mitochondria was exploited to separate the two structures (Meldolesi, 1971a). Mitochondria sedimented through 1 M sucrose while the granule membrane, ZGM-2, floated to the 1 M - 0.3 M sucrose interface. The high amylase specific activity of this fraction necessitated a final purification step. Brief sonication in

Table 3. Amylase and cytochrome c oxidase activities of zymogen granule subfractions.

Fraction	Protein	Amylase		Cytochrome c oxidase	
		Total Activity	Specific Activity	Total Activity	Specific Activity
	%	%	units/mg protein ($x10^{-3}$)	%	milliunits/ mg. protein
Intact zymogen granules	(100)	(100)	101 (4)	(100)	1.0 (5)
Crude membrane fraction (ZGM-1)	3.8	2.2	8.0 (2)	157	10.3 (1)
Membranes from discontinuous gradient (ZGM-2)	1.4	1.2	12.1 (3)	16	4.7 (3)
Membranes after NaBr extraction (ZGM-3)	0.54	0.1	0.8 (3)	0.4	0.2 (5)

Each fraction was prepared as described in Methods.

Amylase and cytochrome c oxidase activities were measured as described in Methods. Values are the means of the number of experiments given in parentheses. Cytochrome c oxidase specific activity of purified mitochondrial membrane was 21 milliunits/mg protein.

Figure 1. Electrophoretic analysis of the intermediate fractions obtained during zymogen granule membrane isolation.

Electrophoresis was performed under standard conditions (9% acrylamide, 1% SDS, pH 7.4) as described in Methods. The following amounts of protein were applied: A, granule contents, 34 µg; B, ZGM-1, 51 µg; C, ZGM-2, 36 µg; D, ZGM-3, 42 µg. Gels were stained with Coomassie blue R (CbR) and scanned at 550 nm. TD marks the distance of migration of the tracking dye, pyronin B.

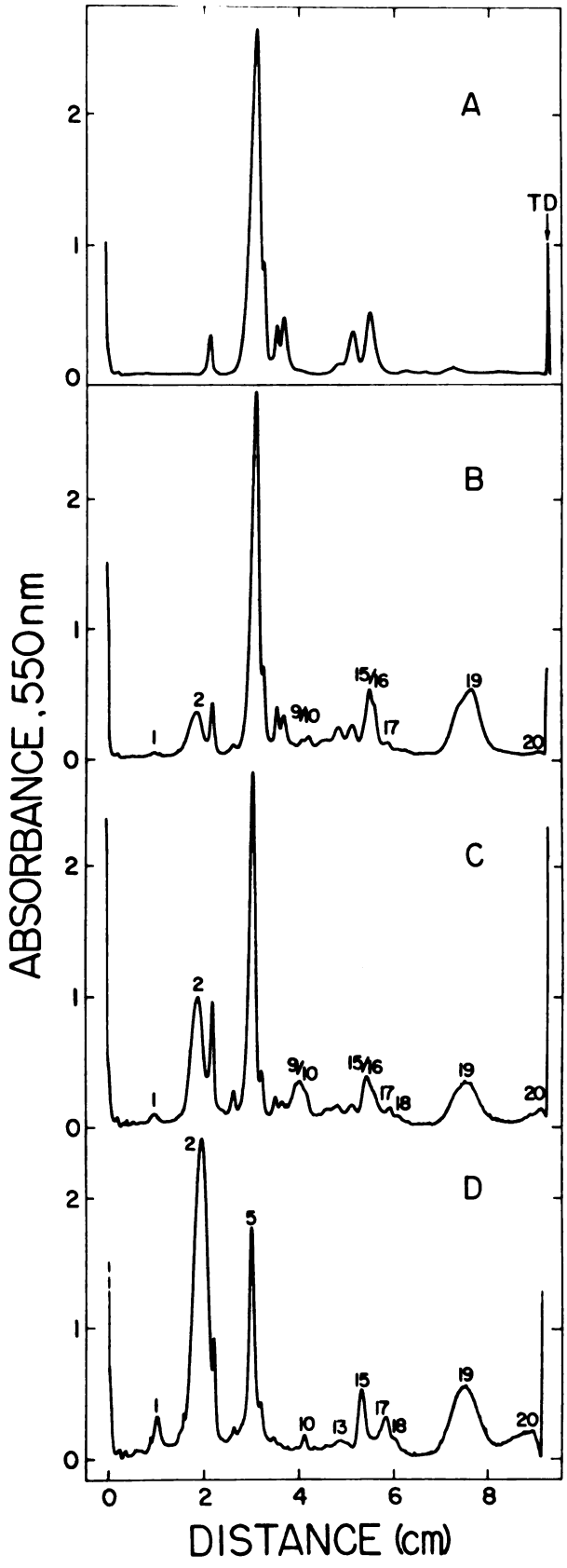

A
TD
B
1
2
9/10
15/16
17
19
20
C
1
2
9/10
15/16
17
18
19
20
D
1
2
5
10
13
15
17
18
19
20
ABSORBANCE, 550nm
0
1
2
DISTANCE (cm)
0
2
4
6
8

Figure 1

0.25 M NaBr, a procedure which did not inactivate amylase, reduced the specific activity of the final membrane preparation, ZGM-3, to less than 1% of the original level in zymogen granules. The final yield of granule membrane was approximately 0.5% of the total granule protein.

Mitochondrial cytochrome c oxidase activity after the rigors of this purification was 21 milliunits/mg protein. Since the cytochrome c oxidase activity of the purified granule membrane was 0.2 milliunits/mg protein (Table 3), mitochondrial contamination was estimated to be less than 1%.

Contamination by inactivated granule contents was evaluated by mixing (^{14}C)leucine labeled soluble granule protein with unlabeled intact zymogen granules (MacDonald and Ronzio, 1974). From the known specific radioactivity of the soluble proteins, it was estimated that less than 3% of the membrane protein isolated from the mixture was adsorbed granule contents.

The removal of mitochondrial and soluble secretory proteins and the enrichment of membrane-associated polypeptides during granule membrane isolation were monitored by SDS polyacrylamide gel electrophoresis. Several polypeptides were consistently enriched in the membrane fraction at each stage of purification. The bands representing these polypeptides are designated in Figure 1D as 1, 2, 5, 10, 13, 15, 17, 18, 19, and 20. The stain intensity of band 2, the major membrane polypeptide, increased six-fold during purification from the crude membrane fraction.

The distributions of mitochondrial membrane and zymogen granule membrane polypeptides are compared in Figure 2. A

Figure 2. Comparison of mitochondrial and zymogen granule membrane polypeptides separated by electrophoresis.

Polyacrylamide gels, run under standard conditions, were stained with CbR and scanned at 550 nm as described in Methods. MITO: 65 µg mitochondrial membrane protein; M denotes the major band. MITO - ZGM-3: a mixture of mitochondrial membrane (36 µg protein) and purified granule membrane (15 µg protein). ZGM-3: 39 µg granule membrane protein; the positions of bands 2 and 15 are indicated. Insert: portion of a scan of a gel containing 40 µg of granule membrane protein and a smaller amount of mitochondrial membrane (14 µg protein). The absorbance spike at the far right marks the migration of the tracking dye.

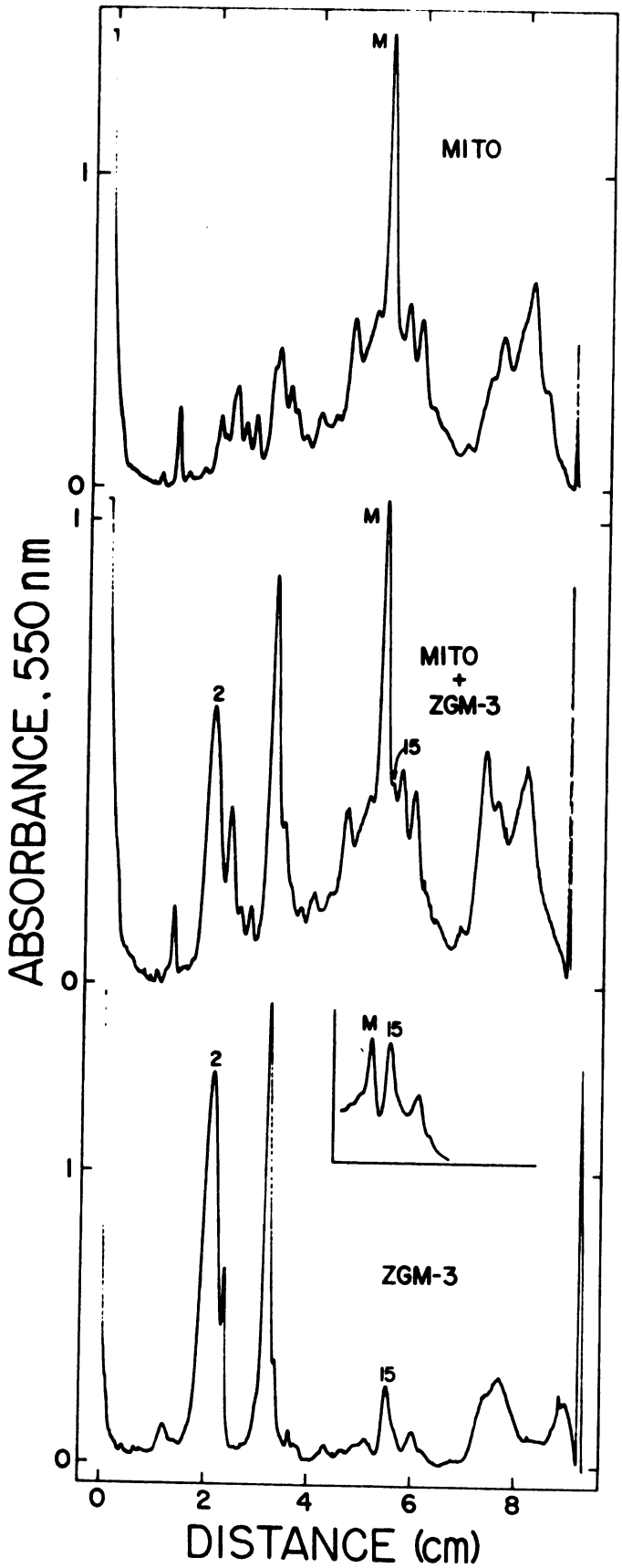

MITO
MITO + ZGM-3
ZGM-3
M
2
15
ABSORBANCE, 550 nm
DISTANCE (cm)
0
2
4
6
8
1
0
1
0
1
0

Figure 2

characteristic mitochondrial band, designated M, had a molecular weight of 26,000 and accounted for 10 to 15% of the profile. Band M and band 15 of ZGM-3 had nearly identical mobilities and it was considered whether band 15 represented mitochondrial contamination. When a mixture of purified granule membrane and similarly treated mitochondrial membrane was subjected to electrophoresis as shown in the middle scan of Figure 2, band 15 migrated slightly faster than the M band. The difference in mobility was readily demonstrated when a smaller amount of mitochondrial membrane was added to ZGM-3 so that both components were of equal staining intensity, and when electrophoresis was prolonged (Insert, Figure 2). On this basis, in addition to the absence of significant cytochrome c oxidase activity, it was concluded that mitochondrial contamination of the final granule membrane preparations was negligible. However, the mitochondrial contamination of ZGM-1 was high. Frames B and C of Figure 1 illustrate that mitochondria were removed by the discontinuous gradient centrifugation step. Most of the stain intensity in bands 15/16 of the crude membrane preparation disappeared at this step; apparently band M accounted for most of the stain intensity in this region. The doublet nature of band 19 in ZGM-1 and the rather high stain intensity relative to the other bands suggest that this band contained both the zymogen granule component as well as low molecular weight polypeptides of mitochondrial membranes.

The loss of adsorbed secretory proteins from the membrane during purification is also documented in Figure 1. The majority of the stain intensity in ZGM-1 is associated with identifiable secretory

proteins, particularly amylase. The enrichment of membrane species was monitored by an increase of band 2. Although significant loss of soluble protein occurs at the discontinuous gradient step, the release of adsorbed contents was essentially complete after 0.25 M NaBr extraction.

Strong chaotropic agents have been shown to disaggregate membrane protein complexes (Hatefi and Hanstein, 1969). 0.25 M NaBr was chosen as a mildly chaotropic reagent which might selectively elute adsorbed contaminations without affecting intrinsic membrane polypeptides (S.D. Aust, personal communication). Figure 3 illustrates that this assumption was borne out. Polypeptides with mobilities identical to components of the granule contents were selectively eluted from ZGM-2 with 0.25 M NaBr, and apparently were secretory proteins which had adhered to the membrane. Bands 8, 12, 14, and 16 were quantitatively extracted, while other content components, bands 3, 5, 6 and 7 were partially extracted. The only polypeptides which were preferentially solubilized, but could not be correlated with secretory proteins, were minor bands 9, 11 and 19.

Figure 3 demonstrates that ZGM-3 contained several components that were masked by NaBr solubilized bands. ZGM-2 contained two minor overlapping bands, designated 9/10. By comparing mobilities of these bands in the 0.25 M NaBr solubilized fraction (ZGS-3, Figure 3) and in the final membrane fraction (ZGM-3, Figure 3), it was concluded that band 9 partitioned with ZGS-3, and band 10 with ZGM-3. The partially resolved bands 15/16 were similarly separated. In addition, removal of bands 12 and 14 by the NaBr wash revealed band 13 with an intermediate mobility.

Figure 3. Identification of membrane-bound and adsorbed polypeptides of ZGM-2.

Electrophoresis of the intermediate fractions was performed under standard conditions with the following amounts of protein: ZGM-2, 36 µg; ZGS-3, the 0.25 M NaBr solubilized fraction, 40 µg; ZGM-3, 42 µg. Gels were stained with CbR and scanned at 550 nm. The absorbance spike at the far right denotes the migration of the tracking dye.

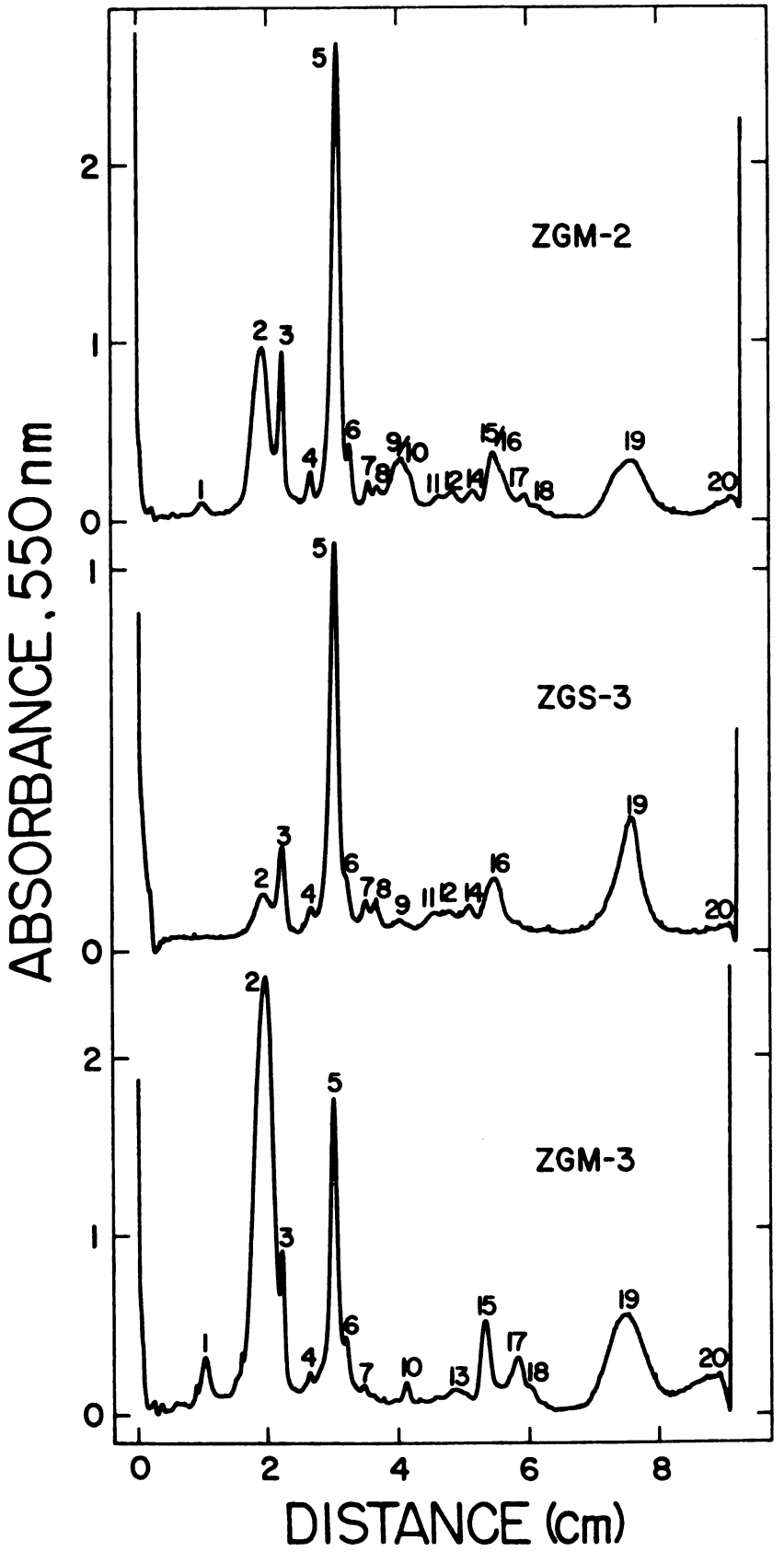


Figure 3

Further Characterization of the Granule Membrane Components

The remaining polypeptides of ZGM-2 which were not significantly extracted by 0.25 M NaBr and were enriched in ZGM-3 were examined further. Estimated molecular weights of the intrinsic granule membrane polypeptides are given in Figure 4. The standard curve was constructed from duplicate analyses of proteins of known molecular weights in 9% polyacrylamide, 1% SDS gels at pH 7.4. Mobilities are recorded relative to the tracking dye, pyronin B. Membrane polypeptides were analyzed in parallel gels and in gels which included standard proteins. Line placement within the linear portion of the standard curve was determined by computer least squares analysis. The non-linear regions above 70,000 and below 15,000 were not unexpected (Weber and Osborn, 1969), and increase the uncertainty of molecular weight estimates of polypeptides of unknown size with mobilities within this region.

In order to limit the uncertainty of the molecular weight estimates, mobilities of granule membrane components relative to standard proteins were analyzed in two other SDS-polyacrylamide gel systems. Increasing the monomer concentrations without altering other conditions has been shown to reveal inaccurate estimates for glycopolypeptides (Bretscher, 1971; Segrest et al., 1971). Electrophoresis in 1% SDS at pH 2.4 has also been shown to detect changes in mobility for specific membrane polypeptides relative to proteins of known molecular weight (Fairbanks and Avruch, 1972). Molecular weight values for the major polypeptide species of ZGM-3 obtained in the

Figure 4. Estimation of the molecular weights of zymogen granule membrane components by electrophoresis in 1% SDS at pH 7.4.

Proteins of known molecular weights were analyzed on 9% acrylamide gels under standard conditions. Mobilities are expressed relative to the tracking dye (TD). The relative migration of each ZGM-3 band is indicated by its number (refer to text and Figures 1 and 3). The molecular weight values for the standard proteins (designated a through t) were obtained from surveys by Klotz and Darnall (1960), Weber and Osborn (1969) and Smith (1968), unless noted otherwise. The table below identifies the standard proteins in the figure.

	Protein		Molecular weight
a	thyroglobulin	pig	165,000[1]
b	β-galactosidase	Escherichia coli	130,000
c	phosphorylase a	rabbit muscle	94,000
d	serum albumin	beef	68,000
e	pyruvate kinase	rabbit skeletal muscle	57,200
f	catalase	beef liver	60,000
g	leucine aminopeptidase	hog kidney	53,000
h	alkaline phosphatase	bacterial	40,000
j	aldolase	rabbit muscle	40,000
k	glyceraldehyde phosphate dehydrogenase	rabbit muscle	36,000
l	alcohol dehydrogenase	yeast	37,000
m	α-chymotrypsinogen A	beef pancreas	25,700
n	trypsin	beef pancreas	23,000
o	trypsin inhibitor	soybean	21,600
p	β-lactoglobulin	human	18,400
q	myoglobin	horse skeletal muscle	17,200
r	ribonuclease IIa	beef pancreas	13,700
s	lysozyme	egg white	14,300
t	cytochrome c	horse heart	12,400

[1]DeCrombrugghe et al. (1966).

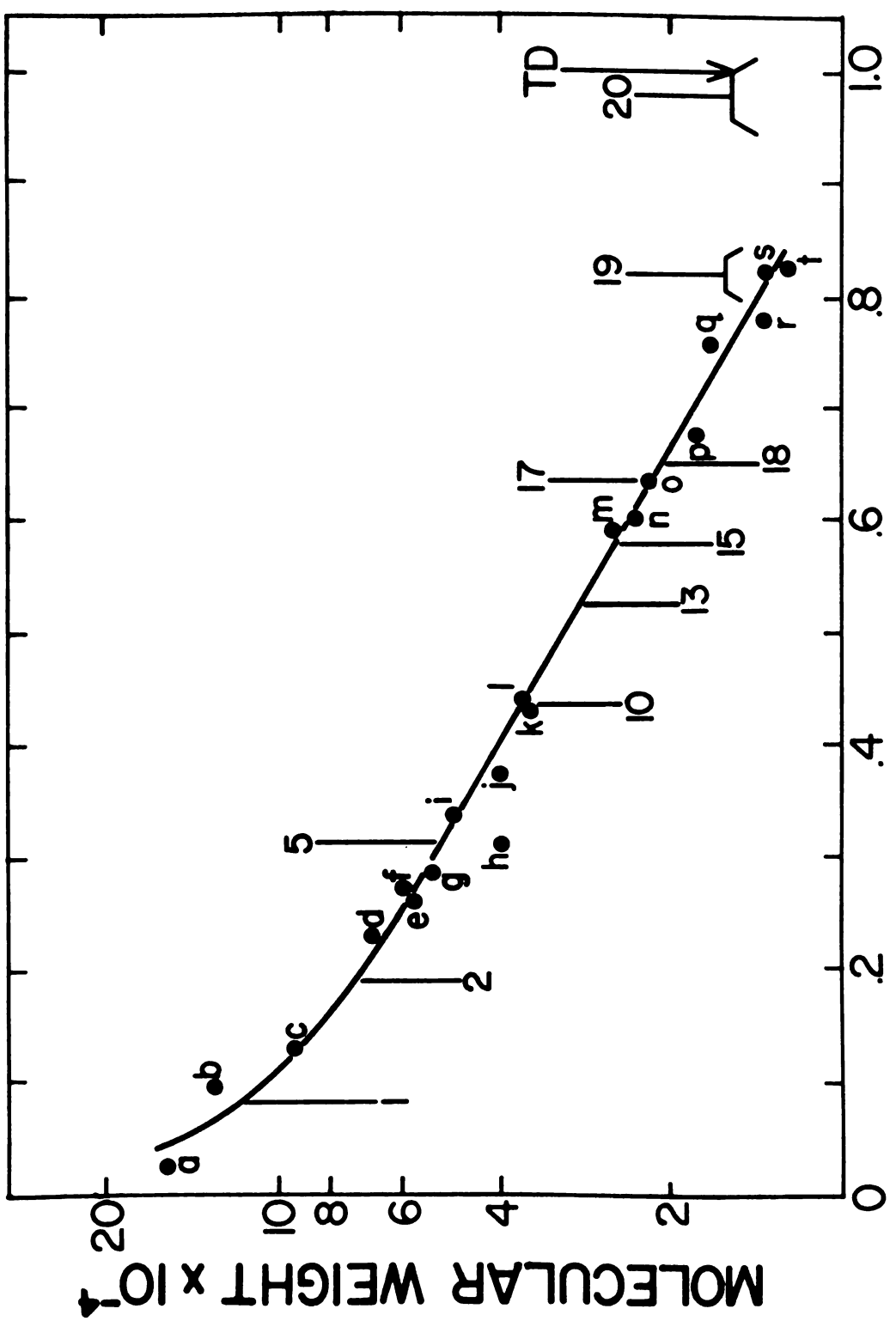

MOLECULAR WEIGHT x 10⁻⁴
20
10
8
6
4
2
0
.2
.4
.6
.8
1.0
RELATIVE MOBILITY
TD
20
19
17
18
15
13
10
5
2
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t

Figure 4

three systems are compared in Table 4. Although heterogeneity of band 5 was revealed, gross differences were not observed.

Component 2 was the major granule membrane polypeptide, accounting for approximately 37% of the total protein stain intensity of Coomassie blue stained gels (Table 4). The unusually broad molecular weight distribution (70,000 to 83,000) of band 2 suggested that it contained a heterogeneous population of molecules.

Band 5 appeared to be a mixture of two polypeptides. The level of amylase activity in ZGM-3 (Table 2) implied that amylase comprised no more than 1% of the gel profile, however, contribution by inactive adsorbed enzyme cannot be rigorously excluded. Mixtures of purified granule membrane and soluble granule contents in different ratios were subjected to prolonged electrophoresis (8 hours) in an attempt to differentiate between band 5 and the amylase band. These electropherograms revealed a much broadened band 5, which was not caused by overloading; however, two bands could not be completely resolved.

Furthermore, amylase has a small periodic acid-Schiff (PAS) positive component (cf. Figure 11) which is near the minimum level of detection in polyacrylamide gels. As will be demonstrated (cf. Figure 9), granule membrane component 5 has a much stronger periodic acid-Schiff reaction, not accountable by the presence of amylase alone. Figure 5 illustrates subfractionation of band 5 by electrophoresis in gels of higher acrylamide concentration and in acidic buffer. The covalent association of significant carbohydrate to one polypeptide would be expected to shift its migration toward a lower apparent molecular weight in these two electrophoresis systems (Segrest et al., 1971; Fairbanks and Avruch, 1972).

Table 4. Relative abundance and molecular weight of zymogen granule membrane polypeptides.

Component	Molecular Weight[b]			Relative Abundance (%)[c]	
	9% acrylamide pH 7.4	12% acrylamide pH 7.4	9% acrylamide pH 2.4	9% acrylamide pH 7.4 (n=12)[e]	7.5% acrylamide[d] acetic acid-urea (n=1)[e]
1	120,000	130,000	>130,000	2.6 ± 1.1	(2.7)
2	74,000	76,000	78,000	38.2 ± 5.9	39.9
5	52,000	55,000[f] 53,000	56,000[f] 53,000	17.9 ± 4.9	9.4
15	25,000	28,000	28,000	5.1 ± 1.8	-
17	22,000	24,000	19,000	2.5 ± 0.9	-
19	12,000-14,000	12,000-14,000	10,000-12,000	12.5 ± 7.9	(19.7)

[a]Since Band 20 is lipid, its staining contribution was not included in estimating relative abundance.

[b]Molecular weights were estimated as described for Figure 4 for each gel system.

[c]Coomassie blue stained gels were scanned and the per cent relative abundance was estimated by cutting out and weighing peaks from the scans; standard deviations are given.

[d]The relative abundance of each band for this individual zymogen granule preparation in the 9% acrylaminde-pH 7.4 system was: Component 1, 1.9; Component 2, 31.4; Component 5, 18.0; Component 19, 26.2.

[e]n is the number of separate zymogen granule membrane preparations examined.

[f]Separation into two bands (cf. Figure 5).

Figure 5. Subfractionation of zymogen granule membrane band 5.

Purified granule membrane was subjected to electrophoresis under the following conditions: A) 9% acrylamide, 1% SDS, pH 7.4 (standard conditions); B) 12% acrylamide, 1% SDS, pH 7.4 (high acrylamide concentration); C) 9% acrylamide, 1% SDS, pH 2.4 (acidic conditions); details of each procedure are given in Methods. Only the area of the scan containing band 5 is shown. Relative absorbances of the three scans are not comparable.

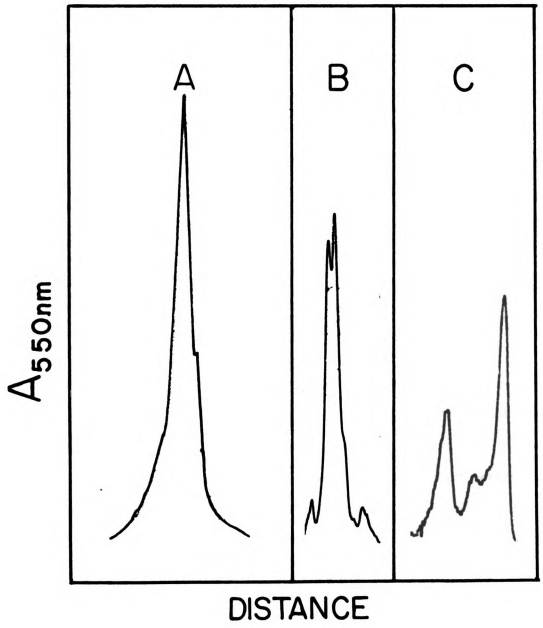


Figure 5

Band 19, a broad band containing polypeptides of molecular weight less than 14,000, was detected in the membrane fraction at each stage of purification (Figure 1). Several lines of evidence suggest that it represents proteolytic products which remain associated with the membrane. The appearance of band 19 was extremely variable (Table 4). In several cases its contribution to the stain intensity of granule membrane electropherograms was less than 2%; generally it accounted for approximately 13%. Storage of intact zymogen granules in ice for 17 hours prior to membrane purification resulted in a polypeptide profile with a reduction of bands 2 and 5 and a significant induction of band 19, when compared to a profile of freshly prepared membrane from the same zymogen granule preparation. Trypsin digestion of isolated granule membrane led to identical results. The isolation of cytochrome b_5 and cytochrome b_5 reductase after limited proteolytic treatment of microsomes illustrates what may be an analogous phenomenon (Spatz and Strittmatter, 1971, 1973). Proteolysis preferentially occurs at a site linking a hydrophilic head portion to a hydrophobic tail imbedded in the membrane. Thus, band 19 appears to represent the hydrophobic portions primarily of components 2 and 5 which remained associated with the membrane after limited proteolysis.

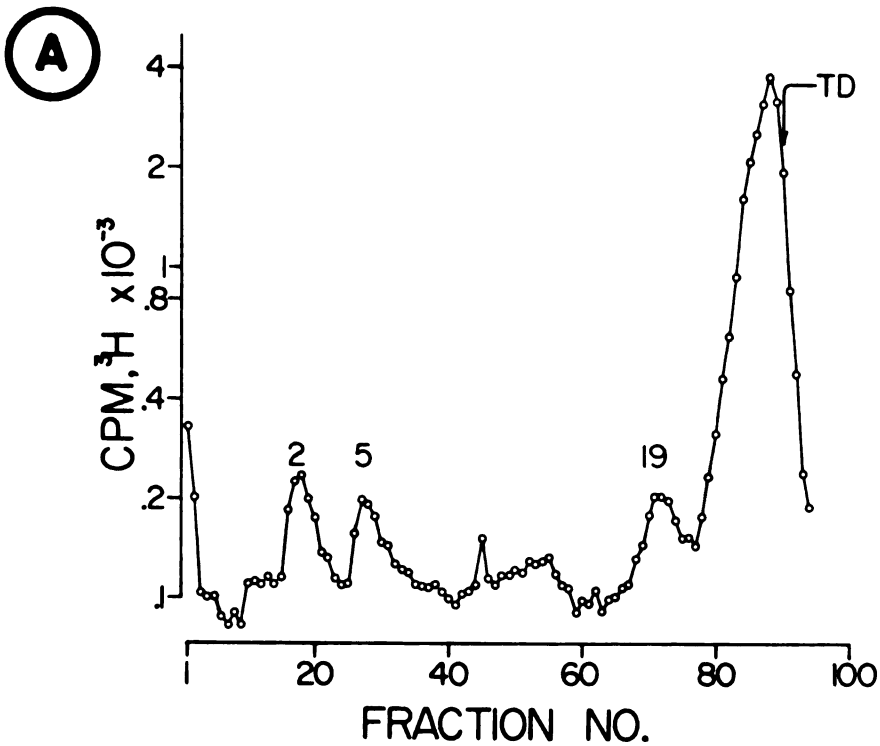
The material within band 20 appeared opalescent when viewed by indirect lighting. In addition, Coomassie blue R stain was preferentially lost from this band during prolonged destaining with 33% methanol-10% TCA. Comparison with the mobilities and staining properties of sphingomylin, phosphatidyl choline, and glycolipids

suggested that band 20 represented membrane lipids. (^{3}H)$NaBH_4$ reduction of Schiff base intermediates formed by the addition of formaldehyde to purified zymogen granule membrane solubilized in 1% SDS yielded a labeled preparation containing approximately 2 x 10^4 cpm per μg protein. Analysis of the membrane components associated with the majority of the label provided further evidence of the lipid nature of band 20. The radioactivity associated with low molecular weight material, primarily (^{3}H)methanol, was removed by chromatography of the labeled membrane sample through a Bio Gel P-4 column equilibrated with 1% SDS. Electrophoretic analysis of the labeled membrane is presented in Figure 6A. Significant label was associated with membrane components 2, 5 and 19. Eighty-three percent of the label, however, migrated with band 20. When the labeled membrane was extracted with chloroform-methanol (2:1), 88% of the total radioactivity was soluble and 12% was retained in a pelletable residue, presumably membrane protein. Essentially all of the radioactivity of the chloroform-methanol soluble fraction migrated on a silicic acid thin layer plate developed with chloroform-methanol-water (100:42:6) (Figure 6B). Thus, band 20 appears to be membrane lipid rather than low molecular weight polypeptides.

The recalcitrant nature of many membrane proteins to dissolution into individual species prompted additional, more rigorous solubilizing conditions in an attempt to simplify or alter the granule membrane polypeptide profile. Membranes were suspended to 2 mg protein/ml in 1% SDS sample solvent buffer without 2-mercaptoethanol, then treated at 23° by the following procedures: a) dialysis against

Figure 6. Labeling of zymogen granule membrane components with $(^3H)NaBH_4$.

Purified granule membrane was treated sequentially with formaldehyde and $(^3H)NaBH_4$ as described in Methods. A) Distribution of radioactivity of ^3H-labeled membrane subjected to electrophoresis under standard SDS conditions is shown; bands 2, 5 and 19 are indicated. B) A chloroform-methanol (2:1, v/v) extract of the ^3H-labeled membrane was analyzed by silicic acid thin layer chromatography. Radioactivity on the thin layer plate was detected with a Berthold Radioscanner. The phospholipid standards are S, sphingomylin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; and PS, phosphatidylserine. Lipids were detected by reaction with iodine vapor.


A
CPM, ^{3}H x 10^{-3}
4
2
.8
.4
.2
.1
2
5
19
TD
1
20
40
60
80
100
FRACTION NO.

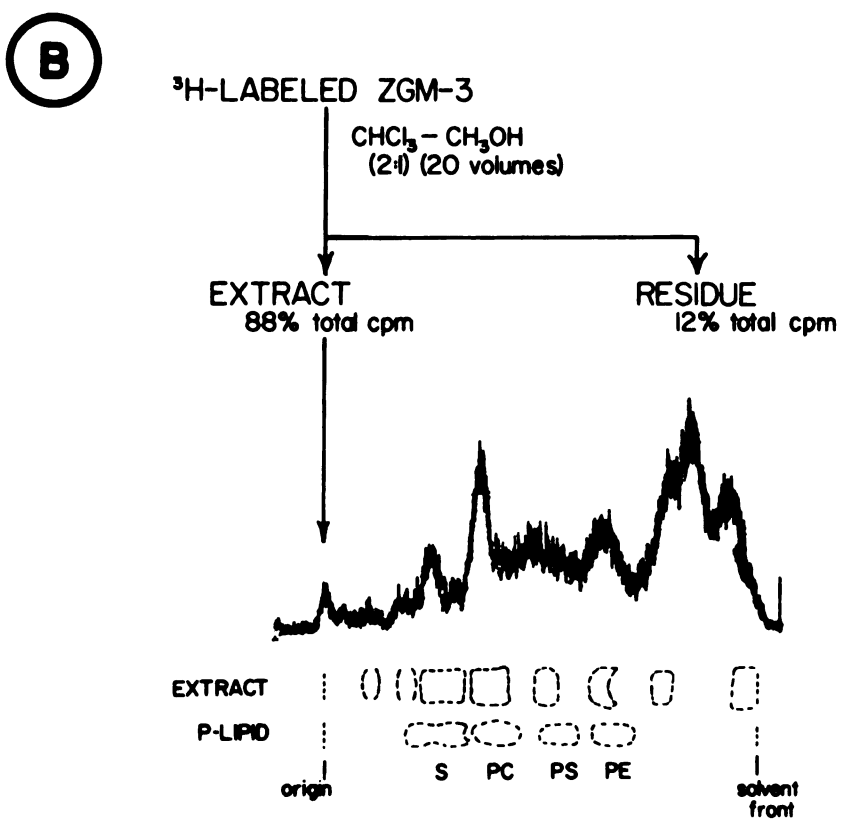

B
^{3}H-LABELED ZGM-3
$CHCl_3 - CH_3OH$
(2:1) (20 volumes)
EXTRACT
88% total cpm
RESIDUE
12% total cpm
EXTRACT
P-LIPID
origin
S
PC
PS
PE
solvent front

Figure 6

sample solvent for 12 hours; b) dialysis against sample solvent containing 6 M urea for 12 hours; c) addition of 2 volumes of 2-chloroethanol at 0° for 2 hours followed by dialysis as in a); d) extraction with chloroform-methanol (2:1) and solubilization of the residue by dialysis as in a). The membrane samples were then reduced by the addition of 2-mercaptoethanol to 1%, heated at 60° and subjected to electrophoresis under standard conditions. None of the treatments altered the mobilities of the major polypeptides.

In another experiment, granule membranes were dissolved in phenol-acetic acid-urea-water (2:1:1:1, w/v/w/v) at a protein concentration of 1 mg/ml and subjected to polyacrylamide gel electrophoresis in 7.5% acrylamide gels according to the procedure of Zahler et al. (1970). After 10 hours at 14 volts/cm, one gel was briefly stained with CbR. This electropherogram was also characterized by a simple profile (Figure 7). The regions in an unstained, unfixed gel corresponding to the two major species were estimated by comparison to the stained gel. After sectioning, the gel segments containing the two major species were equilibrated with 1% SDS sample solvent at room temperature for 4 hours, then placed on top of standard pH 7.4, 1% SDS polyacrylamide gels and subjected to electrophoresis as usual. The slower migrating broad band from the acetic-acid urea gel gave rise only to band 2 of the SDS profile of ZGM-3, while the other major component contributed only band 5.

Detergents other than sodium dodecyl sulfate were tested for their ability to solubilize granule membrane polypeptides. Membrane samples were suspended in detergent solutions by brief sonication.

Figure 7. Analysis of zymogen granule membrane polypeptides by acetic acid-urea polyacrylamide gel electrophoresis.

Details of the electrophoresis procedure are given in Methods. A is a Coomassie blue stained profile of 44 μg of granule membrane protein separated in the acetic acid-urea gel system; the major two bands are designated I and II. Gel sections containing bands I and II from a parallel gel run as in A were subjected to electrophoresis under standard conditions: B contains band I; C contains band II. D, 39 μg membrane protein run simultaneously with gels B and C but without prior fractionation by acetic acid-urea electrophoresis.

Figure 7

The proportion of band 2, determined by SDS electrophoresis, which remained in suspension after centrifugation at 150,000 xg for 90 minutes was used to assess solubilization. Table 5 lists the effects of several detergents. Even though detergents were present in great excess (12.5 to 188:1 w/w), little solubilization was effected. Two percent Tween 20 plus 1% deoxycholate, which has been shown to solubilize outer nuclear membrane and fucose-containing components of HeLa cell surface membrane (Atkinson and Summers, 1971; Penman, 1966) was the most effective of the series.

Comparison of Membrane Polypeptides of Microsomes, Mitochondria and Zymogen Granules

The total number of granule membrane polypeptides and their molecular weight distribution are clearly distinct from those of microsomal and mitochondrial membranes. Figure 8 compares the CbR stained polypeptides for all three membranes prepared similarly by $NaHCO_3$ and NaBr extractions. Electrophoresis of membranes from total pancreatic microsomes revealed 35 discrete bands. Since the isolation procedure removed most of the microsomal RNA (R. A. Ronzio, unpublished observations), the contribution of ribosomal proteins to this profile was minimal. By mixing microsomal and granule membranes prior to electrophoresis, the unique mobility of component 2 was demonstrated. Less than 2% of the stain intensity of the total microsomal profile occurred in the region around 74,000 molecular weight. Band 5 migrated with a major microsomal component, which may also contain amylase. The low molecular weight polypeptides characteristic of microsomes were absent from granule membranes.

Table 5. Detergent solubilization of zymogen granule membrane protein.

Samples of zymogen granule membrane (ZGM-2) were added to polyethylene microfuge tubes (Beckman) containing the detergents indicated below and sonicated at maximum setting (Biosonik, Bronwill Scientific) for 10 seconds. Solubilized material is defined as that which did not sediment after centrifugation at 150,000xg for 90 minutes. Total protein was measured according to Lowry et al. (1951). The relative amount of component 2 solubilized was determined by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) of the soluble and pellet fractions. The presence of other detergents did not affect electrophoresis. Values given in the table represent solubilization above the minimum observed upon sonication in distilled water and centrifugation.

Detergent	Detergent:protein (w/w)	% protein solubilized	% component 2 solubilized
2% Tween 20	125:1	0	none
0.2% deoxycholate	13:1	26	12
0.2% Tween 20 plus 0.1% deoxycholate	19:1	10	none
2% Tween 20 plus 1% deoxycholate	188:1	31	16
2% lubrol	125:1	21	none
2% NP40	125:1	16	none

Figure 8. Comparison of the polypeptides of zymogen granule membranes, mitochondrial membranes and total microsomal membranes.

Membranes were prepared by extraction with $NaHCO_3$ and NaBr as described in Methods. Electrophoresis was performed under standard conditions, and the gels were stained with CbR. Mobility is expressed relative to the dye pyronin B. The following samples are compared: a) granule membrane, ZGM-3 (39 µg protein); b) mitochondrial membrane (65 µg); c) a mixture of granule membrane (15 µg) and mitochondrial membrane (36 µg); d) membrane from total microsomes (63 µg); e) a mixture of granule membrane (28 µg) and total microsomal membrane (42 µg).

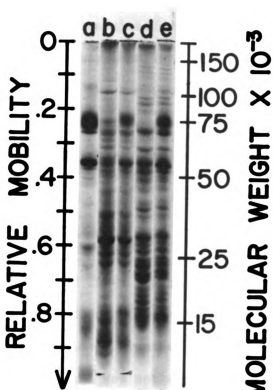

a b c d e
0
.2
.4
.6
.8
RELATIVE MOBILITY
150
100
75
50
25
15
MOLECULAR WEIGHT X 10^{-3}

Figure 8

As described earlier, characteristic mitochondrial membrane components were not detected in purified granule membrane preparations. Conversely, Figures 2 and 8 demonstrate the absence of granule membrane components in mitochondrial membranes and emphasize the difference in complexity of the two membranes.

A dozen polypeptide bands appeared common to mitochondrial and microsomal membranes, but the relative amounts of these bands differed, and each membrane contained unique bands. Both contained a major component with a mobility identical to band 5. The staining of the lipid region of these two membranes was consistently lower than granule membrane for comparable amounts of protein, indicating that the granule membrane may have a relatively low protein to lipid ratio.

Carbohydrate-Containing Components of Pancreatic Intracellular Membranes

Electropherograms of ZGM-3 stained for carbohydrate by the periodic acid-Schiff (PAS) procedure reproducibly gave the absorbance profile illustrated in the lower frame of Figure 9. One band barely penetrated the gel surface and was not evident in the scan. The intense staining immediately behind the tracking dye has been attributed to lipid (Fairbanks et al., 1970; Lenard, 1970a). When a mixture of glycolipids was subjected to electrophoresis and the gel stained with PAS, the only region with stain was just behind the dye marker. The two major species at 74,000 and 52,000 molecular weight correspond to bands 2 and 5 of the CbR profile (upper frame, Figure 9). Thus,

Figure 9. Glycoprotein nature of zymogen granule membrane components.

The periodic acid-Schiff (PAS) and sialic acid profiles of zymogen granule membrane (lower frame) are compared to the CbR profile (upper frame) of a second polyaerylamide gel run simultaneously. Techniques are described in Methods. Upper frame: 50 µg of ZGM-3 on a 5 mm diameter polyacrylamide gel. Lower frame: PAS (A_{560}), 50 µg of ZGM-3 on a 5mm gel; sialic acid profile, approximately 200 µg of ZGM-3 on a 6 mm gel.

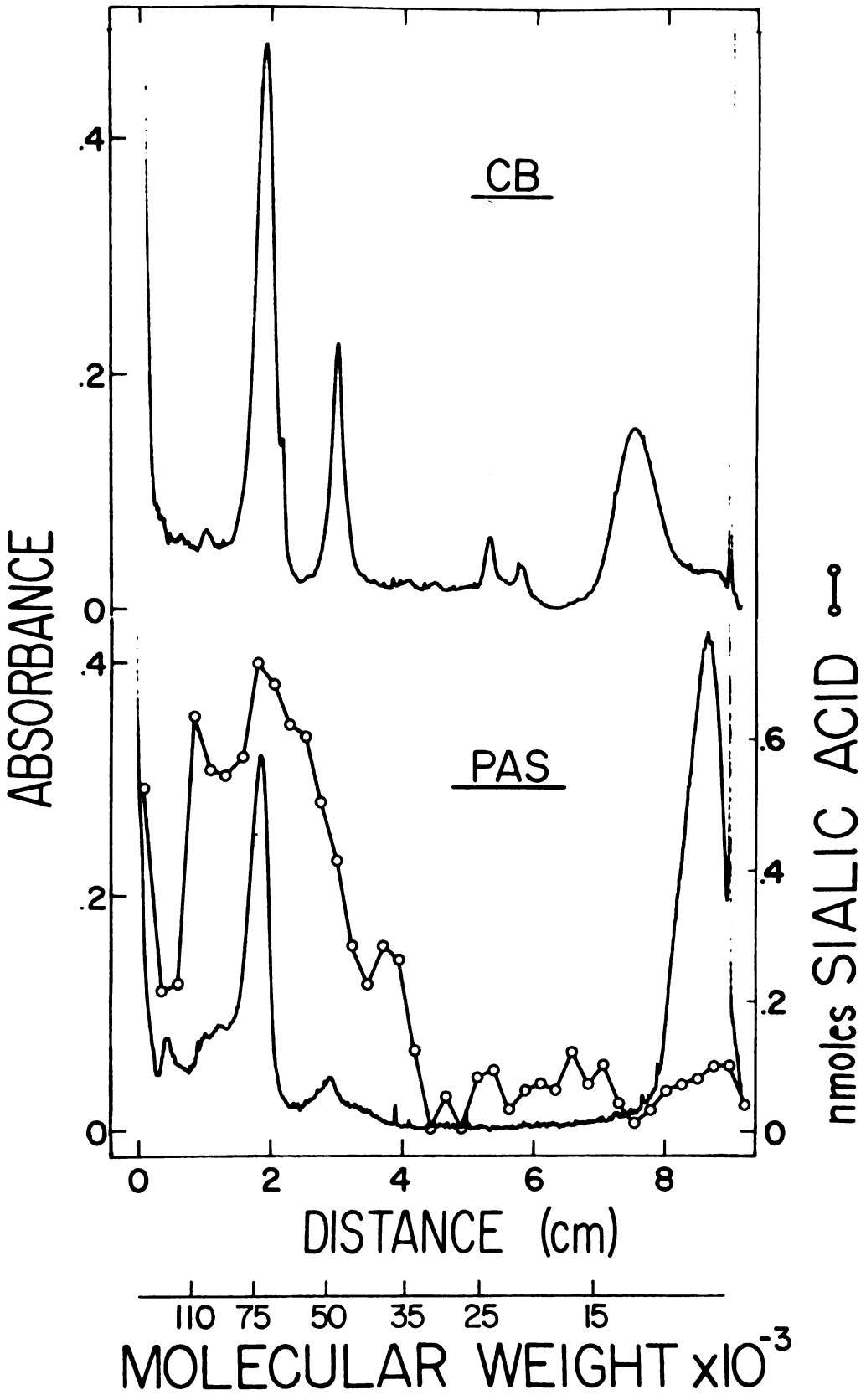

CB
PAS
ABSORBANCE
.4
.2
0
.4
.2
0
.6
.4
.2
0
nmoles SIALIC ACID
0
2
4
6
8
DISTANCE (cm)
110 75 50 35 25 15
MOLECULAR WEIGHT x10-3

Figure 9

the major polypeptide species of the granule membrane appear to be extensively glycosylated.

The sialic acid content of ZGM-3 was found to be approximately 130 nmoles per milligram protein. The distribution of sialic acid components separated by electrophoresis is shown in the lower frame of Figure 9. The recovery of sialic acid from sectioned gels was variable, with an average of 30%. The diffuse nature of the high molecular weight zone containing 70% of the membrane sialic acid may be due to overloading the gel. Nevertheless, the peak fractions of sialic acid were coincident with components 1 and 2.

Membranes were prepared from rough and smooth microsomes isolated by isopycnic sucrose gradient centrifugation. Only minor differences, most notable in the molecular weight region 35,000 to 70,000, were observed in the distribution of CbR stained polypeptides (Figure 10). These differences were not due to contribution of ribosomal proteins, since a preparation of ribosomes analyzed separately did not contain predominate bands in this region and since the majority of ribosomes are lost from rough microsomes during $NaHCO_3$ and NaBr extractions.

In contrast, the two membranes contained markedly different molecular weight classes of carbohydrate-containing components. Aside from the intense staining of the lipid region, smooth microsomal membrane preparations consistently demonstrated a major peak of PAS stain at a polypeptide equivalent molecular weight of 75,000 (Figure 10). Rough microsomes contained a heterogeneous distribution of ten PAS-positive bands; the presence of several bands in the low

Figure 10. Comparison of the polypeptides and glycopolypeptides of mitochondrial and rough and smooth microsomal membranes.

Details of membrane isolation and electrophoresis on 6 mm diameter polyacrylamide gels under standard conditions are given in Methods. Samples in each frame are A) smooth microsomal membrane, B) rough microsomal membrane, and C) mitochondrial membrane. Legend: Dotted lines outline CbR profiles (A_{550}); samples contained 50 µg protein. Solid lines outline PAS profiles (A_{560}); samples contained 150 µg protein.

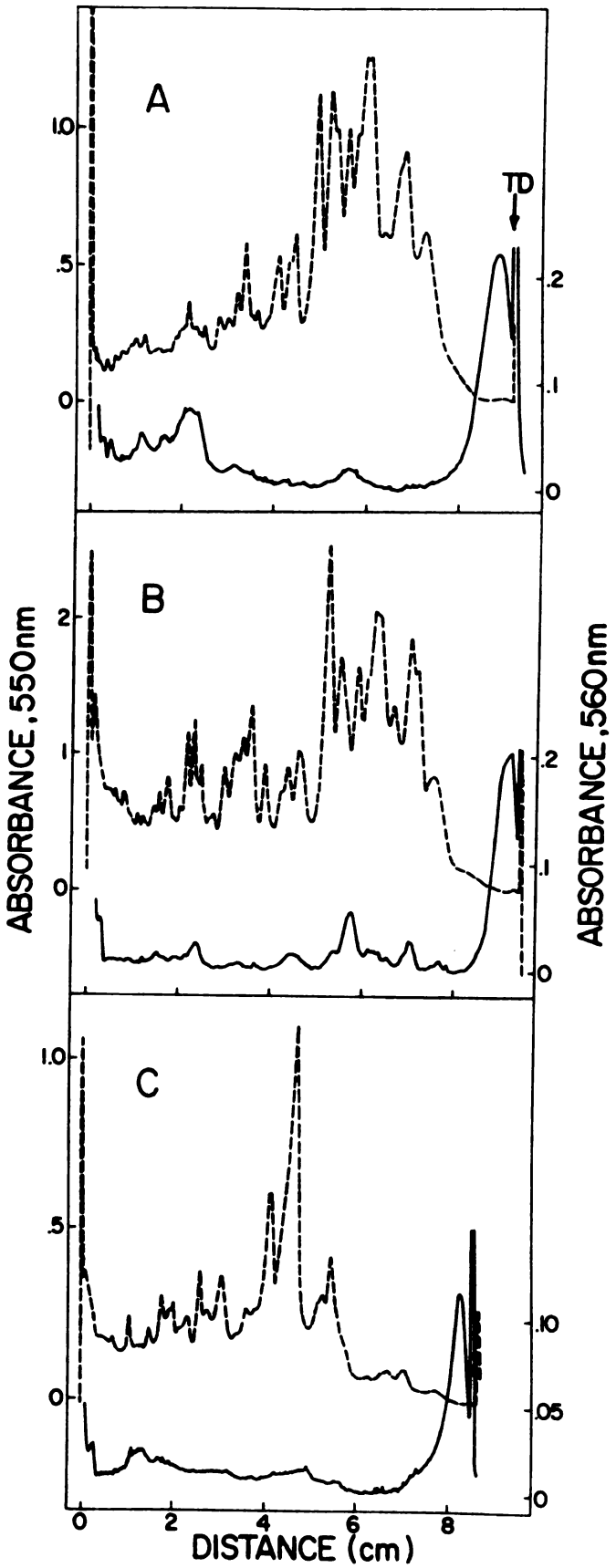

A
B
C
TD
ABSORBANCE, 550nm
ABSORBANCE, 560nm
DISTANCE (cm)
0
2
4
6
8

Figure 10

molecular weight region was distinctive. Mitochondrial membranes stained with PAS gave a pattern characterized by a major component with a relative mobility significantly slower than the major zymogen granule membrane glycopolypeptide.

The postmicrosomal supernatant and the granule contents, two major soluble protein fractions, exhibited two minor glycopolypeptides (Figure 11). Both fractions contained a PAS-positive component which barely penetrated the gel surface, in addition to components at approximately 68,000 (postmicrosomal supernatant) and 54,000 molecular weight (granule contents). Under the conditions employed, 5 μg of ovalbumin, equivalent to 0.17 μg of carbohydrate, could be readily detected. Therefore, glycoproteins which contained 5% carbohydrate and represented 2% of the total protein should have been detectable. The paucity of carbohydrate-containing polypeptides in the soluble cellular fractions indicate that glycopolypeptides are selectively associated with membranes, principally the zymogen granule membrane.

Interspecies Comparison of Zymogen Granule Membrane Components

The granule membrane polypeptide compositions of five mammals other than rat were investigated to determine the extent the major characteristics of the rat granule membrane profile are conserved in other systems. The results of the survey which included membrane from bovine, canine, porcine, and leporine pancreas are illustrated in Figure 12. The major polypeptide species of each profile was similar to band 2 of the rat granule membrane. By mixing rat granule

Figure 11. Electrophoretic analysis of the glycopolypeptide distribution in the post-microsomal supernatant and zymogen granule contents.

The upper scan of each pair represents the absorbance profile of CbR stained polypeptides at 550 nm. The lower scan of each pair represents the absorbance profile of a second polyacrylamide gel with PAS stained glycopolypeptides. A) Post-microsomal supernatant, obtained as the soluble fraction after the series of differential centrifugation steps described in Methods; upper scan contained 50 µg protein, lower scan, 150 µg. B) Zymogen granule contents; upper and lower scans contained 43 µg protein.

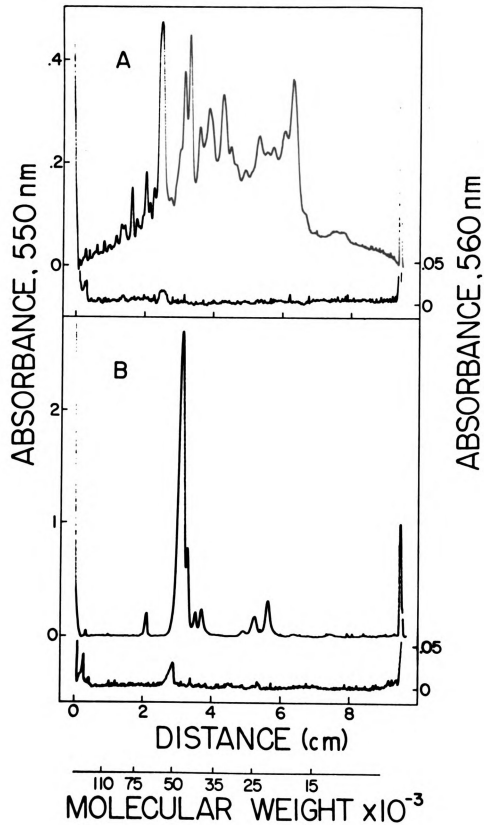

A
B
.4
.2
0
2
1
0
.05
0
ABSORBANCE, 550 nm
ABSORBANCE, 560 nm
0
2
4
6
8
DISTANCE (cm)
110 75 50 35 25 15
MOLECULAR WEIGHT x10⁻³

Figure 11

Figure 12. Comparison of zymogen granule membrane polypeptides and glycopolypeptides from different mammalian species.

Gels marked CB were stained with Coomassie blue R; those marked PAS were stained by the periodic acid-Schiff procedure. 40 μg of granule membrane protein from the following animals were analyzed: a) rat, b) beef, c) dog, d) pig, and e) rabbit. The arrows indicate band 2. The ordinate indicates approximate molecular weights ($X10^{-3}$).

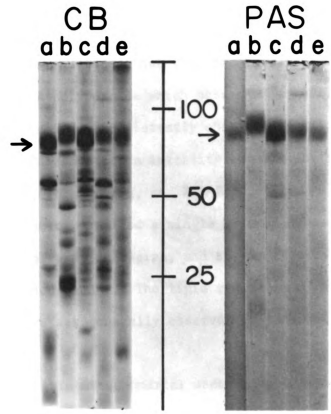


Figure 12

membrane with granule membrane from each of the other species prior to electrophoresis, it was established that rat, dog, pig, and rabbit band 2 had identical mobilities. Bovine band 2 had a slightly slower mobility equivalent to an apparent molecular weight of 85,000. In addition, the beef profile, unlike those of the other species, contained an intensely stained pair of bands near 23,000. Although purification of sheep granule membrane was difficult and yields were low, preliminary results indicated that this membrane resembles beef rather than rat. A polypeptide which possessed a mobility very similar to that of rat band 5 was consistently observed in all species. The relative amount of band 2 stain intensity was 37% for rat, 20% for beef, 62% for dog, 18% for pig, and 24% for rabbit.

In all species examined a single major PAS-positive band was detected in the polypeptide region, and it was invariably associated with the CbR stained band 2. The lipid region immediately adjacent to the tracking dye was generally observed by both CbR and PAS staining.

The pancreatic mitochondrial membrane polypeptide distribution was distinct from the microsomal membrane polypeptide distribution within each species, but each membrane was similar across species. The profiles consistently contained a complex array of polypeptides which contrasted with the relatively simple distribution of granule membrane polypeptides. The band 2 region always accounted for less than 5% of the CbR stain intensity of either microsomal or mitochondrial membranes. Conversely, the lower molecular weight polypeptides of the more complex membranes were absent from the granule

membrane profiles. Mitochondrial contamination, measured by cytochrome c oxidase activity, of dog and pig granule membrane preparations was found to be negligible.

Isolation of the Major Zymogen Granule Membrane Glycopolypeptide (Component 2)

Calculations on the Surface Distribution of Rat Granule Component 2

The observation that component 2 represented the major protein portion of zymogen granule membranes of all species examined led to further investigations of the nature of this component. Neuraminidase treatment of glutaraldehyde-fixed rat zymogen granules released much of the bound sialic acid (R. Hsieh and R. A. Ronzio, unpublished observation). Since component 2 accounts for most of the membrane sialic acid, the data imply that component 2 is present on the cytoplasmic face of the granule membrane, or that it extends through to the surface. Assuming that component 2 is roughly a globular protein positioned on the cytoplasmic face, its distribution and influence on the surface properties of the granule can be calculated. Table 6 lists the results of these calculations beginning with an estimation of the amount of component 2 per granule. All calculations are subject to significant uncertainty due to the likelihood of proteolysis during granule membrane isolation. Thus, if the stain intensity of band 19 (cf. Figure 1D and Table 4) represents degradation of the major component, the calculated percent of granule protein as this component may be low by a factor of two. Regardless of the degradation which may occur, the estimated number of component 2 molecules per

Table 6. Calculations of the component 2 content of rat zymogen granule membrane.

The number of rat zymogen granules in a suspension of known protein concentration was estimated with the aid of a Petroff-Hausser bacteria counter (A. H. Thomas Co.). The yield of zymogen granule membrane protein was 0.55% of the total granule (cf. Table 2), but due to losses during isolation represented approximately 0.7%. Assuming equivalent staining of all membrane protein species, component 2 was estimated to be 38% of the rat granule membrane (cf. Table 3). The number of component 2 molecules per granule was then calculated from the amount per granule (4.6×10^{-15} gm) and its estimated molecular weight of 74,000 (cf. Table 3).

The average diameter of a zymogen granule was estimated from a negative of an electron micrograph of known magnification containing a field of isolated, glutaraldehyde-fixed zymogen granules sprayed onto a carbon coated grid with an atomizer (R. A. Ronzio, unpublished data). The negative was sandwiched between two glass plates and displayed on a white screen with the aid of a lantern slide projector. The observed diameters of 55 individual granules were averaged and the actual diameter was calculated from the magnifications of each manipulation.

The surface area covered by a component 2 molecule was estimated from the Stokes radius for a globular protein of 74,000 molecular weight (approximately 35 Å).

Total protein per zymogen granule	1.7×10^{-12} gm
% granule protein as component 2	0.30%
Total number of component 2 molecules per zymogen granule	38,000
Average zymogen granule diameter	1.2 μ
Surface area of a spherical zymogen granule 1.2 μ in diameter	4.6 μ^2
Membrane density of component 2	8,000/μ^2
Membrane area available per component 2	12,000 Å^2
Maximum area covered by a single globular component 2	3,000-4,000 Å^2
Maximum % total surface coverable by a globular component 2	25-33%

granule (3.8×10^4) and their contribution to the total surface area were low. On the basis of similar calculations, there were 1.1×10^7 amylase molecules per granule.

Isolation of Dog Component 2

Since SDS-polyacrylamide gel electrophoresis had been demonstrated to effectively separate the granule membrane components and the procedure was straightforward, the technique was applied to the isolation of the major granule membrane component. Several observations indicated dog pancreas as the optimal source of component 2. Dog pancreas was available in large quantities, pure zymogen granule preparations were easily isolated in high yield, and component 2 accounted for approximately 60% of the granule membrane protein. Figure 13 illustrates a reconstructed polyacrylamide slab gel used for preparing dog component 2. The glycopolypeptide within the gel slices was eluted into dialysis bags as described in Methods, and dialyzed against two changes of 2.5 liters of distilled water containing 3 gm of washed Dowex AG-1X anion exchange resin, and 2 ml of chloroform or toluene. After 12 hours of dialysis the presence of small particulate material was observed; the quantity increased with prolonged dialysis. When viewed under a microscope, the particles appeared birefringent and crystalline. Dialysis was usually stopped after 18 hours to avoid bacterial growth. The particulate material was collected by centrifugation at 7000xg for 15 minutes, and washed once by resuspending in distilled water and centrifuging. The soluble fraction was lyophilized. The protein recovered in the

Figure 13. Preparative polyacrylamide gradient slab gel separation of dog zymogen granule membrane components.

Preparation of the gel and electrophoresis in 0.1% SDS are described in Methods. 6.3 mg of dog granule membrane protein in 3.0 ml was applied in the trough at the top. Electrophoresis was for 18 hours. The three slices marked A, B and C were quick-stained with CbG to locate the protein bands. After removal of the gel sections containing component 2 (marked C2), the remaining gel pieces were stained with CbG, then re-assembled for the photograph. The intensely stained region near the bottom represents lipid.

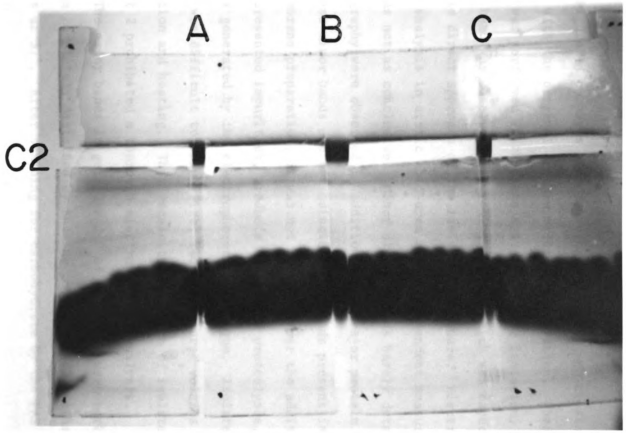


Figure 13

combined fractions ranged from 28-44% of the protein applied for five preparations. The crystalline material accounted for between 15 and 35% of the isolated protein measured according to Lowry et al. (1951).

Purity

Figure 14 compares electropherograms of the non-crystalline material with the original granule membrane preparation. When electrophoresis was performed in the same system used for isolation, a single discrete band was observed (Figure 14, gel B), which was expected from the simple direct approach of the isolation procedure. Electrophoretic analysis in acetic acid-urea as an independent measure of purity was not as conclusive. Minor diffuse bands barely detectable by photography were observed in addition to the major protein band. Since these minor bands did not coincide with bands present in the whole membrane preparation, it was not clear whether the additional bands represented impurities, products of limited proteolysis, or artifacts generated by the electrophoretic technique. Isolated component 2 was difficult to solubilize, requiring large volumes of 1% SDS solution and heating. This recalcitrant nature of isolated component 2 prohibited a thorough electrophoretic analysis.

The major bands in gels C and D of Figure 14 were marked with ink, then completely destained by shaking in 10% TCA-33% methanol for nine days at 37°. After staining for carbohydrate by the PAS procedure, the only stain observed was located precisely at the ink mark, i.e. C2, on both gels. These results indicate that the carbohydrate moiety remains associated with the major Coomassie blue

Figure 14. Purity of isolated dog granule membrane component 2.

A) Center strip of a preparative slab gel stained with CbG illustrating the separation and distribution of dog zymogen granule membrane components.

B) After excising and electroeluting the region of gel containing component 2 for the preparation shown in A, the sample was electrophoresed on a second preparative slab gel. B shows the CbG stained center slice of the gel containing isolated component 2. The duration of electrophoresis for the second slab gel was much shorter, thus the mobilities of components in the two gels are not directly comparable.

C) Separation of dog zymogen granule membrane (80 μg) components by acetic acid-urea gel electrophoresis (cf. gel system II, Table 1), stained with CbR.

D) Acetic acid-urea gel electrophoresis of isolated component 2 (96 μg protein). Compare with whole membrane in gel C.

E) Acetic acid-urea gel electrophoresis of proteins of known molecular weight, from top to bottom: β-galactosidase, phosphorylase a, bovine serum albumin, alcohol dehydrogenase and lysozyme.

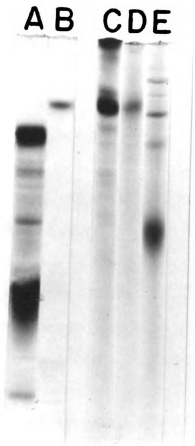


Figure 14

stained band during purification and under distinctly different dissociating and electrophoresis conditions.

The acetic acid-urea system was employed to estimate the molecular weight of isolated component 2. In Figure 15 the mobility of several proteins relative to lysozyme is plotted against the logarithm of their molecular weight. Although not precisely linear, the curve could readily be used to estimate the molecular weight of proteins between 94,000 and 14,000. By this method the middle of the component 2 band (Figure 14, gel D) corresponded to a molecular weight of 72,000 (Figure 15), very similar to the estimate obtained in 1% SDS.

The crystalline material obtained during dialysis contained a high proportion of bound SDS, measured according to Reynolds and Tanford (1970). The mean SDS content of two preparations was 8.7 mg/mg protein. Electrophoresis of 150 μg of the particulate protein yielded only faint staining limited to two diffuse bands with higher mobilities than component 2. Less protein could not be visualized. In addition, the two bands did not stain by the PAS procedure. By these criteria the material was not identical to component 2.

Zymogen Granule Mg^{2+}-dependent Adenosine Triphosphatase (Mg^{2+}-ATPase)

Mg^{2+}-dependent adenosine triphosphatase activity is distributed throughout the subcellular fractions of rat (Ronzio, 1973b and unpublished observations) and guinea pig (Meldolesi et al., 1971c) pancreas. Although the zymogen granule accounted for only 1 percent of the total homogenate activity, the specific activity of the granule membrane was extremely high (Ronzio, 1973b; Meldolesi et al., 1971c).

Figure 15. Estimation of the molecular weight of isolated dog granule membrane component 2 by acetic acid-urea polyacrylamide gel electrophoresis.

Protein standards and purified dog component 2 were dissolved in phenol-acetic acid-urea-water-2-mercaptoethanol and subjected to electrophoresis at 13 volts/cm in acetic acid-urea as described in Methods. Mobilities are expressed relative to lysozyme. The standards are a) β-galactosidase, b) phosphorylase a, c) bovine serum albumin, d) alcohol dehydrogenase and e) lysozyme. The relative mobility of component 2 is noted as C2.

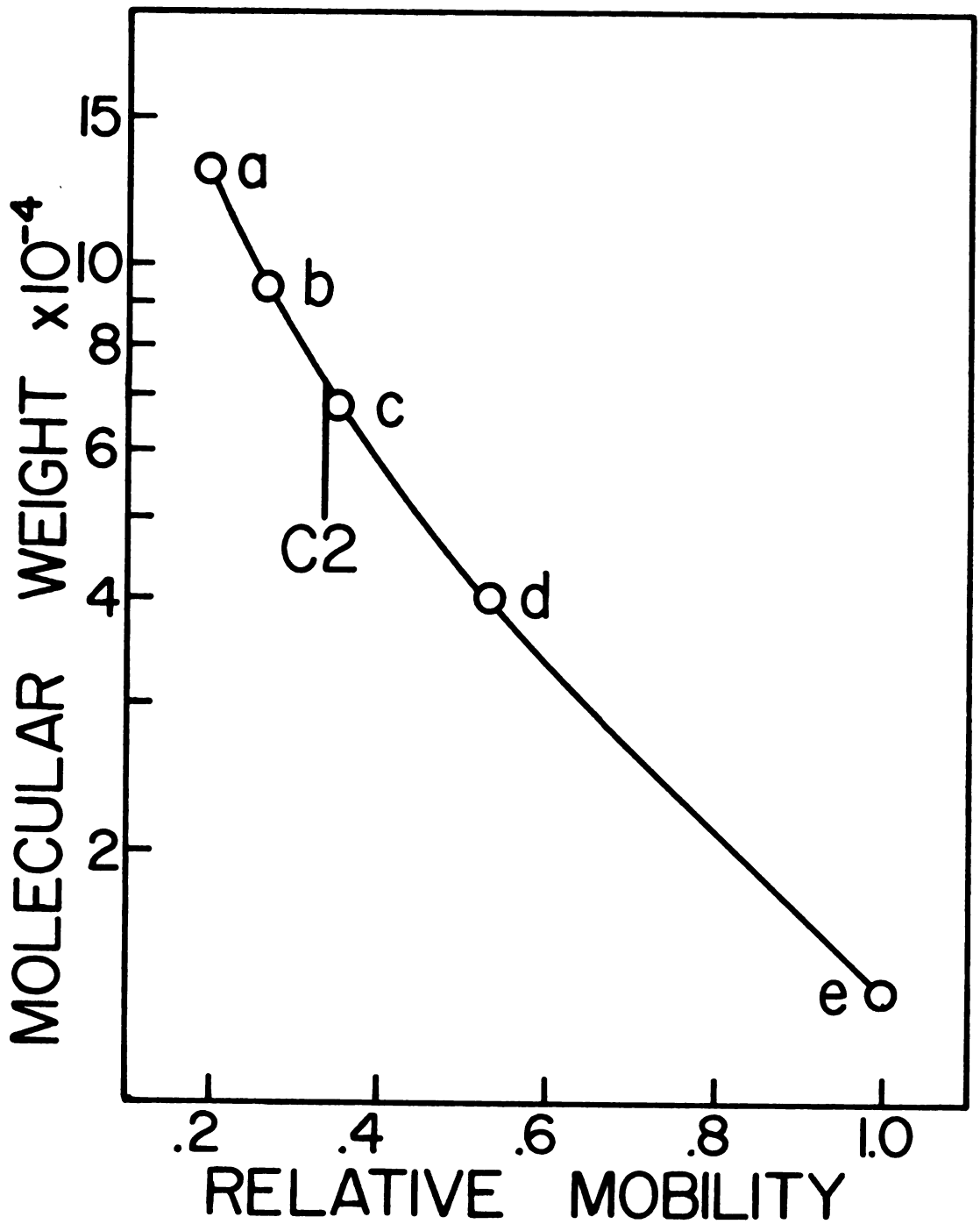

MOLECULAR WEIGHT x10⁻⁴
15
10
8
6
4
2
a
b
c
C2
d
e
.2
.4
.6
.8
1.0
RELATIVE MOBILITY

Figure 15

The Mg^{2+}-ATPase is Tightly Bound to the Granule Membrane

The extent of association of the granule activity with the membrane was demonstrated by sedimentation of a lysed granule preparation through a 0.2 M to 1.2 M sucrose gradient. Large cellular debris and nuclei were removed from a pancreas homogenate by a brief centrifugation at 600xg. A granule fraction rich in mitochondria was then collected by centrifuging the supernatant at 1600xg for 30 minutes. The brown mitochondrial layer was removed from the pellet and the zymogen granules were lysed by resuspension in 0.17 M NaCl containing 0.05 M Tris-Cl, pH 8.2. The lysate was layered above a 0.2 M to 1.2 M sucrose gradient containing a 1 ml cushion of 1.75 M sucrose. Intact rat zymogen granules and mitochondria band at densities of approximately 1.219 and 1.215 (about 1.67 M sucrose), respectively (R. A. Ronzio, unpublished observations). Figure 16 illustrates the results of sucrose gradient centrifugation of the lysed granules. Mitochondria monitored by cytochrome c oxidase activity were found at the 1.2 M- 1.75 M sucrose interface (arrow, Figure 16). The low level of amylase activity detected at the interface indicates that lysis was essentially complete.

Two peaks of Mg^{2+}-ATPase activity, containing more than 75% of the total, were coincident with two opaque granule membrane bands observed near the middle of the gradient. The separation of granule membrane into two peaks appears to be caused by adsorption of secretory proteins and mitochondria to a fraction of the membrane population, resulting in an increased equilibrium density of that fraction of membrane.

Figure 16. Association of Mg^{2+}-ATPase activity with the zymogen granule membrane.

0.7 ml containing 0.8 mg of protein of a lysed granule suspension was layered over a 9.7 ml sucrose gradient (0.2 M to 1.2 M sucrose) on a 1 ml 1.75 M sucrose cushion. After centrifugation for three hours at 192,000xg (SW 41 rotor, Beckman) and 0°, 0.33 ml fractions were collected and assayed for amylase (—o—), cytochrome c oxidase (—□—), and Mg^{2+}-ATPase (assay A) (—Δ—) as described in Methods.

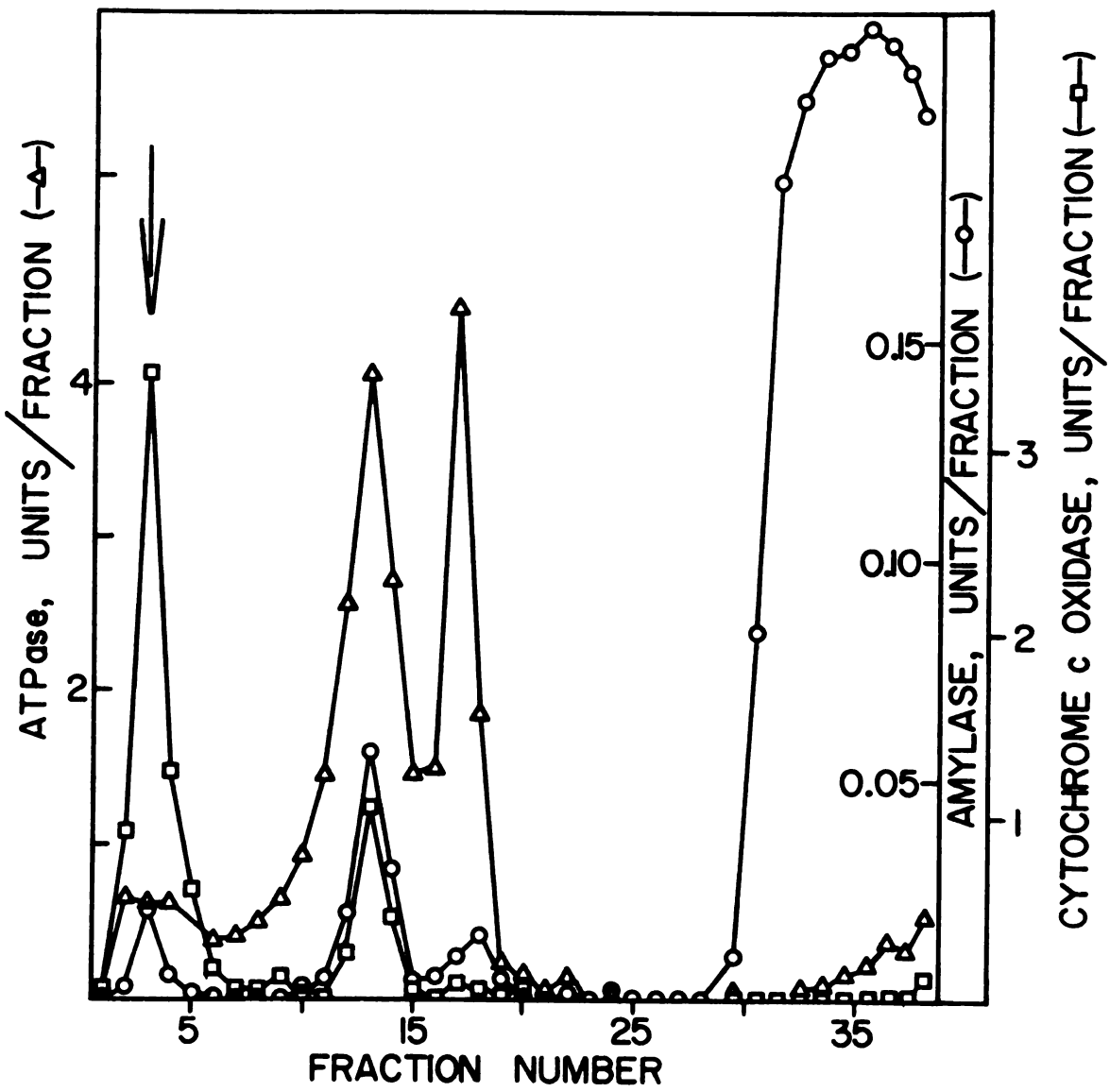

ATPase, UNITS/FRACTION (—△—)
AMYLASE, UNITS/FRACTION (—○—)
CYTOCHROME c OXIDASE, UNITS/FRACTION (—□—)
FRACTION NUMBER
5
15
25
35
2
4
0.05
0.10
0.15
1
2
3

Figure 16

The low level of Mg^{2+}-ATPase in the main cytochrome c oxidase peak at the sucrose interface (arrow, Figure 16) indicates the low specific activity of mitochondrial ATPase. Only a very small fraction of the Mg^{2+}-ATPase was soluble, indicated by the low level of activity at the top of the gradient.

Table 7 summarizes the purification of Mg^{2+}-ATPase activity during granule membrane isolation. Activity was measured by (γ^{32}P)ATP hydrolysis (assay B); essentially identical results were obtained by analysis of orthophosphate release from ATP (assay A). The average specific activity of three preparations was 77 µmoles/hour/mg ZGM-3 protein. The contribution of mitochondrial ATPase, removed at the discontinuous gradient step, was 1-2% of the total.

The data contained in the lower section of Table 7 demonstrate that less than 3% of the original (γ^{32}P)ATPase activity was extracted with 0.2 M $NaHCO_3$ and 0.25 M NaBr (containing 2 mM $MgCl_2$ and 1 mM ATP). In the absence of Mg^{2+} and ATP in the NaBr extraction, 29% of the enzyme activity was solubilized from ZGM-2 (Table 8).

Further studies of the Mg^{2+}-ATPase reaction were conducted with membrane prepared with 2 mM $MgCl_2$ and 1 mM ATP in the NaBr wash. Electropherograms of membranes prepared by the two NaBr extractions contained no differences which could be related to the selective loss of one or more components.

Partial Characterization of the Granule Mg^{2+}-ATPase Activity

Table 9 lists effects of cations on granule membrane Mg^{2+}-ATPase. The presence of a divalent cation was shown to be

Table 7. Purification of zymogen granule nucleoside triphosphatase.

The preparation of each granule subfraction is described in Methods. Bicarbonate lysis of the granule suspension resulted in a 1.2 to 1.5 fold stimulation of the Mg^{2+}-ATPase activity. Thus the data presented for fold-purification and % activity recovered are relative to the activated enzyme.

Fraction	Protein	$(\gamma^{32}P)$ATP hydrolysis		
	% Total	Specific Activity[a]	Fold-Purification	% Total Activity
Zymogen granules lysed in $NaHCO_3$	(100)	0.84	--	(100)
Crude membrane fraction (ZGM-1)	2.5	15	18	45
Membranes from discontinuous gradient (ZGM-2)	0.85	38	45	38
Membranes after NaBr extraction (ZGM-3)[b]	0.55	48	57	33
Lysate supernatant	98	0.02	--	1
Pellet from discontinuous gradient (mitochondria)	0.25	3.6	--	1.1
NaBr extract[b]	0.20	10	--	1.9

[a] units/mg protein.

[b] NaBr (0.25 M) solution contained 2 mM $MgCl_2$ and 1 mM ATP.

Table 8. NaBr extraction of the granule membrane Mg^{2+}-ATPase.

Treatment	Activity-Units		
	ZGM-2	ZGM-3	Soluble
0.25 M NaBr	36.0	24.6(71%)	9.8(29%)
0.25 M NaBr plus 2 mM Mg^{2+} and 1 mM ATP	36.0	28.4(94%)	0.9(6%)

Number in parentheses indicate percent of total recoverable activity.

Table 9. Effects of cations on the granule Mg^{2+}-ATPase.

Modifications	% Activity
none[a]	(100)
minus $MgCl_2$	5
plus 5 mM EDTA, minus $MgCl_2$	1
plus 3.4 mM $MnCl_2$	73
plus 3.4 mM $MnCl_2$, minus $MgCl_2$	73
plus 3.4 mM $CaCl_2$	112
plus 3.4 mM $CaCl_2$, minus $MgCl_2$	118
plus 5.0 mM $CaCl_2$, minus $MgCl_2$	121
plus 10 mM $CaCl_2$, minus $MgCl_2$	102
plus 0.142 M NaCl and 0.02 M KCl	99
plus 10^{-4}M oubain	113

[a]The unmodified assay mixture contained 3.4 mM $MgCl_2$ and 3.4 mM disodium ATP.

essential for activity. Manganous ion can partially replace magnesium ion, but in the presence of magnesium ion, manganous ion was slightly inhibitory. Calcium ion, whether in the presence or absence of magnesium ion, activated. Concentrations of calcium ion above 5 mM were less effective. Zymogen granule adenosine triphosphatase activity was neither stimulated by Na^+ and K^+ nor inhibited by oubain.

Zymogen granule membranes were stored at -20°C or as a suspension in 20 mM imidazole-Cl, pH 7.1, at 4°C for at least two weeks with minimal loss of Mg^{2+}-ATPase activity. An 18% loss of activity was observed after a 10-minute incubation in 20 mM imidazole-Cl, pH 7.1, at 37°C prior to the assay. Therefore the enzyme may be slightly unstable under assay conditions. Ninety-six percent of the activity was lost during incubation at 50°C for 10 minutes. The activity had a broad pH optimum with peak activity near pH 8 (Figure 17).

To assess the progress of ATP hydrolysis catalyzed by zymogen granule membrane enzymes, assay mixtures containing (8-^{14}C)ATP were terminated at various time intervals and analyzed by paper chromatography. As shown in Figure 18, (^{14}C)ADP did not accumulate appreciably during the incubation; the majority of the radioactivity in reaction products appeared as (^{14}C)AMP. Hence it appeared likely that ADP formed by the hydrolysis of ATP was subsequently hydrolyzed to AMP. The stability of AMP in the assay, as judged by the lack of adenosine formation, indicated that little 5'-nucleotidase was present.

Figure 17. The effect of pH on the rate of ATP hydrolysis catalyzed by the zymogen granule membrane Mg^{2+}-ATPase.

Orthophosphate release was measured by assay A as described in Methods. The assays contained either 0.05 M imidazole-Cl (—o—) or 0.05 M Tris-Cl (—□—) buffer.

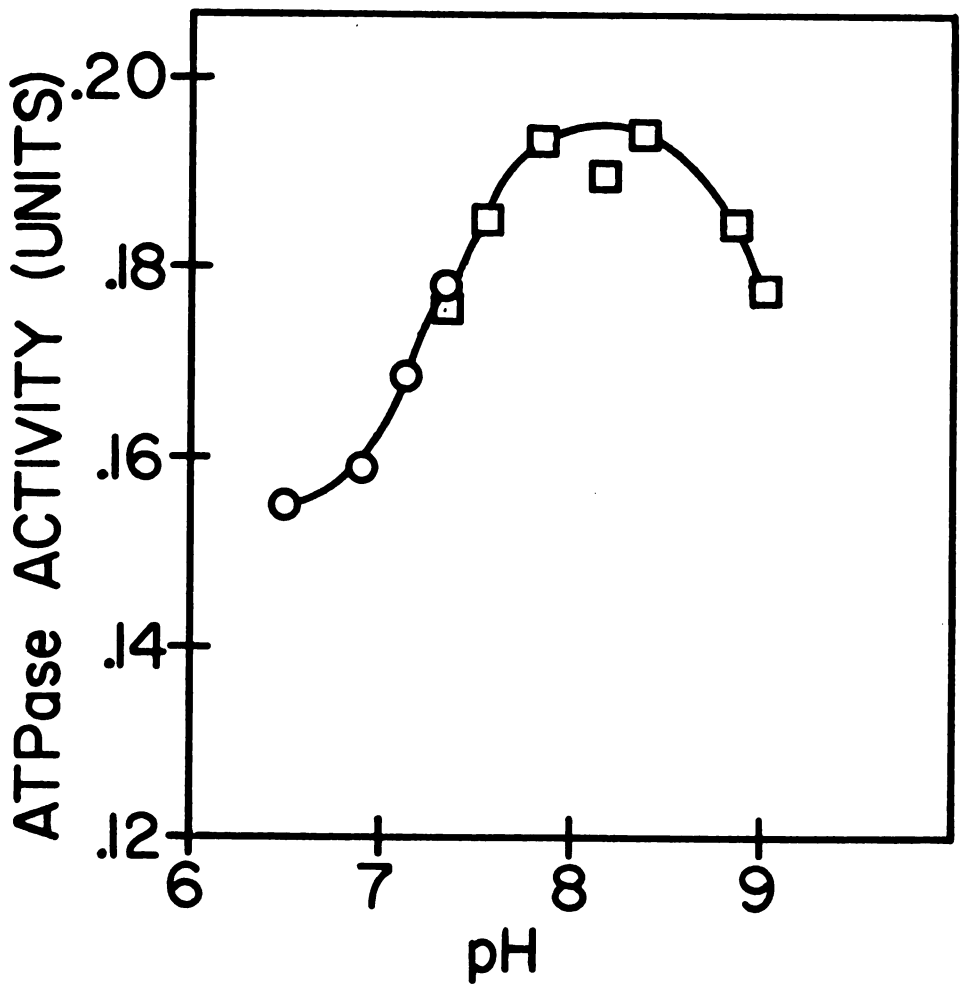


Figure 17

Figure 18. Chromatographic analysis of the products of ATP hydrolysis catalyzed by enzymes associated with the zymogen granule membrane.

The standard reaction mixture contained 3.3 mM ATP, 140,000 cpm (8-^{14}C)ATP, and 0.16 units of ZGM-3 Mg^{2+}-ATPase activity. Each assay was initiated by removing the reaction mixture from an ice bath and placing in a water bath at 37°. Assays were terminated by adding one-fifth volume of 10% TCA at A) 0, B) 10, and C) 30 minutes. Carrier ADP, AMP and adenosine and assay mixtures were then spotted on Whatman No. 1 filter paper. The chromatograms were developed by descending chromatography with isobutyric acid-H_2O-concentrated NH_4OH (66:33:1, v/v/v). Radioactivity was monitored with a Packard Radiochromatogram Scanner model 7201.

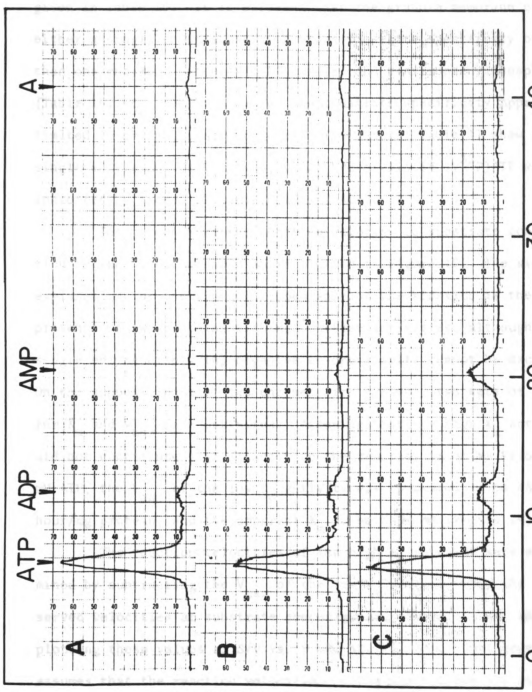

ATP
ADP
AMP
A
A
B
C
0
10
20
30
40
DISTANCE (cm)
RELATIVE RADIOACTIVITY

Figure 18

The relative rates of hydrolysis of several nucleotides are given in Table 10. It is apparent that the granule membrane contains either a single phosphatase with broad substrate specificity or more than one enzyme. Since AMP (Figure 18) and p-nitrophenylphosphate (Table 10) were not hydrolyzed, the substrate specificity appears limited to pyrophosphate linkages. The data of Table 11 show that a single enzyme may be involved, since hydrolysis of (γ^{32}P)ATP was inhibited by excess UTP, GTP, CTP and ADP.

The response of the reaction velocity to substrate concentration between 0.05 mM and 6 mM is plotted in Figure 19. The simplest explanation for the biphasic appearance of the kinetics is the presence of two proteins with Mg^{2+}-ATPase activities, although a single enzyme displaying negative co-operativity cannot be excluded. On the supposition that two enzymes were present, two sets of values for K_m and V_{max} were estimated. Assuming that the high K_m activity did not contribute significantly to the reaction velocity below 0.05 mM ATP, the V_{max} of the low K_m activity was estimated to be 48 μmoles/hour/mg protein, and the apparent K_m to be 0.04 mM (Figure 19).

The reaction velocities of the high K_m activity were determined by subtracting the V_{max} of the low K_m activity from the observed velocities at substrate concentrations above 1.5 mM, and by plotting these values as S/v vs. S and 1/v vs. 1/S. This procedure assumes that the reaction velocities of the two enzymes are additive, and at ATP concentrations above 1.5 mM an increase in the ATP concentration does not significantly increase the velocity of the low K_m activity. By this method the K_m of the high K_m activity was estimated to be between 2 and 6 mM.

Table 10. Relative rates of hydrolysis of various nucleotides by zymogen granule membrane-bound activities.

Orthophosphate release from nucleotide substrates was measured by assay A as described in Methods. p-Nitrophenylphosphate hydrolysis was monitored spectrophotometrically at 420mμ according to Robinson (1969).

Substrate	% Relative Activity
3.3 mM ATP	(100)
3.1 mM ADP	103
3.1 mM GTP	115
3.3 mM UTP	163
3.2 mM CTP	105
2 mM p-nitrophenylphosphate	2

Table 11. Nucleotide inhibition of (γ^{32}P)ATP hydrolysis.

^{32}P-orthophosphate release was measured by assay B as described in Methods. (γ^{32}P)ATP was used at 0.6 mM to permit the addition of a ten-fold excess of competing nucleotide. The addition of 6 mM nucleotides shifts the substrate concentration from well below saturation to near the level for maximum velocity. The effect of the shift was to lessen the apparent inhibition. $MgCl_2$ was included in the assay at concentrations equivalent to the nucleotides.

Additions	$^{32}PO_4$ released (cpm)	% of the activity in the absence of added nucleotides
none (0.6 mM ATP)	38,750	(100)
6 mM ATP	6,340	16.4
6 mM UTP	18,270	47.2
6 mM GTP	11,650	30.2
6 mM CTP	16,540	42.8
6 mM ADP	10,000	25.9

Figure 19. Kinetic data for zymogen granule Mg^{2+}-ATPase. ($\gamma^{32}P$)ATP hydrolysis was measured at substrate concentrations between 0.05 and 6 mM. Assays contained 1.25 µg ZGM-3 protein. Values for points on the graph were calculated from observed velocities (expressed as nmoles/minute) at the various substrate concentrations. A) v vs. S. B) 1/v vs. 1/S.

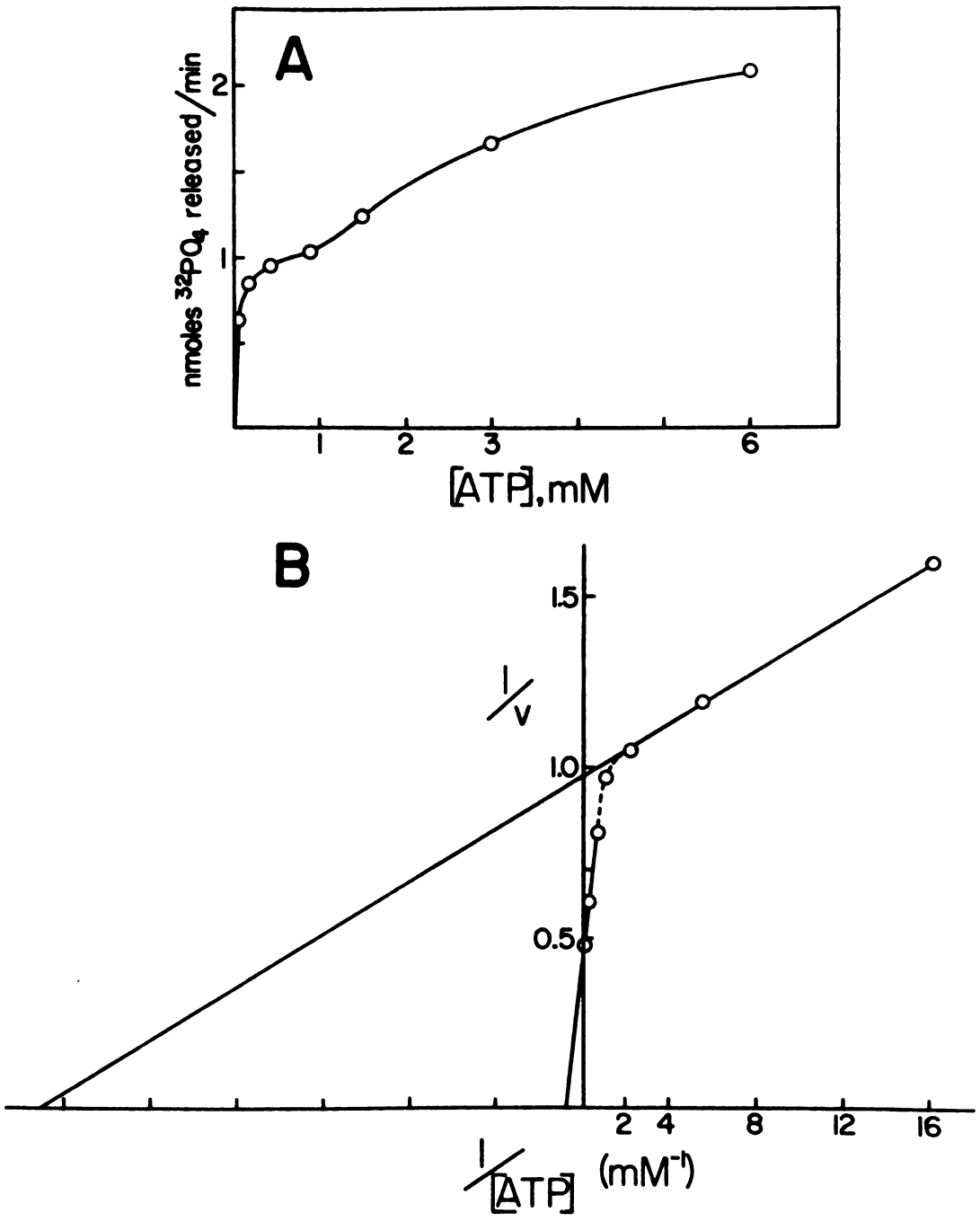

A
nmoles $^{32}PO_4$ released/min
2
1
1
2
3
6
[ATP], mM
B
1.5
1/V
1.0
0.5
2
4
8
12
16
1/[ATP] (mM^{-1})

Figure 19

Phosphorylation of Zymogen Granule Membranes

Incorporation of $^{32}PO_4$ Into a Single Granule Membrane Polypeptide

The occurrence of a second ATP requiring enzyme activity in purified zymogen granule membrane was investigated. Protein kinase activities have been shown to phosphorylate an endogeneous protein acceptor in secretory granules from the anterior pituitary (Labrie et al., 1971) and adrenal medulla (Trifaro and Dworkind, 1971). The activity in chromaffin granules of adrenal medulla was localized in the membrane and phosphorylated membrane associated protein.

The transfer of $^{32}PO_4$ form ($\gamma^{32}P$)ATP to a single component in zymogen granule membrane by an endogeneous protein kinase is illustrated in Figure 20A. ^{32}P-labeled membranes were prepared as described in Methods. Although ATP was rapidly degraded by the endogeneous nucleoside triphosphatase, ATP was not rate limiting during the incubation. Maintaining acidic conditions at a lowered temperature during membrane isolation (0°) and electrophoresis (15°) stabilizes both ester and acyl-phosphate bonds, thus facilitating analysis of the type of linkage by employing discriminatory hydrolysis conditions to labeled membranes prior to electrophoresis. Formation of amide phosphate bonds, such as imidazole-phosphate which is unstable in acid solutions, would not be observed under these conditions.

The separation of polypeptides in 1% SDS at pH 2.4, visualized by staining with Coomassie blue R, was similar to the separation under standard conditions (compare Figures 10 and 20A). The major component migrates with an apparent molecular weight of 74,000

Figure 20. Distribution of protein stain and $^{32}PO_4$ in electropherograms of phosphorylated zymogen granule membrane.

1A: Purified granule membranes were phosphorylated, washed and subjected to electrophoresis in 1% SDS at pH 2.4 as described in Methods. One gel containing 200 µg membrane protein was sliced and counted; a parallel gel containing 100 µg protein was stained with Coomassie blue. 1B: Phosphorylated granule membranes (200 µg) were incubated in 0.01 N HCl with 1 mg/ml pepsin at 23°C. for 10 minutes, then subjected to electrophoresis. The radioactivity profile of membranes incubated in 0.01 N HCl minus pepsin was similar to 1A.

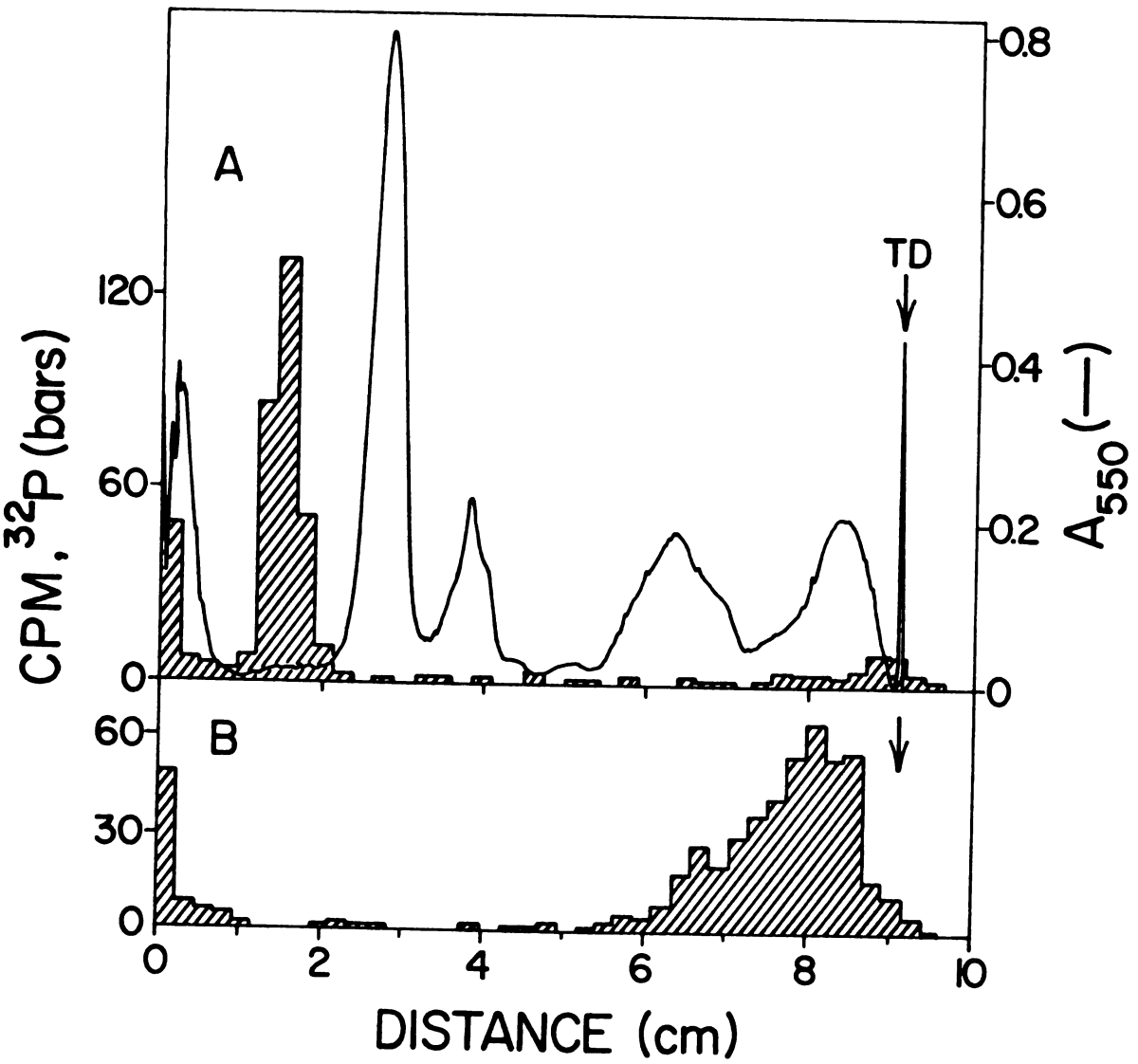

A
B
TD
120
60
0
60
30
0
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
CPM, ^{32}P (bars)
A_{550} (—)
DISTANCE (cm)

Figure 20

(Table 4). Comparison with gels stained for carbohydrate with periodic acid-Schiff reagent demonstrated that the predominant band detected by Coomassie blue was also the major glycopolypeptide. Visual inspection of the gel revealed that the distorted band at approximately 3.8 cm appeared as a tightly spaced triplet and represented further fractionation of component 5 as demonstrated previously in 12.5% acrylamide gels at standard pH (cf. Figure 5). The broad band at 6.5 cm intensified in proportion to the length of storage of the membrane prior to labeling, and therefore correlates with band 19 under standard electrophoresis conditions. The band immediately left of the tracking dye represents lipid (Avruch and Fairbanks, 1972). Oxidation of membrane protein by the residual perchloric acid in the washed membrane preparations coupled with the relatively mild reducing conditions employed in these experiments account for the increased staining material at the gel top.

Characterization of the Phosphorylated Components

Figure 20A reveals that the majority of the $^{32}PO_4$ transferred to membrane macromolecules migrates as a single component with an apparent protein molecular weight of 130,000. Other electropherograms contained a more readily identifiable Coomassie blue stained band which was precisely related to the radioactivity. In six different membrane preparations the average rate of phosphate incorporation into this component as determined after electrophoresis was 0.6 pmole per mg membrane protein per five second incubation. Treatment of labeled granule membrane with pepsin at acid pH eliminated label from

this region (Figure 20B). The radioactivity migrated with low molecular weight species generated by proteolytic digestion, indicating that the $^{32}PO_4$ was protein bound.

The radioactivity in the first gel fraction, although resistant to hydrolysis by pepsin (Figure 20B), was sensitive to hydrolysis under the identical alkaline conditions employed to release the $^{32}PO_4$ bound to the 130,000 molecular weight component (see below). These observations are consistent with the supposition that the $^{32}PO_4$ incorporation represents aggregation of the major phosphorylated component which became nonspecifically trapped at the gel surface, and does not represent a distinct phosphorylated component. Nevertheless, radioactivity at the gel surface was not included in the calculations of subsequent experiments. Consequently, the specific activity of the protein kinase is a minimum value.

Electrophoresis of (γ^{32})ATP and $^{32}PO_4$ under identical conditions indicated that the radioactivity near the tracking dye was substrate and orthophosphate generated by the nucleoside triphosphatase activity, although slight incorporation into lipid, which also migrates in this region, cannot be ruled out. In six independent experiments the amount of label observed near the tracking dye varied between 10% and 200% of the incorporation into the polypeptide. This variability in the efficiency of washing individual membrane preparations required that each assay for membrane phosphorylation be analyzed by electrophoresis.

Characterization of the Phosphoryl-Polypeptide Bond

A pulse-chase experiment first indicated that the phosphate transfer to the membrane polypeptide represented the formation of a stable rather than transient product. After incubation under standard assay conditions for five seconds, 20-fold excess unlabeled Mg-ATP was added and the incubation was continued for an additional seven seconds before quenching with perchloric acid solution. No loss of radioactivity from the membrane was observed, indicating that the label did not turn over.

Further analysis of the stability of the product is summarized in Table 12. As expected no hydrolysis was observed after a brief incubation under electrophoresis conditions. Similarly, little or no loss of label occurred during incubation at pH 10 or at pH 5.4 in the presence of hydroxylamine. These latter two conditions hydrolyze acyl-phosphates such as those known to occur as phosphorylated enzyme intermediates (Hokin et al., 1965). Complete hydrolysis was obtained by more rigorous alkaline conditions at 37°, indicative of the stability of peptidyl serine or threonine phosphate esters (Plummer and Bayliss, 1906; Riley et al., 1968; Labrie et al., 1971). More direct evidence of phosphate esters such as acid hydrolysis of labeled membrane followed by separation and identification of serine or threonine phosphate by paper electrophoresis was not feasible because of low levels of $^{32}PO_4$ incorporation and limited amounts of available granule membrane.

Table 12. Stability of the phosphorylated granule membrane component.

Washed, phosphorylated zymogen granule membranes were prepared as described in Methods, then resuspended under conditions listed below. After the second incubation, membranes were collected by reprecipitation with 0.3 N $HClO_4$, 5 mM H_3PO_4 and 2.5 mM ATP, washed once with water, and analysed by electrophoresis in 1% SDS at pH 2.4. Each gel was sliced and counted; only the radioactivity in the polypeptide region is recorded.

Treatment	$^{32}PO_4$ Incorporation cpm
Experiment 1 (10 min., 23°C.)	
none (control)	324
pH 2.4, 0.1 M sodium phosphate	319
pH 10, 0.1 M sodium borate	256
pH 5.4, 0.2 M sodium acetate plus 0.8 M hydroxylamine	365
Experiment 2 (30 min., 37°C.)	
none (control)	213
1 N NaOH	5

Partial Characterization of the Endogeneous Protein Kinase Activity

Several properties of the kinase activity investigated for comparison with other membrane bound protein kinases are shown in Table 13. Addition of EDTA to the assay mixture significantly reduces, but does not completely inhibit, phosphate transfer to membrane protein. The recalcitrant activity may be due to residual Mg^{2+} tightly bound to the membrane. The pronounced inhibitory affect of Ca^{2+} on the protein kinase activity of erythrocyte membrane (Rubin et al., 1972; Guthrow et al., 1972), cardiac sarcoplasmic reticulum (Wray et al., 1973), and adenohypophyseal granule (Labrie et al., 1971) is not observed with zymogen granule membrane protein kinase. Neither cyclic AMP nor cyclic GMP significantly enhanced the rate of phosphorylation of the granule membrane polypeptide.

Phosphorylation of Mitochondrial Membrane

Mitochondrial membrane was examined as a possible source of contamination of small amounts of a potent protein kinase activity in zymogen granule membrane preparations. Figure 21 illustrates $^{32}PO_4$ transfer to four distinct components exclusive of material at the dye marker. In addition to label at the gel surface, radioactivity migrated with membrane species of approximate molecular weights 120,000, 75,000, and 17,000. The rate of incorporation into the high molecular weight species was 1 pmole per mg membrane protein per five seconds. The low specific activity precludes a mitochondrial protein kinase contamination of granule membranes.

Table 13. Partial characterization of the granule membrane protein kinase activity.

Membrane phosphorylation was performed as described in Methods with reaction mixtures supplemented as indicated below. Only radioactivity in the polypeptide region is recorded.

Addition	% Incorporation
none	(100)
2.5 mM EDTA, minus $MgCl_2$	40
2.5 mM $CaCl_2$	86
1.5 mM UTP plus 3 mM $MgCl_2$	21
1.5 mM ADP plus 3 mM $MgCl_2$	117
2.5 μM cyclic AMP plus 2.5 mM theophylline	93
2.5 μM cyclic GMP	113

Figure 21. Distribution of $^{32}PO_4$ in electropherograms of phosphorylated mitochondrial membrane.

200 µg of purified mitochondrial membrane were phosphorylated, washed and subjected to electrophoresis in 1% SDS at pH 2.4 as described in Methods. Gels were then sliced and counted. Approximate polypeptide molecular weights for three phosphorylated species are noted in the figure. Insert A. Control. Membrane was labeled under the standard five second incubation conditions. Insert B. 3 mM ATP was added after five seconds and the incubation was continued for an additional seven seconds. The radioactivity in only the first four centimeters is shown.

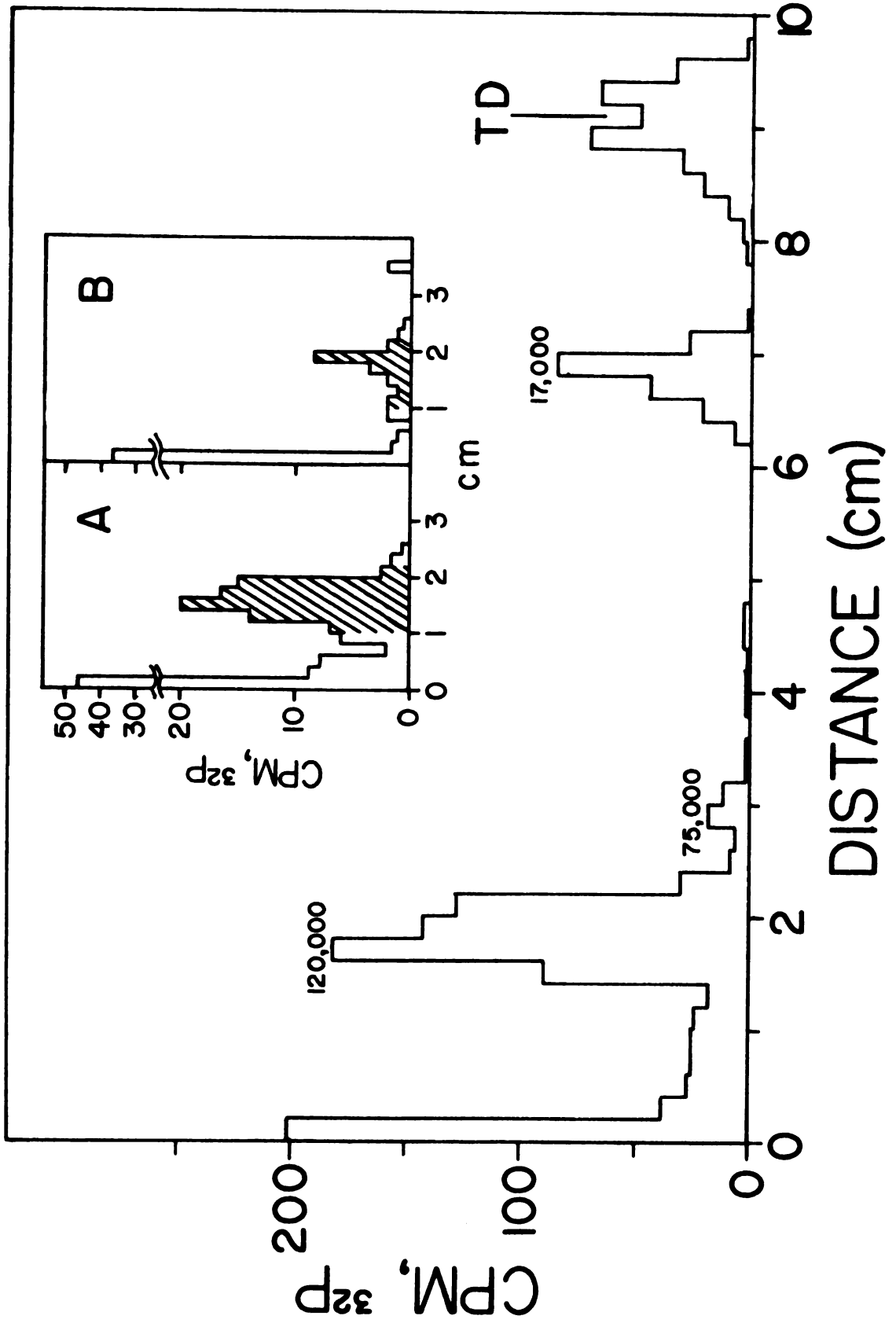

CPM, 32P
200
100
0
0
2
4
6
8
10
DISTANCE (cm)
120,000
75,000
17,000
TD
A
B
CPM, 32P
50
40
30
20
10
0
0
1
2
3
cm

Figure 21

Furthermore, 80% of the $^{32}PO_4$ associated with the mitochondrial 120,000 molecular weight species was released when 20-fold excess unlabeled Mg^{2+}-ATP was added after the initial five second labeling period and incubation continued for an additional seven seconds (Insert B, Figure 21). The rapid turnover of label was distinct from the stability of the phosphorylated granule membrane polypeptide.

DISCUSSION

Analysis of Zymogen Granule Membrane Polypeptides

The SDS polyacrylamide gel electrophoresis procedure of Fairbanks et al. (1970) fulfilled several criteria for precise analysis of membrane polypeptide components, whereas other procedures did not (Kiehn and Holland, 1970; Hoober, 1970). Electropherograms of membranes from zymogen granules, mitochondria, and microsomes were highly reproducible. Peaks accounting for 2% of the protein stain intensity (0.2 to 0.4 μg protein) could be accurately measured. Procedures such as lipid extraction, dissolution in organic solvents, prolonged dialysis and heating in solvent buffer, failed to alter electropherograms of granule membrane polypeptides. These results confirm the earlier report that this method is relatively free of artifacts (Fairbanks et al., 1970). Since traces of secretory proteins proved difficult to remove from purified membranes, it was essential that proteolysis be inhibited during membrane solubilization and during electrophoresis. Dissolution in 1% SDS combined with heating at 100° are sufficient conditions to inhibit most proteases (Fairbanks et al., 1970).

The persistent problem of proteolytic degradation during membrane isolation is particularly acute in studies of pancreatic subfractions. Release of hydrolytic enzymes and activation of zymogens can lead to marked degradation of membrane polypeptides, resulting in a drastic alteration of membrane electropherograms. Control experiments, in which proteolytic inhibitors were not included in the homogenization medium, showed that degradation of microsomal membrane polypeptides lead to a dimunition of major polypeptide bands and the formation of a heterogeneous population of low molecular weight species resulting in poorer resolution of bands (R. A. Ronzio, unpublished observations). Proteolysis of zymogen granule membrane was apparent in a different manner. Under conditions which promote degradation, an increase in small polypeptides with a limited size distribution (11,000 to 14,000 molecular weight) was observed. By this criteria, rat granule membrane was susceptible to a significant but variable degree, while dog granule membrane was isolated with minimal degradation, since a band 19 was not generally detected.

The utility of the first two steps in the purification of zymogen granule membranes has been demonstrated previously. Granule lysis in slightly alkaline solutions was first used by Hokin (1955) to prepare zymogen granule ghosts. More recently, Meldolesi et al., (1971a) separated lysed granules from the more dense contaminating mitochondria by means of discontinuous gradient centrifugation. This step effectively removed mitochondria from granule membranes; however, the remaining amylase activity was appreciable, and electropherograms of these fractions indicated a significant contamination by the

soluble secretory polypeptides representing the granule contents. Extraction by 0.25 M NaBr was incorporated to remove these contaminants. This chaotropic anion has been employed to solubilize mitochondrial membrane proteins at a concentration of 2 to 4 M, approximately 10-fold higher than employed in this study (Hatefi and Hanstein, 1969).

Since the granule membrane accounted for such a small percentage of the zymogen granule protein, it was essential to determine the degree of purity of zymogen granule membranes. Secretory protein contamination was negligible. Since mitochondria are similar in size and density, they represent the most likely particulate contamination of zymogen granules. However, mitochondrial membrane polypeptides were not present in granule membrane electropherograms and purified granule membranes were essentially devoid of cytochrome c oxidase activity. Furthermore, membrane polypeptide profiles of zymogen granules, mitochondria, and microsomal subfractions were distinct from each other, and distinct from the polypeptides in the post-microsomal supernatant and granule contents.

Since a variety of procedures often employed to disaggregate protein complexes failed to alter the profile of the zymogen granule membrane, it was concluded that band 2 was not an aggregate. Band 2 accounted for approximately half of the PAS stain intensity. The granule membrane possesses a high content of sialic acid, associated primarily with high molecular weight polypeptides. These observations suggest that band 2 is an acidic glycoprotein species, and that its broad zone of staining may represent microheterogeneity due to variable carbohydrate content.

In a survey of several soluble proteins, Reynolds and Tanford (1970a) demonstrated that at neutral pH and low ionic strength proteins uniformly bind 1.4 gm of SDS per mg protein. This binding stoichiometry yields a relative constant charge density for protein-SDS complexes, and is partly the basis for the regular relationship between molecular weight and electrophoretic mobility in SDS solutions. On the other hand, membrane proteins, particularly glycoproteins, may not bind predictable amounts of SDS. Since the interaction between SDS and protein is primarily hydrophobic (Rosenberg et al., 1969), membrane polypeptides containing extensive hydrophobic regions bind more SDS (Simons and Kaariainen, 1970; Spatz and Strittmatter, 1973). As a result, the electrophoretic mobility of very hydrophobic proteins increases relative to that of other proteins. The anomalous behavior of glycoproteins in SDS-gel electrophoresis (Bretscher, 1971; Segrest et al., 1971; Russ and Polakova, 1973) is attributed to the lack of SDS binding by attached carbohydrate. More accurate molecular weight estimates are obtained from gels of higher acrylamide concentrations, since under these conditions the charge of the protein-SDS complex becomes less important relative to the sieving effect of the gel. Furthermore, the negative charge contribution by an appreciable number of sialic acid residues associated with acidic glycoproteins would increase electrophoretic mobility. Consequently, the apparent molecular weight estimates of membrane glycoproteins are subject to considerable uncertainty.

Variations of SDS polyacrylamide gel electrophoresis procedures which have been shown to uncover discrepancies of glycoprotein

mobility were applied to the analysis of component 2. Neither increasing the acrylamide content of the gel (Segrest et al., 1971) nor decreasing the pH of the electrophoresis solutions (Fairbanks and Avruch, 1972) altered the observed molecular weight of band 2. It is noteworthy that acetic acid-urea polyacrylamide gel electrophoretic analysis of isolated dog component 2 yielded a molecular weight of 72,000, consistent with all previous estimates.

Band 5, with a molecular weight similar to rat pancreatic amylase, was a major band in ZGM-3, though present at a much reduced level than in ZGM-2. Band 5 was 15 times greater than could be accounted for by amylase activity. Several observations suggested that band 5 was not primarily denatured amylase, but rather a membrane component.

Bands 15 and 17 were sequentially enriched in the membrane during purifications and could be distinguished from bands 14 and 16, with apparent molecular weights of 25,000 and 23,000, respectively. The latter corresponded to polypeptides in the granule contents and may be trypsinogen and chymotrypsinogen, respectively (Sanders, 1970). Band 19 represents a major part of the ZGM-3 profile. Ribonuclease, a relatively minor product of the rat pancreas (Kemp et al., 1972), migrated somewhat less rapidly than the peak of this band. Band 19 may be a family of small membrane polypeptides. Polypeptides of this size have been observed in a variety of membrane classes (Kiehn and Holland, 1970; Swank et al., 1971). As mentioned earlier, band 19 could represent degraded membrane polypeptides retained within the membrane. This species did not stain with PAS, and thus it does

not include carbohydrate containing proteolytic fragments of band 2.

Band 20 stains with both Coomassie blue and PAS and is a prominent part of the zymogen granule membrane profile. Several lines of evidence indicate that this band represents lipid. Reductive alkylation of intact zymogen granule membranes with formaldehyde and (^{3}H)$NaBH_4$ primarily labels lipid, and the same fraction of incorporated radioactivity has the electrophoretic mobility of band 20. Several other investigators (Gahmberg, 1971; Lenard, 1970a; Carraway and Kobylka, 1970; Lopez and Siekevitz, 1973) have confirmed the lipid nature of this band. The high staining intensity in the lipid region of zymogen granule membrane electropherograms relative to gels of other membranes implies a high lipid: protein ratio. This indication has been verified by the observation that the phospholipid content of rat zymogen granule membrane is approximately 2 mg/mg protein. (Ronzio, unpublished data). In a related study, Meldolesi et al. (1971b) noted that zymogen granule membrane from guinea pig contained considerably more phospholipid than other membranes. The high lipid content of membranes from chromaffin granules (Winkler et al., 1970) signifies that this may be a general phenomenon indicative of the functional simplicity of secretory granule membranes.

Meldolesi and Cova (1972) have examined membrane polypeptides from guinea pig pancreas. Zymogen granule membranes from this source seem to be somewhat more complex than those of rat, though distribution of glycosylated membrane components was not reported. The present evidence suggests that the zymogen granule membrane is unusually rich

in glycoproteins. These could simply be plasma membrane precursors, transmitted to the luminal plasmalemma during exocytosis. Alternatively the glycosylated components may possess specific granule functions. Preliminary experiments suggest that a portion of the sialic acid can be cleaved from intact zymogen granules by neuraminidase (R. Hsieh and R. A. Ronzio, unpublished observation). Consequently the membrane glycoproteins are accessible to the cytoplasmic milieu, and may interact specifically with elements of the cytoplasm or with components of the luminal plasmalemma as part of the exocytosis mechanism.

Storage granules have now been isolated from many cell types. In only a few cases have the membrane polypeptides been examined. Chromaffin granule membrane contains approximately 10 polypeptides as judged by SDS polyacrylamide gel electrophoresis (Hortnagl et al., 1971). One of the two major polypeptide species is dopamine β-hydroxylase, an enzyme involved in synthesis of components of the granule contents. Parotid storage granule membrane has been analyzed using acetic acid-urea polyacrylamide gels (Amersterdam et al., 1971). While the level of contamination by granule contents is unclear, the results suggest that the granule membranes are much less complex than other membranes of this tissue. It appears that storage granules represent a class of highly specialized membranes, composed of relatively few polypeptide species. It will be important to establish whether secretory granule membrane from other tissues have a small number of polypeptide components, and whether those which are present are glycosylated.

Comparison of Intracellular Membranes

There have been relatively few attempts to define the intracellular distribution of glycopolypeptides in mammalian tissues. It has been noted that both rough and smooth microsomal membranes from liver contained sialopolypeptides (Helgeland et al., 1972; Larsen, et al., 1972), and membranes of synaptic vesicles and synaptosomal plasma membranes possessed two common glycopolypeptides (Breckenridge and Morgan, 1972). Electropherograms of particulate and soluble polypeptides of adult rat pancreas suggested that glycopolypeptides, though widely distributed, were particularly enriched in zymogen granule membranes (compare Figures 9, 10 and 11).

Glycopolypeptides of the postmicrosomal supernatant and zymogen granule lysate were not readily detected by the carbohydrate stain. However (^{3}H) glucosamine was incorporated into both classes of soluble proteins by tissue slices, although their specific radioactivities were lower than membrane fractions (R. A. Ronzio, unpublished observations). Neither the PAS-positive components nor the (^{3}H) glucosamine labeled components corresponded to the major membrane glycopolypeptides.

Polypeptides of rough and smooth microsomal membranes from rat pancreas were quite similar, though there were differences in the relative amounts of the common polypeptides. A similar conclusion was made for microsomal subfractions from liver (Zahler et al., 1970; Helgeland et al., 1972). The compositions of the smooth microsomal membranes and membranes of rat pancreas Golgi-rich fractions were similar to each other (Ronzio, 1973b), and it is likely that the

former were derived from the latter. The glycopolypeptide profiles of rough and smooth microsomes and mitochondria were considerably less complex than their Coomassie blue profiles and were distinctive for a given membrane class. The glycopolypeptides of smooth microsomal membranes were quite similar to zymogen granule membranes. A glycopolypeptide with the mobility of band 2 was particularly prominent. The restriction of this component to smooth microsomes exclusive of mitochondria and rough microsomes suggests a specific function. If the smooth microsomal component bears some relation to the major granule membrane glycopolypeptide, two explanations are envisaged. The hypothesis that zymogen granules form from coalescing vesicles originating from the Golgi apparatus (Jamieson and Palade, 1967b) requires a pre-existing pool of zymogen granule membrane polypeptides within the Golgi membranes. The occurrence of the glycopolypeptide in smooth microsomal membrane preparations, which are primarily derived from fragmented and resealed Golgi cisternae for such a pool. Alternatively, since membranes from granules ruptured during homogenization would be expected to collect in the smooth membrane fraction, the PAS stain may represent contamination. Based on the specific radioactivity of (^{3}H) glucosamine labeled granule membrane and smooth microsomal membrane fractions and the total amount of cellular granule membrane relative to smooth microsomes, it can be calculated that insufficient granule membrane exists to account for the level of (^{3}H) glucosamine found in smooth microsomes (R. A. Ronzio, unpublished observations). Therefore, the latter alternative is excluded.

The differences in polypeptide compositions of the major membrane classes from pancreas support the proposal that these structures are biochemically, hence, functionally distinct (Meldolesi and Cova, 1972; Ronzio, 1973a). The limited number of zymogen granule membrane polypeptides may be considered to mirror limited functional responsibilities. Unlike the endoplasmic reticulum which partially serves as a matrix for attached enzymes functioning in discrete pathways, thus facilitating reaction rates by limiting the enzymes to diffusion in two rather than three dimensions, the granule membrane does not appear to serve such a function. Another inference is that the mechanism for generating zymogen granule membranes requires the segregation of membrane polypeptides associated with the Golgi complex. This selection process may be linked to the glycosylation of granule membrane polypeptides. Finally, the uniqueness of each membrane species indicates that membrane mixing does not occur during the intracellular transport of secretory proteins.

Zymogen Granule Membrane Mg^{2+}-ATPase

Conceptual models of the secretory granule release reaction have been elucidated in detail by Poste and Allison (1969, 1974), Woodin and Wieneke (1970) and Mathews (1970). A single enzymatic activity, a Ca^{2+}- or Mg^{2+}-ATPase associated with many secretory granules, has been postulated to catalyze the fusion reaction of granule and plasma membrane. Mg^{2+}-ATPase has been previously demonstrated in zymogen granule membranes from guinea pig pancreas (Meldolesi, 1971c). A Mg^{2+}-ATPase of high specific activity has been found to be firmly bound to rat pancreatic granule membrane

(Figure 16; Table 7). The activity is slightly stimulated by the addition of high levels of Ca^{2+} in either the presence or absence of Mg^{2+} and is slightly inhibited by Mn^{2+}. Because of the small magnitude of these effects, it is not likely that these observations reflect the basis for stimulation of granule release by Ca^{2+} or the inhibition by Mn^{2+} (Heisler et al., 1972), although more specific effects manifested in vivo cannot be excluded.

The most simple, but not singular, explanation of the kinetics observed for the granule Mg^{2+}-ATPase is the presence of two enzymes with similar activities. The broad substrate specificity observed may also be indicative of more than one enzyme. However, a single enzyme displaying negative cooperativity (Levitski and Koshland, 1969; Teipel and Koshland, 1969) cannot be excluded at present. The observed kinetics do not appear compatible with models proposed for enzyme reactions which involve substrate and metal modifier interactions (London and Steck, 1969).

A straightforward rationale for the presence of two granule membrane Mg^{2+}-ATPase activities may be postulated from the data of Jamieson and Palade (1971) on the energy requirements of pancreatic secretion. The concentration of granule contents during condensing vacuole maturation did not demonstrate a definite requirement for continued intracellular ATP synthesis. On the basis of this observation, Jamieson and Palade excluded the possibility of an exergonic ion pump, such as the plasma membrane Na^+, K^+-stimulated ATPase, functioning to remove ions, and thereby water, from the granule contents as a mechanism of maturation, unless the putative

enzyme had a high affinity for ATP. The diminishing ATP concentration during exposure to antimycin A and sodium fluoride can be calculated from the data of Jamieson and Palade (1971) assuming an initial uniform cellular ATP concentration of 2 mM (Baudin et al., 1969; Tani and Ogata, 1970). Ten, 20, 40 and 60 minutes after the addition of the metabolic inhibitors, the estimated ATP levels are 0.8, 0.3, 0.2 and 0.1 mM, respectively. An enzyme with a Km for ATP between 0.1 and 0.2 mM would retain approximately 60% of its maximum activity under these conditions. During this 60-minute interval, condensing vacuole conversion decreased to 60% of the uninhibited level (Jamieson and Palade, 1971). The zymogen granule Mg^{2+}-ATPase activity with high affinity for substrate had an apparent Km for ATP of 0.04 mM (Figure 19). If this activity were functional in condensing granule maturation, it would be expected to perform nearly optimally even in the presence of antimycin A and sodium fluoride during the interval investigated.

Jamieson and Palade (1971) observed that the final step of granule release was quickly and effectively inhibited upon addition of the metabolic inhibitors. The energy requirement is most probably involved in fusion of the granule with the apical plasmalemma. The high Km Mg^{2+}-ATPase activity (Figure 19) would be expected to be very sensitive to changes of ATP concentration below 2 mM. Indeed at this level it would be only partially active, and if involved in membrane fusion may require a local rise in the ATP level as part of the secretion process. The activity of the high Km enzyme would

be inhibited upon antimycin A and sodium fluoride treatment, parallel to the inhibition of secretion.

Zymogen Granule Membrane Protein Kinase Activity

Since cyclic AMP has been indicated as an intermediate in the secretion stimulus for several cell types, including exocrine pancreas (Kulka and Sternlicht, 1968; Baudin et al., 1971; Ridderstap and Bonting, 1969), it is of obvious interest to investigate the possible involvement of cyclic AMP-dependent phosphorylation of the structures immediately involved in the secretion process, i.e., secretory granules and plasma membranes. A most plausible site for cyclic AMP intervention is membrane fusion. Alterations of the granule or inner plasmalemma surface properties, such as the phosphorylation of specific sites by a protein kinase, could profoundly alter the rate of granule release.

The initial observation that the terminal phosphate of (γ^{32}P)ATP was transferred to a protein component of the zymogen granule membrane did not distinguish between the action of a protein kinase or the capture of an acyl-phosphate enzyme intermediate. Since trapping a phosphorylated intermediate of the granule Mg^{2+}-ATPase was very possible, experiments to determine the phosphate linkage were conducted. The phosphorylated protein was stable to hydrolysis in dilute base and acid, while greater than 97% hydrolysis occurred in 1 N NaOH at 37° for 30 minutes. These results are characteristic of protein O-phosphoserine or O-phosphothreonine residues, products of a protein kinase reaction.

The initial rate of the protein kinase of purified granule membrane preparations was comparable to several other membrane-bound kinase activities (Guthrow et al., 1972; Wray et al., 1973; Korenman et al., 1974), and varied between 3 and 10 pmoles/min/mg protein. The rigorous washing procedure involved in the membrane isolation, including alkaline sodium bicarbonate extraction of the granules and a high salt wash of the membranes, indicates that both the protein kinase and the phosphorylated proteins were firmly associated with the granule membrane and were not adventitous components. Mitochondrial membrane, the most likely source of contamination, did not contain a protein kinase of sufficient activity to account for the observed granule membrane phosphorylation.

Further investigations of the granule protein kinase require an improved assay. $(\gamma^{32}P)ATP$ hydrolysis by the potent granule Mg^{2+}-ATPase severely limits the incubation time. Addition of a selective ATPase inhibitor such as sodium fluoride (Ueda et al., 1974) should permit the use of much less membrane protein per assay and yet increase the total $^{32}PO_4$ incorporation. ADP, which acts as a competitive inhibitor of the granule membrane catalyzed $(\gamma^{32}P)ATP$ hydrolysis without affecting protein phosphorylation, may also be employed.

Assuming that the phosphorylated component was a single polypeptide species of 130,000 molecular weight constituting 2.6% of the granule membrane (Table 1), 200 μg of membrane protein (the amount in each assay) contained approximately 40 picomoles. The 0.6 picomoles of $^{32}PO_4$ transferred during the five-second incubations therefore

represents a very small fraction of the available sites. Long term assays are required to determine whether all the sites can be phosphorylated. Alternatively, the membrane may be partially phosphorylated prior to isolation. A reciprocal relationship between the number of available phosphorylation sites and the rate of hormone-induced secretion would imply a direct role for the granule membrane protein kinase in the control of secretion.

No significant cyclic nucleotide stimulation of the granule membrane protein kinase was observed. The absence of cyclic AMP stimulation of several membrane-bound protein kinases has been recently incorporated into a scheme which accounts for alteration of membrane properties as an important physiologic response during hormone-induced increases in cyclic AMP levels (LaBrie et al., 1971; Lemay et al., 1974; Korenman et al., 1974). Upon stimulation of uterine adenylyl cyclase by isoproterenol, the number of unsaturated cyclic AMP binding sites were reduced, and the concentration of cyclic AMP independent protein kinase increased at the expense of cyclic AMP dependent activity. The appearance of the independent form of protein kinase was due to the well known cyclic AMP-induced dissociation of the protein kinase cyclic AMP-receptor/catalytic subunit complex (Krebs, 1972). Unlike the distribution prior to stimulation, the kinase activity was not found in the soluble fraction of the homogenates, but instead was recovered bound to membrane. Korenman et al. (1974) proposed that the cyclic AMP-induced kinase translocation is responsible for the uterine response to β-adrenergic stimulation. Likewise, it is reasonable to postulate as a working hypothesis that in the stimulated exocrine

pancreas cyclic AMP induced dissociation of a cytoplasmic receptor-catalytic complex results in the binding of the active catalytic subunit to the zymogen granule surface.

APPENDIX

APPENDIX

A PRELIMINARY STUDY OF MEMBRANE FORMATION DURING PANCREATIC DIFFERENTIATION IN THE RAT EMBRYO

Abstract

Differentiation of the exocrine pancreas in the rat embryo between 15 and 20 days of gestation is characterized by greatly increased rates of synthesis of secretory enzymes (Kemp et al., 1973). Proliferation of the rough endoplasmic reticulum occurs during this period. Zymogen granules appear late and are prominent by 19 days of gestation. To compare these observations with membrane protein synthesis, maximal rates of amino acid incorporation into total protein and particulate protein, were determined by incubating 14 to 20 day old pancreatic rudiments in minimum essential medium containing ^{3}H-leucine. If the specific activity of the leucine precursor pool is taken into account, the rate of particulate protein synthesis (nmoles/mg protein/hr) varies about 2-fold during this interval. When expressed on a per cell basis, however, the rate of ^{3}H-leucine incorporation increased nearly 4-fold from day 14 to day 20. ^{3}H-leucine labeled subcellular components from 15, 16 and 20 day old rudiments were fractionated; the relative rate of incorporation into the zymogen granule fraction increased by approximately 70-fold. These results suggest that formation of specialized membranes is accompanied by a comparatively small increase in the rate of membrane protein synthesis.

Introduction

Biochemical studies of the differentiative process, primarily concerned with relating the appearance and accumulation of tissue specific proteins, have led to possible schemes of cellular control (Rutter et al., 1968b; Papaconstantinou, 1967; Palmiter et al., 1970). Morphological studies of this same process have described ultrastructural changes, primarily the elaboration of membrane systems and the appearance of storage and secretion granules common in many differentiated cell types (Flickinger, 1969; Mills and Topper, 1969). Few attempts have been made to define the biochemical events involved in elaboration of membrane systems and their functions during differentiation. Hence, the relationship between regulation of membrane formation and regulation of the synthesis and secretion of cell specific products has not been established.

The increase of functional membrane is an integral part of the differentiative process. In mature pancreas exocrine cells, secretory products are thought to be synthesized preferentially on polysomes attached to rough endoplasmic reticulum (Takagi et al., 1971; Redman, 1969). Protein glycosylation and "packaging" may be performed by the Golgi apparatus (Jamieson and Palade, 1967a; Morre et al., 1969; Schacter et al., 1970). Cell-specific products, e.g., amylase, chymotrypsinogen, and ribonuclease, are stored and transported to the luminal plasma membrane by membrane-bound secretory vesicles, zymogen granules (Jamieson and Palade, 1967a). Prior to differentiation, exocrine cells possess little rough endoplasmic reticulum, and no

granules. Therefore, quite extensive membrane development must occur to attain the differentiated state.

Kallman and Grobstein (1964) have observed that the first detectable ultrastructural change in developing mouse pancreas, the accumulation of cytoplasmic ribosomal aggregates, occurs at the beginning of the secondary transition. Elaboration of rough endoplasmic reticulum begins one day later at the time of pronounced zymogen synthesis. The appearance of zymogen granules evidence the accumulation of secretory product three days after the initial observation of the accumulation of cytoplasmic ribosomes.

As yet only the rates of synthesis and accumulation of secretory products have been characterized (Sanders, 1970; Rutter et al., 1968b; Kemp et al., 1972). This report contains initial experiments designed to correlate the appearance of cell-specific secretory products with the synthesis and assembly of components of intracellular membranes. The results have led to the insight that, to be fruitful, such a study requires the analysis of precursor incorporation into specific, well-defined and isolatable membranes.

Methods and Materials

Methods specific for each experiment are included in the figure and table legends.

Sprague-Dawley rats (Spartan Research Animals, Haslett, MI) were paired in breeding cages; conception was noted in the morning by dropped vaginal plugs (day 0). After decapitation, placentas from pregnant rats were removed and placed in Earle's balanced salt solution (EBSS) (Earle, 1943). Dissected pancreas tissue equivalent

to approximately five 15-day rudiments (70 to 100 µg tissue protein) were cultured at 37° in a minimum of 0.2 ml minimum essential medium (MEM) (Eagle, 1959) buffered with 20 mM HEPES (the kind gift of N. Good), pH 7.0. The increase of the osmolality of the medium due to the addition of 20 mM HEPES was measured with a clinical osmometer and found to be negligible. Sterile conditions were maintained. Twelve hour cultures contained penicillin (100 units/ml), streptomycin (100 µg/ml) and fungizone (0.25 µg/ml) (Grand Island Biological Company).

Prior to use (4,5-^{3}H)L-leucine (22 Ci/mmole; Amersham/Searle) was characterized using an amino acid analyzer. Although the total recovery of leucine applied to the amino acid analyzer column was low, 89% of the radioactivity recovered eluted with L-leucine added as carrier. The TCA soluble fraction from homogenates of thoroughly washed tissues obtained after a 30-minute incubation with ^{3}H-leucine was also analyzed and was found to contain 88% of the counts recovered as leucine. Hence, negligible conversion to other soluble molecules occurred, and the radioactivity recovered in the TCA precipitates was assumed to represent protein ^{3}H-leucine.

Radioactive samples were added to 10 ml of fluid containing (per liter) 667 ml toluene, 333 ml Triton X-100, 5.5 gm PPO and 0.1 gm POPOP, and counted in a Packard Tri-Carb scintillation spectrometer.

Results

Estimation of the Rates of Membrane Synthesis at Different Developmental Ages by ^{3}H-leucine Incorporation

Figure 22 illustrates the soluble and particulate protein accumulation of embryonic pancreas cells between day 15 of gestation

Figure 22. Changes in the soluble and particulate protein content of cells of embryonic pancreas during development.

Pancreases from embryos of different ages were sonicated briefly in polyethylene microfuge tubes containing 300 µl of 0.2 M $NaHCO_3$, pH 8.2, then centrifuged at 100,000xg for 1 hour. The pellet was washed once by brief sonication in 0.25 M NaBr and recentrifuged. Particulate protein refers to the final pellet; soluble protein refers to the combined supernatants. The distribution of particulate and soluble protein was calculated from the known total cellular protein content assuming a 7 pg DNA per cell (Rutter et al., 1968a).

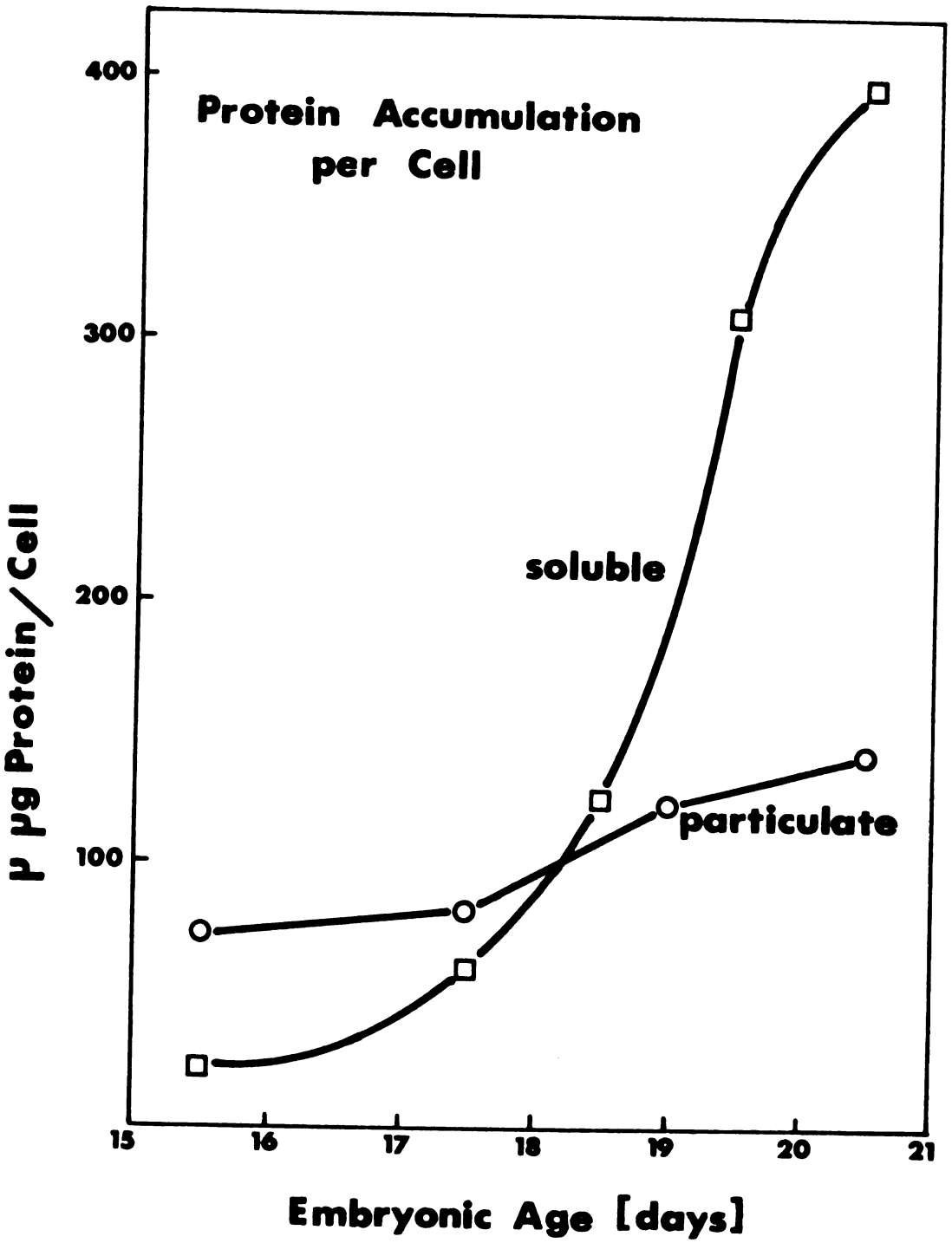

Protein Accumulation
per Cell
soluble
particulate
μ μg Protein/Cell
400
300
200
100
15
16
17
18
19
20
21
Embryonic Age [days]

Figure 22

and birth. While soluble protein accumulates to a level of 20-1/2 days that is 19-fold greater than at 15-1/2 days, the particulate protein contribution per cell appears to only double. The appearance of extensive endoplasmic reticulum and the accumulation of zymogen granule membrane is masked by the large contribution of nuclei and mitochondria to the total particulate material. Therefore, the increase of individual intracellular membranes is more significant than implied in Figure 22. Further experiments were performed to determine the rate of synthesis of membrane components and their assembly into subcellular structures.

Preliminary experiments determined the optimum ^{3}H-leucine labeling conditions for 15 to 20 day pancreatic rudiments in culture. Using Earle's minimum essential medium with HEPES buffer, the pulse label was initiated by the addition of ^{3}H-leucine to 10 μCi/ml. Under these conditions the soluble leucine pool equilibrated within 20 minutes and incorporation into cellular protein is linear after 10 minutes (Figure 23) and as long as 10 hours (data not shown).

Figure 24 illustrates the incorporation of ^{3}H-leucine under the conditions just described into particulate and non-particulate fractions of embryonic pancreas of various ages. The slope of each line is indicative of the rate of incorporation of precursor. The rates of ^{3}H-leucine incorporation into <u>particulate</u> protein are initially high at 15-1/2 and 17-1/2 days of gestation, then decrease during development. This initial elevated rate could be responsible for the increase of particulate protein between 17 and 19 days of gestation (Figure 22). Incorporation into the <u>soluble</u> fraction, which

Figure 23. ^{3}H-leucine uptake and incorporation into macromolecules by embryonic pancreas cultured in vitro.

Pancreatic rudiments from 19 day rat embryos were cultured for varied intervals in 0.5 ml HEPES buffered MEM containing 0.4 mM leucine and 10 µCi/ml ^{3}H-leucine. ^{3}H-leucine incorporation was terminated by the addition of 3 ml of ice cold EBSS. The rudiments were rinsed three times in 4 ml of EBSS, sonicated in 400 µl polyethylene microfuge tubes (Beckman) containing 200 µl $NaHCO_3$, and precipitated with an equal volume of cold 10% TCA. The precipitates were collected on glass fiber filters (Whatman GC/C), washed with 5 ml of 10% TCA, placed in scintillation vials with 0.5 ml 1 N NaOH, and heated 2 hours at 100°C. Aliquots of the homogenate (—o—) and TCA soluble fraction (—Δ—) were counted as well as the TCA precipitate (—□—).

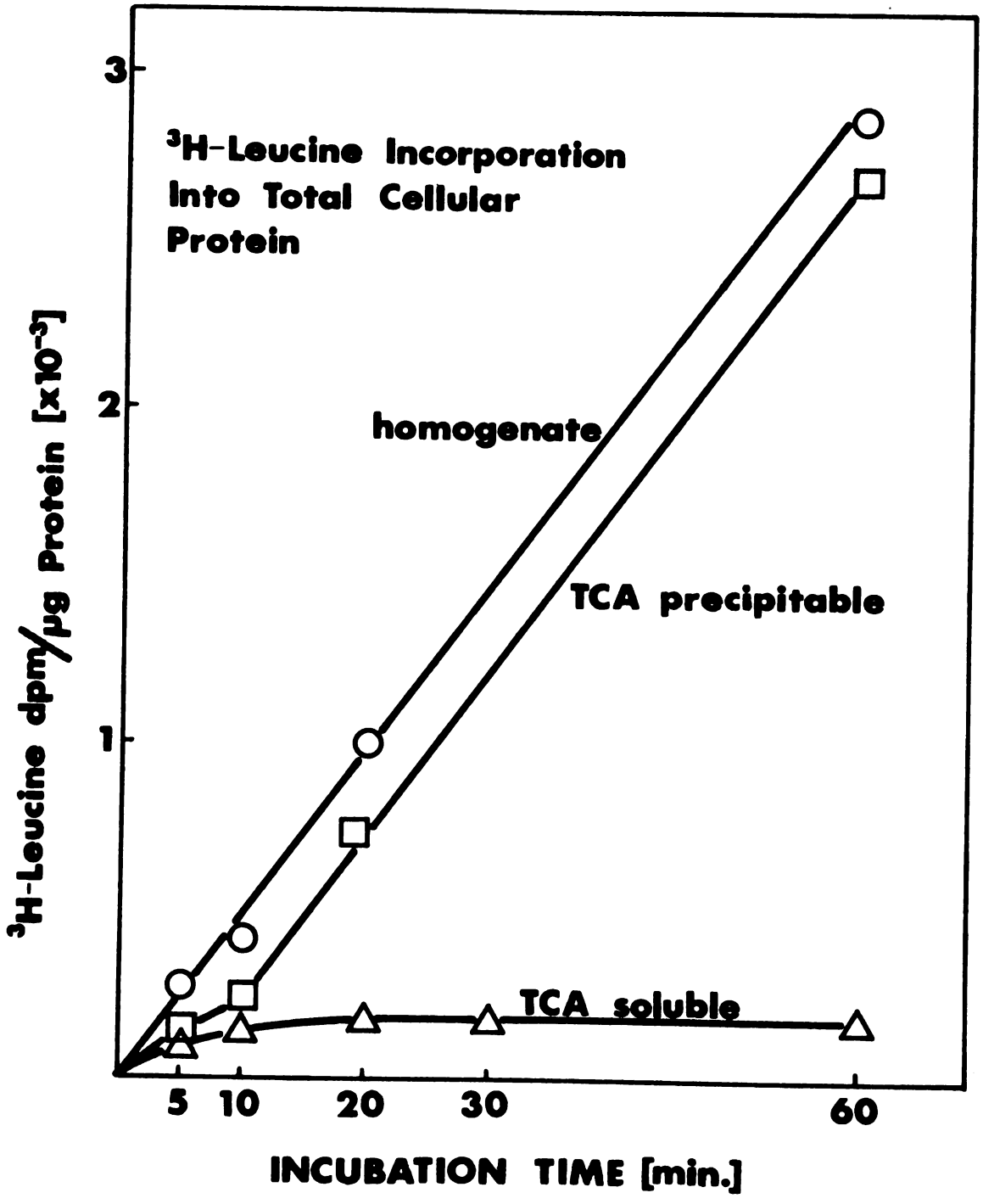

3
2
1
^{3}H-Leucine Incorporation
Into Total Cellular
Protein
homogenate
TCA precipitable
TCA soluble
^{3}H-Leucine dpm/µg Protein [x10^{-3}]
5
10
20
30
60
INCUBATION TIME [min.]

Figure 23

Figure 24. Rates of ^{3}H-leucine incorporation into particulate and nonparticulate (soluble) cellular fractions.

Pancreatic rudiments from embryos at 15-1/2, 17-1/2, 18-1/2, and 20-1/2 days of gestation were incubated in HEPES buffered MEM containing ^{3}H-leucine and terminated by the addition of 3 ml of ice cold EBSS. The rudiments were rinsed three times in 4 ml of EBSS and subsequently fractionated into particulate and soluble fractions as follows: The rinsed pancreases were sonicated briefly in polyethylene microfuge tubes containing 300 µl of 0.2 M $NaHCO_3$, pH 8.2, then centrifuged at 100,000xg for 1 hour. The pellet was washed once by brief sonication in 300 µl 0.25 NaBr and recentrifuged. Particulate protein refers to the final pellet; soluble protein refers to the combined supernatants.

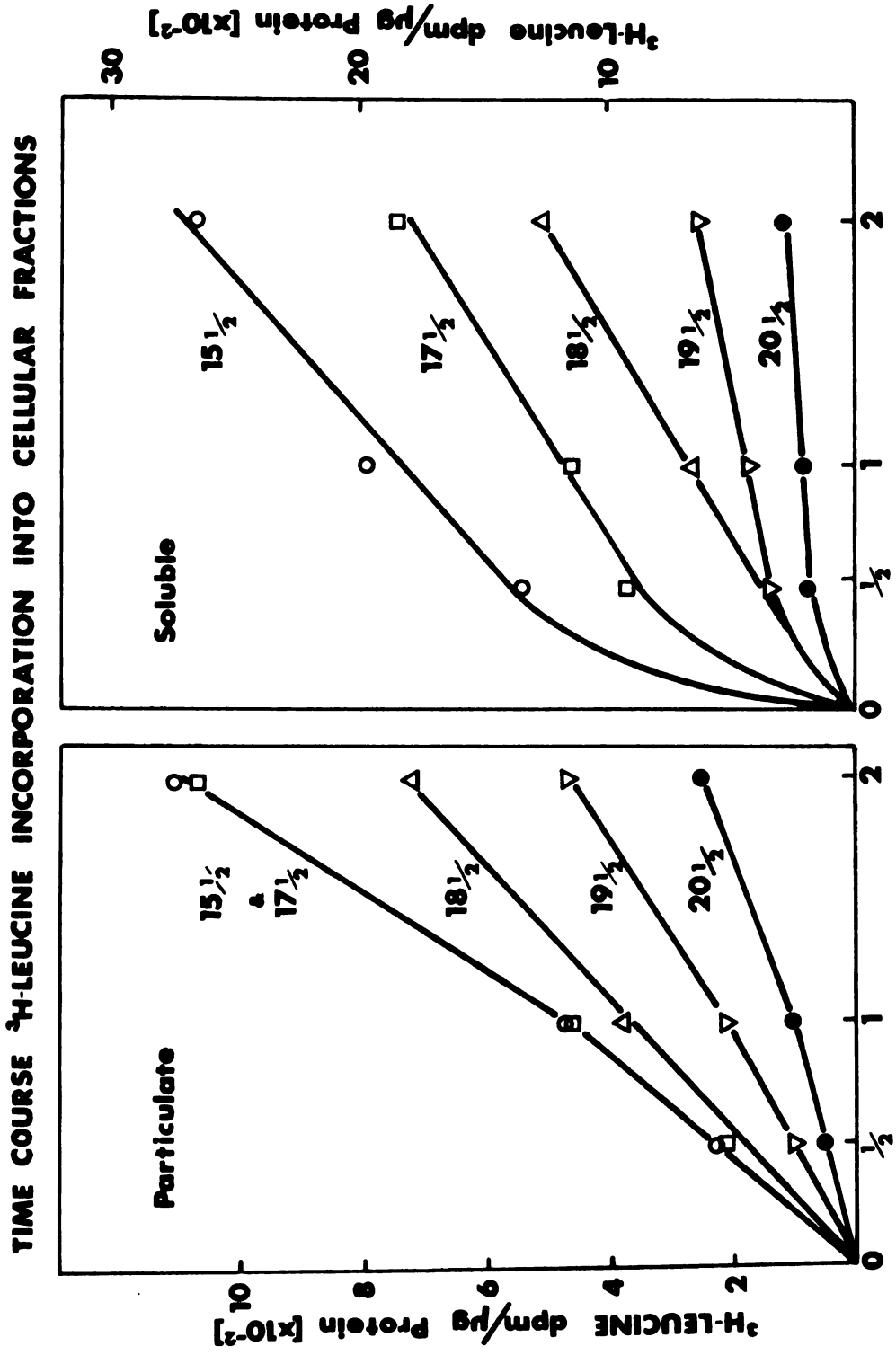

TIME COURSE ³H-LEUCINE INCORPORATION INTO CELLULAR FRACTIONS
Particulate
Soluble
15½ & 17½
18½
19½
20½
³H-LEUCINE dpm/µg Protein [x10⁻²]
³H-Leucine dpm/µg Protein [x10⁻²]
INCUBATION TIME [hours]
0
½
1
2
2
4
6
8
10
10
20
30

Figure 24

includes both the leucine intracellular pool and all soluble labeled protein, follows a similar pattern.

The apparent augmented rate of particulate protein synthesis may reflect a differential response of rudiments of different ages to culture and labeling conditions rather than true changes due to the mechanisms of differentiation. If more developed rudiments are less capable of maintaining sufficient intracellular amino acid pools in culture, the rate of protein synthesis could be limited by precursor levels. In addition, the apparent rate of protein synthesis is directly related to the specific radioactivity of the precursor pool, so that differences of specific activity in these studies could alone account for the observed differences in rate.

To determine the influence of these two possibilities on the apparent rates of particulate protein synthesis, pancreatic rudiments, dissected from embryos at different gestation times, were cultured for 30 minutes in HEPES buffered MEM containing ^{3}H-leucine, and the sizes and specific radioactivities of the intracellular leucine pool were determined. Table 14 summarizes these data. As can be seen, the size of the pool does not change significantly during the developmental interval observed. The specific radioactivity of the pool, however, declines at day 20 to one-third the value it was at day 14.

The corrected rates of protein synthesis for 14, 17 and 20 day rudiments can be calculated from the relative rates of incorporation (Figure 24; expressed as dpm/μg cell protein) and the specific radioactivity of intracellular leucine (Table 14; expressed as dpm/μμmole leucine). The rates thus calculated (last column, Table 14) indicate

Table 14. Rates of ^{3}H-leucine incorporation into particulate fractions of pancreatic rudiments.

Rudiments of different ages were incubated in HEPES buffered MEM containing 0.4 mM ^{3}H-leucine (10 μCi/ml) for 30 minutes. The tissues were then collected, washed three times with EBSS, sonicated, assayed for protein content, and precipitated with 10% TCA. The TCA soluble fraction was exhaustively extracted with ether. The aqueous phase was then evaporated under vacuum to dryness, redissolved in citrate buffer, and subjected to amino acid analysis. Specific radioactivity of the leucine pool was calculated from the radioactivity applied to the column and the nmoles of leucine calculated from the chromatogram peak heights relative to p-chlorophenylalanine. Eighty-eight per cent of the radioactivity detected in the eluate was confined to the leucine peak.

Embryonic Age (days)	TCA soluble leucine (nmole per mg protein)	Specific activity (dpm/μμmole)	Rate (nmole/mg protein/hr)
14	7.0(±1.0)[a]	64	10
15	8.3(±0.1)[a]	-	-
17	12.7[b]	42	13
20	10.8[b]	23	6

[a]Two determinations.

[b]A single determination.

that the rate of protein synthesis and subsequent assembly in a membrane structure does not change appreciably during or prior to the accumulation of intracellular membrane.

Figure 25 contrasts the results obtained when intracellular and extracellular leucine specific radioactivities are used to calculate the rates. The differences between Figures 25A and 25B can be attributed to the dramatic increase in protein per cell during development (Figure 22). As differentiating acinar cells increase in size and membrane content, their rate of membrane protein synthesis increases nearly 4-fold (Figure 25B).

^{3}H-Leucine Labeling of Individual Subcellular Fractions During Development--The Appearance of a Distinct Membrane Class

Table 15 gives the results of an experiment designed to measure the appearance of zymogen granules in developing cells. At 15 and 16 days the extent of synthesis of zymogen granule protein is less than 0.1% of the total cell protein synthesized; this radioactivity may actually represent a small contamination by mitochondria. At 19-1/2 days, however, more than 7% of protein synthesized is incorporated into zymogen granules. This estimate of incorporation into zymogen granules is probably low by a factor of 3, since only 25% of the total granule population was isolated in this fraction. These results corroborate electron microscope observations (Kallman and Grobstein, 1964; Pictet et al., 1972) and suggest that the appearance of zymogen granules may result in a 100-fold increase in the rate of synthesis of the zymogen granule membrane.

Figure 25. Comparison of the calculated rates of particulate protein synthesis.

A) —□—, rates calculated from intracellular leucine specific radioactivity (Table 14); —o—, rates calculated from the leucine specific radioactivity of the incubation medium (53 dpm/μμ mole leucine) (relative rates taken from Figure 24).

B) Rates, calculated as in A), are expressed per 10^6 cells rather than per mg protein. The number of cells per milligram protein for pancreas of different ages was taken from Rutter et al. (1968a).

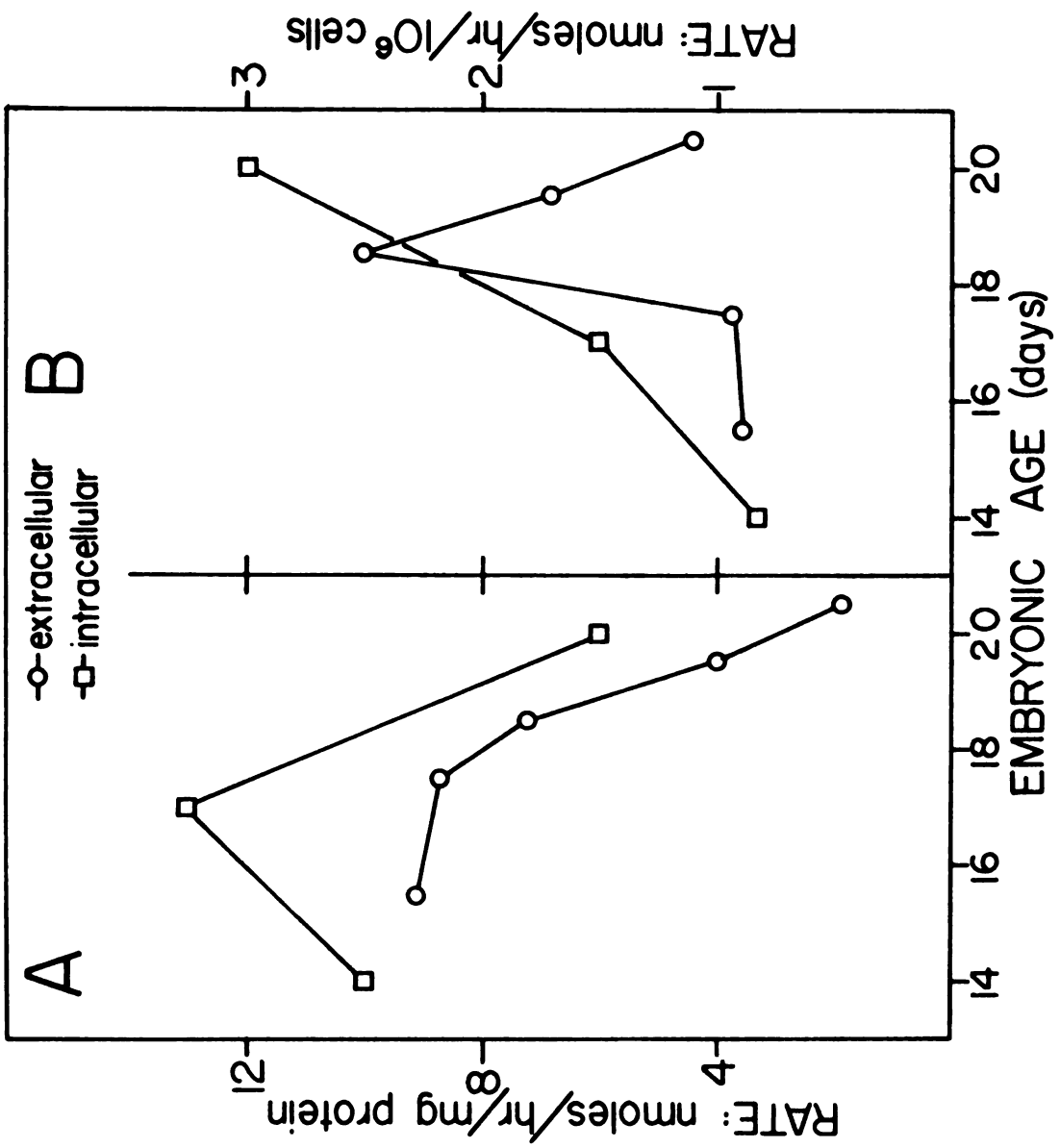

A
B
extracellular
intracellular
RATE: nmoles/hr/mg protein
RATE: nmoles/hr/10⁶ cells
EMBRYONIC AGE (days)
14
16
18
20
4
8
12
1
2
3

Figure 25

Table 15. Distribution of ^{3}H-leucine incorporation into subcellular fractions of embryonic pancreas.

Fifteen, 16 and 19 1/2 day pancreatic rudiments were incubated for 10 hours in HEPES buffered MEM containing 0.02 mM leucine (10 µCi/ml). After rinsing in cold EBSS, the tissues were homogenized with a small glass-teflon Potter-Elvehjem homogenizer, combined with carrier homogenate from a single adult rat pancreas, and fractionated into five subcellular fractions by differential centrifugation (Methods).

Fraction	Percent total dpm incorporated		
Embryonic age:	15 day	16 day	19-1/2 day
Debris	20	18	16
Zymogen granules	0.05	0.08	7
Mitochondria	11	11	12
Microsomes	21	19	9
100,000xg supernatant	48	52	56

Discussion

The transitions which occur within a presumptive pancreatic acinar cell during differentiation are dramatic. In order to compare the rates of protein synthesis at individual time points in a continuously changing system, several criteria must be fulfilled. For all developmental stages (a) the intracellular pool of labeled precursor must attain a steady state level quickly, relative to the total labeling period, (b) the steady state conditions must be maintained during the labeling period, (c) incorporation into protein must be linear throughout the labeling period, and (d) the specific activity of the intracellular pools must be monitored, and variations observed must be incorporated into calculations to obtain more accurate rates of protein synthesis.

Requirements (a), (b) and (c) were fulfilled under the conditions employed for pancreatic rudiments of embryos from 15 through 20 days of gestation. In the absence of information on the specific activity of intracellular leucine, the relative rates of pancreatic membrane protein synthesis appeared uniformly to decrease from 15-1/2 days of gestation until term. When the rates of synthesis of particulate protein were corrected from the observed relative rates using the measured specific activity of the intracellular leucine, the rates appear more constant, fluctuating only 2-fold. The rates of membrane assembly monitored independently by ^{3}H-choline incorporation gave similar results (preliminary results, data not given).

These results of leucine and choline incorporation studies suggest that the increase of total embryonic pancreatic membranes,

which is particularly significant for rough endoplasmic reticulum and zymogen granule membranes (Pictet et al., 1972), may involve only a slight increase in the rate of synthesis of membrane protein and lipid. It should be noted, however, that since acinar cell development results in an approximate 10-fold increase in protein per cell, that there was up to a 4-fold increase in the rate of membrane protein synthesis when expressed on a per cell basis.

The steady state leucine pool specific activity in cultured 14-day embryonic pancreas was approximately the same as the extracellular leucine of the labeling medium. The steady state specific activities of more developed pancreatic rudiments were significantly lower. This observed decrease may reflect the presence of two distinct intracellular amino acid pools (Righetti et al., 1971; Mortimore et al., 1972) and either the relative changes which occur between them during development, or differential effects of culture conditions on the two pools at the different stages of development. The difficulties encountered in interpretation of results which reflect temporal changes in the contribution by two independent precursor pools may be experimentally overcome. Since the size, and therefore influence, of the pool in equilibrium with extracellular leucine can be enhanced several-fold by increasing the external leucine concentration, the specific activity of the total internal leucine pool can be made to reflect that of external leucine by increasing the concentration of leucine in the medium above 2 mM (R. J. MacDonald, unpublished observation; Mortimore et al., 1972).

Analysis of pancreatic development by culturing 14-day pancreatic rudiments has been shown to mirror the in vivo rates of synthesis and accumulation of the tissue specific hydrolytic enzymes (Rutter et al., 1968b; Kemp et al., 1972). Parallel changes in other important parameters of exocrine development are not observed, however. The extent of cell protein accumulation is low, presumably due to enhanced mesenchymal tissue growth of cells with a low protein to DNA ratio (Kemp et al., 1972). Similarly, neither the apparent changes in the rate of ^{3}H-leucine incorporation nor the decrease in specific activity of the leucine intracellular pool is observed for rudiments in extended culture. The utilization of freshly dissected embryonic pancreas to monitor rates of macromolecular synthesis at distinct developmental stages and the use of a continuous culture system during which development occurs must be viewed as two distinct experimental approaches to the same system, and caution must be exercised in the comparative analyses of their results.

The appearance of zymogen granules prior to 19-1/2 days gestation (Pictet et al., 1973; Kallman and Grobstein, 1964; and Table 15) clearly indicates the emergence of a functionally distinct membrane species as indicative of differentiation as the appearance of pancreatic digestive enzymes and pro-enzymes. The concept that the zymogen granule membrane represents part of the differentiated characteristics of an acinar cell (i.e., part of the mechanism of secretion of tissue specific products) has led to the premise that the rate of synthesis and assembly of zymogen granule membrane may be employed as another index of pancreatic development. An analysis

of the rate of assembly would be free of many of the ambiguities of investigating the synthesis of bulk intracellular membranes.

Analysis of several physical and enzymatic parameters of the zymogen granule membrane for the purpose of identifying distinctive characteristics useful in measuring rates of synthesis and assembly during development formed the basis of the studies reported in the main text of this thesis.

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