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ZYMOGEN GRANULE MEMBRANE PHOSPHORYLATION AND
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ZYMOGEN GRANULE MEMBRANE PHOSPHORYLATION AND GLYCOPROTEIN TOPOLOGY IN THE EXOCRINE PANCREAS

By

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ABSTRACT

ZYMOGEN GRANULE MEMBRANE PHOSPHORYLATION AND GLYCOPROTEIN TOPOLOGY IN THE EXOCRINE PANCREAS

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There is considerable interest in the role of cyclic nucleotides in stimulus-coupled secretion. A possible function is the regulation of protein kinases and the phosphorylation of zymogen granules, the secretory vesicles of the exocrine pancreas. Zymogen granule membranes contain a protein kinase capable of phosphorylating endogenous acceptors. To examine the possible translocation of a soluble protein kinase to granules or modulation of the endogenous granule kinase, I studied the subcellular distribution of protein kinase activity in the adult rat pancreas. Protein kinase activity in granules and the post-microsomal supernate was stimulated by 2 μM cyclic AMP and cyclic GMP. Preincubation of zymogen granules and the post-microsomal supernate in the presence of theophylline and cyclic AMP led to a five-fold increase of granule protein kinase activity; 90% of this activity could be removed by extraction with 0.15 M KCl. Cyclic GMP had no significant effect. Zymogen granules isolated from tissue pieces, incubated with either carbachol (10^{-5} M) or pancreozymin (0.1 U/ml) for 2 to 60 min did not exhibit increased levels of cyclic nucleotide-dependent or independent protein kinase activity, when compared to granules from resting tissue. From this

observation I conclude that zymogen secretion in the exocrine rat pancreas does not involve significant changes in zymogen granule protein kinase activities.

However, increased $^{32}PO_4^{-3}$ incorporation into zymogen granule membrane phospholipids during stimulus-coupled secretion was observed. Nearly 100% of the $^{32}PO_4^{-3}$ counts, accounting for the observed increase, was associated with phosphatidylinositol as determined by two-dimensional thin layer chromatographic analysis. Increased phosphatidylinositol turnover appears to account for the increase in zymogen granule membrane phosphorylation.

To explore the proposal that a membrane glycoprotein (GP-2) is a general feature of storage granule membranes of the mammalian exocrine pancreas, I have isolated GP-2 from zymogen granule membranes of adult dog pancreas. GP-2 accounted for 50-60% of the total granule membrane protein. Electrophoretic mobilities in several membrane solvents suggested a single component with an apparent molecular weight of 72,000. Enzymatic labeling studies of intact granules and granule membranes suggested that GP-2 was located on the inner (cisternal) side of the zymogen granule. Furthermore, GP-2 was localized in granule membranes, not other subcellular fractions.

The presence of GP-2 on the internal side of zymogen granules allowed the possibility of detecting GP-2 on the acinar cell surface as zymogen granules fuse with the plasmalemma during exocytosis. Pancreatic acinar cells, stimulated with carbachol for 2 h, were able to incorporate ^{3}H two-fold over control cells into oxidized galactose residues of glycoproteins located on the cell surface,

presumably due to the appearance of granule membrane glycoproteins (i.e., GP-2).

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BPA	bovine plasma albumin
BSA	bovine serum albumin
Cyclic AMP	adenosine 3'5' cyclic monophosphate
Cyclic GMP	guanosine 3'5' cyclic monophosphate
Dibutyryl cyclic AMP	$N^{6}O^{2}$'-dibutyryl adenosine 3'5' cyclic monophosphate
EDTA	disodium ethylenediamine tetraacetate
$(\gamma-^{32}PO_4)ATP$	adenosine triphosphate containing phosphorus 32 in the gamma phosphate
GP-2	zymogen granule membrane glycoprotein with a molecular weight of 72,000
IRB	Krebs Ringer buffer
PAS	periodic acid-Schiff
$^{32}PO_4-^{3}$	orthophosphorus-32
SBTI	soybean trypsin inhibitor
SDS	sodium dodecyl sulfate

LITERATURE REVIEW

The mammalian pancreas possesses two major classes of cells, each serving a different function. Acinar and duct cells, comprising one group, have an exocrine function involving the release of digestive enzymes (Greene et al., 1963; Hokin, 1955), electrolytes and sodium bicarbonate fluid. The second class of pancreatic cells, B cells, regulate blood sugar levels (Howell et al., 1969). Subsequent discussions will concern only pancreatic acinar cells.

Pancreatic Protein Secretion

Upon hormonal stimulation, pancreatic acinar cells secrete digestive enzymes and proenzymes into the duodenum via secretory ducts. This secretory process can be divided into the following steps: i) synthesis and segregation of secretory proteins, ii) intracellular transport of these proteins to the Golgi complex, and iii) intracellular storage and discharge of secretory proteins.

Proteins destined for export in the pancreas are synthesized on polysomes bound to the endoplasmic reticulum membrane (Siekeveitz and Palade, 1960). Newly synthesized secretory proteins are then extruded into the cisternae space of the endoplasmic reticulum, where they are segregated (Redman et al., 1966; Redman and Sabatini, 1966; Blobel and Sabatini, 1970). The use of autoradiographic and electron microscopic techniques by Caro and Palade (1964) allowed the first glimpse of intracellular transport of secretory proteins from the

cisternae space to condensing vacuoles of the Golgi complex (Jamieson and Palade, 1967). This transport process is dependent upon ATP derived from oxidative phosphorylation (Jamieson and Palade, 1968). Following concentration in the condensing vacuoles, secretory proteins are packaged into secretory vesicles, designated zymogen granules. It is these secretory granules that fuse with the plasmalemma membrane to discharge their content. This process of exocytosis is absolutely dependent upon energy and calcium (Douglas and Rubin, 1961; Schramm, 1967; Douglas, 1968).

Secretory Vesicles

A large number of secretory tissues contain membrane vesicles, denoted secretory granules. The importance of these granules, aside from segregating secretory proteins, is their ability to interact with the cell plasma membrane during exocytosis. Exocytosis is the process by which secretory granules fuse with the plasma membrane in order to discharge their content. Two of these secretion granules have been extensively studied, the chromaffin granule (from the adrenal medulla) and the zymogen granule of the pancreas. Morris (1974) has proposed an experimental model for exocytosis of chromaffin granules. The model entails the following stages: i) activation of the adrenal medulla secretion response by a suitable stimulus, ii) chromaffin granule fusion with the plasma membrane, followed by release of the granule contents, and iii) removal of the granule membrane from the plasma membrane with possible recycling of granule membrane components. Electron microscopic techniques have illustrated a similar exocytosis process between zymogen granules and the pancreatic acinar cell plasmalemma (Palade, 1975). This apparent

interaction of membranes during secretion emphasizes the importance of secretory granule membranes.

Meldolesi et al. (1971) partially purified zymogen granule membranes from guinea pig pancreas. Using modifications of the procedure employed by Meldolesi et al., MacDonald and Ronzio (1972) obtained Coomassie blue polypeptide profiles of zymogen granule membranes from a variety of species. Analysis of these profiles showed a relatively simple composition of membrane proteins. Other secretory granules, i.e., parotid gland granules (Castle et al., 1975) and chromaffin granules (Winkler et al., 1970), also appear to contain simple membrane polypeptide profiles. If one assumes that secretory granules have one function, that of recognizing that portion of the cell membrane where the granule fuses, the appearance of a simple membrane protein composition would be reasonable.

The most prevalent membrane protein of dog zymogen granules has been isolated by MacDonald (1974). This protein was designated glycoprotein-2 (GP-2) on the basis of migration on SDS-polyacrylamide gels and the presence of sugar moieties typical of mammalian glycoproteins (Lewis et al., 1977). GP-2 is present in zymogen granule membranes from a wide variety of mammalian species (MacDonald and Ronzio, 1972). Comparison of granule membrane gel profiles with those of mitochondria and microsomal membranes suggests that GP-2 is a distinctive feature of the zymogen granule membrane. The origin of GP-2 appears to be in the Golgi complex, the site of zymogen granule synthesis (Jamieson and Palade, 1967). The lack of zymogen granule membrane glycosyltransferases and the presence of a protein, similar to GP-2, in the Golgi complex (Ronzio and Mohrlok, 1977) suggests that GP-2 is glycosylated at the site of zymogen granule

synthesis. It is unknown whether GP-2 is involved in granule formation or in the mechanism of secretion.

The composition of zymogen granule membrane-bound enzymatic activities is presented in Table 1. Chromaffin granules and adenohypophyseal secretion granules (from the anterior pituitary) are included in Table 1 for comparison. MacDonald (1974) and MacDonald and Ronzio (1974) have reported Mg^{2+}-ATPase and protein kinase activities associated with the zymogen granule membrane. These enzymes, with similar specific activities, are also associated with chromaffin and adenohypophyseal granules. As of yet, granule membrane-bound protein kinases have not been isolated, although Trifaro and Warner (1972) have solubilized the chromaffin granule Mg^{2+}-ATPase. The relationship of these enzymes to pancreatic protein secretion will be discussed later.

The lipid composition of zymogen granule membranes, as well as chromaffin granules, is characterized by a high content of lysophosphatides (Meldolesi et al., 1971). Whether this high lysophosphatide content is due to lipase action during cell homogenization (Meldolesi et al., 1971) is unclear. Zymogen granules, when compared to other pancreatic subcellular membranes, have a higher sphingomyelin and cholesterol content (Meldolesi et al., 1971). These increased amounts of sphingomyelin and cholesterol are also common in chromaffin granules (Blaschko et al., 1967), adenohypophyseal granules (Tesar, 1967) and hepatic lysosomes (Thines-Sempoux, 1967).

Table 1. Membrane-bound enzymatic activities of several secretory granules

Enzyme	Chromaffin granules[a]	Zymogen granules[b]	Adenohypophyseal granules
Mg^{2+}-ATPase	(Banks, 1965)-3 1.1 μmol $^{32}PO_4$/mg protein/min[3]	(MacDonald, 1974) 77 μmol/mg protein/h	ND
Phosphatidylinositol kinase	(Phillips, 1973; Muller and Kirshnar, 1975)	ND	ND
Protein kinase	(Trifaro and Dworkind, 1970) -3 26 pmol PO_4/mg protein/min[3]	(MacDonald and Ronzio, 1974) -3 7.2 pmol PO_4/mg protein/min[3]	(Labrie et al., 1971)

[a]Other membrane enzymes present: dopamine β-hydroxylase, cytochrome b-561 and NADH oxidoreductase (Winkler, 1976).

[b]Zymogen granules also contain a membrane-bound lipase activity (Rutten et al., 1975).

[c]Specific activities of enzymes (assayed with endogenous substrates), if available.

ND = has not been reported in the literature.

Biochemical Mechanisms of Secretion

Cyclic AMP

Most secretory cells are regulated by stimuli ranging from polypeptide hormones to increased serum concentration of potassium ions. Table 2 summarizes most tissues that respond to stimuli in one of three ways: secretion of secretory products via secretory granules, release of secretory products through the plasma membrane, or by changes in membrane permeability to water and various ions. Table 2 also incorporates the possible second messenger involved in the stimulus-coupled process. Earl Sutherland was responsible for much of the work on the second messenger concept, which is discussed in several reviews (Butcher et al., 1972; Robison et al., 1971). The second messenger concept involves a hormone receptor interaction (stimulus), which in turn alters intracellular cyclic AMP levels, which correspondingly leads to a physiological response, e.g., altered membrane permeability or secretion. The mechanism by which polypeptide hormones, cholinergic, and catecholamine transmitters elevate cyclic AMP levels is through the activation of adenylate cyclase, which converts ATP to 3'5' cyclic AMP and pyrophosphate. Several models exist to describe the hormone, hormone receptor and adenylate cyclase interaction. These models are based on the fact that adenylate cyclase is membrane bound (usually to the plasma membrane) and the orientation of adenylate cyclase within the membrane is asymmetric, i.e., the catalytic subunit is located on the interior of the cell membrane with the regulatory subunit on the outer cell surface (Øye and Sutherland, 1966). Robison et al. (1967) postulated that adenylate cyclase consists of two entities: a catalytic subunit

Table 2. Summary of secretory tissues

Target tissue	Process	Inducer	Second messenger	Reference
Adenohypophysis (anterior pituitary)	prolactin and TSH secretion	TRF	↑ cyclic AMP[a]	Oka et al., 1974
	LH secretion	LRF	↑ cyclic AMP	Labrie et al., 1973
		TRF	↑ cyclic AMP	Bowers et al., 1968
	GH secretion	cyclic GMP	---	Cehociv et al., 1973
	inhibitor of GH secretion	somatostatin	↑ cyclic AMP ↓ cyclic GMP	Oka et al., 1974
Posterior pituitary	vasopressin secretion	electrical	---	Rasmussen, 1970
Adrenal cortex	corticosteroid release	ACTH	↑ cyclic AMP	Grahme-Smith et al., 1967; Karaboyas & Koritz, 1965
Adrenal medulla	catecholamine secretion	cyclic AMP	---	Peach, 1972
Bladder, toad	membrane permeability	vasopressin	↑ cyclic AMP	Orloff & Handler, 1967
Corpus luteum	progesterone release	LH	↑ cyclic AMP	Rasmussen, 1970
Gastric mucosa	H^+ transport	gastrin	↑ cyclic AMP	Alonso et al., 1965
Intestine	membrane permeability	cyclic AMP	---	Field et al., 1968
Kidney tubules	membrane permeability	cyclic AMP	---	Grantham & Burg, 1968
Lacrimal gland	peroxidase release	cholinergic	---	Herzog et al., 1976

Table 2 (continued)

Target tissue	Process	Inducer	Second messenger	Reference
Leukocytes	histamine release	catechol-amines, methylxan-thines	↓ cyclic AMP	Lichtenstein & Margolis, 1968
Polymorphonuclear leukocytes	lysosomal enzyme secretion	phagocytic stimuli	↓ cyclic AMP ↑ cyclic GMP	Zurier et al., 1974
Liver	glucose release	glucagon	↑ cyclic AMP	Rasmussen, 1970
Mast cells	histamine secretion	antigenic factors	↑ cyclic AMP	
Neuromuscular	muscle contraction	catechol-amines	↑ cyclic AMP	Breckenridge et al., 1967; Goldberg & Singer, 1969
Oviduct, chick	egg white protein secretion	steroids, i.e., estro-gen	---	Palmiter, 1972; Brant & Nalbander, 1956
Pancreas duct cells	electrolyte release	secretin	↑ cyclic AMP	Rutten et al., 1972; Case & Scratcherd, 1972
Pancreas beta cells	insulin secretion	catechol-amines	↑ cyclic AMP	Malaisse et al., 1967; Howell & Montague, 1974
Parietal cells	acid secretion	gastrin	↑ cyclic AMP	Robison et al., 1971

Table 2 (continued)

Target tissue	Process	Inducer	Second messenger	Reference
Parotid gland	amylase secretion	catechol-amines	↑ cyclic AMP	Rabad et al., 1967
		cholinergic	↑ cyclic GMP	Wojcik et al., 1975
Salivary gland (insect)		5-hydroxy-tryptamine	↑ cyclic AMP	Berridge & Patel, 1968
Submandibular gland	enzyme secretion	neurotrans-mitters	↑ cyclic AMP ↑ cyclic GMP	Albano et al., 1975
Thyroid	thyroid hormone secretion	TSH	↑ cyclic AMP	Bastomsky & McKenzie, 1967
		prostaglan-dins	↑ cyclic AMP	Ensor & Murno, 1969
Ventral skin (frog)	membrane permeability	vasopressin	↑ cyclic AMP	Baba et al., 1967

[a]Arrows refer to changes in intracellular cyclic nucleotide levels: ↑ = increase, ↓ = decrease.

Abbreviations: ACTH, adenocorticotrophic hormone; LH, luteinizing hormone; LRF, luteinizing hormone release factor; TRF, thyroid releasing factor; TSH, thyroid stimulating hormone.

and a regulatory subunit. Interaction of the hormone-receptor complex with the regulatory subunit activates the adenylate cyclase. Cuatrecasas (1974) and Birnbaumer et al. (1974) have proposed that interactions between the hormone-receptor complex and the adenylate cyclase may be dependent upon the fluidity of the membrane. Thus, the regulation of adenylate cyclase is a function of receptor concentration, affinity of hormone receptor complex for adenylate cyclase, and diffusion properties of the hormone-receptor complex within the membrane.

Another way cyclic AMP levels can be regulated is through degradation. Insulin (Loten and Sneyd, 1970) and epinephrine (Pawlson et al., 1974) decrease fat cell cyclic AMP levels through the activation of a cyclic nucleotide phosphodiesterase, which catalyzes the breakdown of cyclic AMP to 5' AMP.

To evaluate the role of cyclic AMP as an obligatory second messenger in hormone responses, criteria established by Robison et al. (1968) are commonly referred to. These criteria include: i) the addition of the stimulus (hormone) causes alteration in cyclic AMP levels, usually within minutes of application, ii) the addition of potent cyclic nucleotide phosphodiesterase inhibitors, i.e., theophylline and caffeine, will potentiate or mimic the physiological response produced by the hormone, and iii) application of exogenous cyclic AMP (usually as dibutyryl cyclic AMP) produces the same physiological effect as the hormone. Most, if not all, of these criteria apply to most polypeptide hormones, cholinergic agents, and catecholamines presented in Table 2.

Cyclic GMP and Calcium

Although the role of cyclic AMP as a secondary messenger is well established, there are other possible mediators of hormone action. Among these messenger possibilities are cyclic GMP and calcium ions.

Cyclic GMP was first discovered in 1963 by Ashman et al. Since then, cyclic GMP was found ubiquitously distributed in mammalian tissues, although at lower levels than cyclic AMP (Goldberg et al., 1969). The presence of guanylate cyclase (Schultz et al., 1969; Schultz et al., 1973; Hardman et al., 1971) and hormones capable of increasing cyclic GMP levels (see Table 2) suggests that cyclic GMP may perform as a second messenger in some stimulus-coupled systems. Since Hardman and Sutherland (1969) have shown that guanylate cyclase is largely soluble in most tissues, the mechanism by which membrane impermeable hormones (i.e., polypeptide hormones) elevate cyclic GMP levels within the cell via a cytoplasmic guanylate cyclase is in question. One possible mechanism is through intracellular calcium ions, which are capable of increasing guanylate cyclase activity (Hardman et al., 1973; Nakazawa and Sano, 1974) and inhibiting adenylate cyclase (Malkinson, 1975). However, in parotid gland (Franks et al., 1974) and brain (Bradham, 1972) tissue, calcium is capable of stimulating adenylate cyclase. Another mode of regulation is the susceptibility of cyclic GMP to cyclic nucleotide phosphodiesterases (Change, 1967; Rosen, 1970). However, calcium often inhibits both cyclic AMP and cyclic GMP phosphodiesterases. The most likely mechanism of action of calcium is the activation of guanylate cyclase (Hardman et al., 1973; Nakazawa and Sano, 1974).

Evidence exists that cyclic GMP functions as an antagonist to cyclic AMP. Acetylcholine, which decreases the rate of contraction in cardiac tissue, increases cyclic GMP levels. Isoproterenol, which increases cardiac muscle contractility, increases cyclic AMP. Another example of cyclic GMP opposing cyclic AMP is in the growth cycle of mammalian cells. During the stationary phase of growth, cyclic AMP levels increase and cyclic GMP levels decrease. During the logarithmic phase, the opposite is true. As shown by Table 2, the opposite effects of cyclic AMP and cyclic GMP are apparent in the inhibition of growth hormone release from the anterior pituitary by somatostatin (cyclic GMP levels are low and cyclic AMP levels are high), and in catecholamine stimulated histamine release from polymorphonuclear leukocytes (cyclic GMP levels are up and cyclic AMP levels are down). These instances are examples of bidirectional processes, in which cyclic AMP participates in one direction and cyclic GMP participates in the other. There also appear to be situations where both cyclic AMP and cyclic GMP cooperate rather than antagonize. Such examples are included in Table 2. Upon hormone stimulation of pancreatic beta cells, parotid gland, and the submandibular gland, both cyclic AMP and cyclic GMP levels increase during the secretion response (for references, see Table 2). Such cooperation between cyclic AMP and cyclic GMP may also be the case for the exocrine pancreas (Table 3).

Cyclic Nucleotides and Secretion in the Exocrine Pancreas

Table 3 represents a summary of the effect of secretagogues upon pancreatic intracellular cyclic nucleotides. There are two factors common among the efficacy of most of the secretagogues:

Table 3. Summary of pancreatic secretagogues

Secretagogue	Calcium ions implicated	Cyclic nucleotide[a]	Reference
Acetylcholine	yes	cyclic GMP	Albano et al., 1976
Caerulein	yes	cyclic GMP	Haymovits & Scheele, 1976
Calcium		---[b]	Heisler et al., 1972
Calcium ionophore A-23187	yes	cyclic GMP	Heisler, 1976
Carbachol	yes	cyclic GMP	Wojcik et al., 1975; Haymovits & Scheele, 1976; Kapoor and Krishna, 1977; Heisler et al., 1974
Carbachol plus dibutyryl cyclic AMP	yes	---[b]	Heisler et al., 1972
Cyclic AMP	yes	---[b]	Ridderstap & Bonting, 1969
Dibutyryl cyclic AMP	yes	---[b] (cyclic AMP)	Ridderstap & Bonting, 1969
Epinephrine	yes	cyclic AMP cyclic GMP	Wojcik et al., 1975
Isoproterenol	yes	cyclic AMP	Wojcik et al., 1975
Methoxamine	yes	cyclic GMP	Wojcik et al., 1975
Myo-inositol	yes	---[b]	Slaby & Bryan, 1976
Pancreozymin	yes	cyclic GMP	Haymovits & Scheele, 1976; Robberecht et al., 1974
Pancreozymin-C-octapeptide	yes	cyclic AMP	Klaverman et al., 1975; Kempen et al., 1975
		cyclic GMP	Albano et al., 1976
Pancreozymin plus theophylline	yes	(cyclic AMP)[c]	Ridderstap & Bonting, 1969

Table 3 (continued)

Secretagogue	Calcium ions implicated	Cyclic nucleotide[a]	Reference
Pilocarpine	yes	cyclic GMP	Robberecht et al., 1974
Secretin	yes	cyclic AMP	Gardner et al., 1976
Secretin plus theophylline	yes	cyclic AMP	Robberecht et al., 1974; Gardner et al., 1976
Somatostatin	yes	cyclic AMP	Robberecht et al., 1975
Theophylline	yes	(cyclic AMP)[c]	Ridderstap & Bonting, 1969; Gardner et al., 1976
Vasoactive intestinal polypeptide	yes	cyclic GMP	Robberecht et al., 1974; Gardner et al., 1976

[a]Hypothetical cyclic nucleotide messenger for each secretagogue.

[b]Unknown.

[c]Cyclic AMP is implicated due to the activity of theophylline or dibutyryl cyclic AMP.

absolute dependence upon extracellular calcium ions, and elevation of a cyclic nucleotide due to the hormone action.

Initial work by Kulka and Sternlicht (1968) suggested that cyclic AMP was directly involved in exocytosis of the exocrine pancreas. Other evidence has accumulated (Ridderstap and Bonting, 1969; Bauduin et al., 1971; Heisler et al., 1972) supporting the idea that cyclic AMP was a probable second messenger of at least some pancreatic secretagogues. Evidence supporting a cyclic AMP second messenger hypothesis was based on three observations: the ability of cyclic AMP (10^{-3} M) and dibutyryl cyclic AMP (10^{-5} M) to initiate protein secretion *in vitro* (Ridderstap and Bonting, 1969; Heisler et al., 1972); the ability of theophylline, a potent cyclic nucleotide phosphodiesterase inhibitor, to stimulate protein secretion alone (Ridderstap and Bonting, 1969) or to combine synergistically with other secretagogues (Ridderstap and Bonting, 1969; Robberecht et al., 1974; Gadner et al., 1976); and the detection of intracellular changes of cyclic AMP during stimulus-coupled secretion (Wojcik et al., 1975; Klaverman et al., 1975; Kempen et al., 1975; Robberecht et al., 1975). The reader should note that these observations satisfy the requirements set by Robison et al. (1968). Perhaps, unfortunately, there are conflicting reports in the literature concerning the observations presented above. The ability of either cyclic AMP or dibutyryl cyclic AMP to stimulate pancreas protein secretion is dubious to some workers (Case and Scratcherd, 1972; Case et al., 1969; Benz et al., 1972). Case and Scratcherd claim that a washout effect is responsible for the apparent secretagogue ability of both cyclic AMP or its dibutyryl derivative. The washout effect is the release of secretory proteins

from the excretory ducts, where they had accumulated prior to the experiment. It must also be realized that experiments in which exogenous cyclic AMP was administered are difficult to evaluate due to the impermeability of cell membranes towards cyclic nucleotides. The elevation of pancreatic cyclic AMP levels during secretion is also controversial. Recently, investigators have reported no cyclic AMP changes in pancreas tissue treated with carbachol (a synthetic derivative of acetylcholine), pancreozymin, or caerulein, a decapeptide derivative of pancreozymin (Haymovits and Scheele, 1976; Gardner et al., 1976; Heisler, 1976; Robberecht et al., 1974). Haymovits and Scheele attribute the contradictory results to: a) methodological problems in the assay for cyclic nucleotides, b) difficulties in distinguishing fluid and electrolyte from protein secretion (duct versus acinar cells), and c) the nonphysiological high concentrations of cyclic AMP administered during secretion experiments. In their studies, Haymovits and Scheele utilized a radioimmune assay system developed by Steiner et al. (1972). This assay is based upon competition of a cyclic nucleotide with isotopically labeled cyclic nucleotide analogues for the binding sites on specific antibodies. Advantages of the assay are its sensitivity and specificity. Using radioimmunoassay, several laboratory groups have detected significant elevations in cyclic GMP in pancreatic acinar cells (Kapoor and Krishna, 1977; Haymovits and Scheele, 1976; Albano et al., 1976) following the addition of acetylcholine, carbachol, pancreozymin and caerulein. Since carbachol and pancreozymin increase pancreatic cyclic GMP levels prior to the release of digestive enzymes, cyclic GMP appears to be directly involved in the

initiation of pancreatic secretion. However, a role for cyclic AMP in stimulus-coupled secretion remains unclear.

Kempen et al. (1977) showed the presence of a pancreozymin-secretin sensitive adenylate cyclase associated with the rat pancreas acinar cell. Secretin is a polypeptide capable of stimulating the release of enzymes from acinar cells and electrolytes from duct cells. Even though pancreozymin C-octapeptide activates pancreatic adenylate cyclase (Klaverman et al., 1975), increased levels of intracellular cyclic AMP were not observed (Albano et al., 1976). One explanation is coordination of both adenylate cyclase and phosphodiesterase. Robberecht et al. (1974b) showed that rat pancreas crude homogenate contained cyclic AMP phosphodiesterase activities with high (32 μM) and low (1.9 μM) Km values. However, at least the high Km cyclic AMP phosphodiesterase appears to have minimal impact on cellular cyclic AMP levels (Kempen et al., 1977).

The ability of polypeptide hormones and neurotransmitters to elevate cyclic GMP levels (see Table 3), usually within the first 5 minutes of application, is probably a secondary event, brought about by the increase of free calcium ions in the pancreatic acinar cell (Meldolesi, 1976). Although there is a continuous influx of calcium ions into the acinar cell, the cytoplasmic concentration of calcium is low (10^{-6} M). This is accomplished by binding or segregating calcium ions to intracellular organelles, such as mitochondria, Golgi, and zymogen granules (Clements and Meldolesi, 1975). Immediately following secretagogue stimulation, the free intracellular level of calcium ions increases (Case and Clausen, 1973; Matthews et al., 1973). Two sources of calcium ions, responsible for the intracellular increase, have been postulated. Several groups have

reported that release of organelle bound calcium ions accounts for the observed increase in cytoplasmic calcium (Peterson and Ueda, 1975; Clemente and Meldolesi, 1975b). Others have reported an increase in the influx of extracellular calcium into the acinar cell during secretagogue stimulation, presumably accomplished by increased plasma membrane permeability (Schreurs et al., 1976). The exact mechanism by which calcium ions and cyclic GMP interact during stimulus-coupled secretion is unknown.

In summary, the pancreas acinar cell responds to different stimuli by secreting digestive enzymes. Neurotransmitters, i.e., acetylcholine, increase intracellular levels of calcium ions and cyclic GMP. Polypeptide secretagogues act in a similar way, but in addition they have been shown to activate pancreatic acinar cell adenylate cyclase.

Protein Kinases in Secretion

Cyclic AMP Dependent Protein Kinases

The enzyme, protein kinase, was first implicated as an integral part of the mechanism of action of cyclic AMP in catecholamine induced glycogenolysis (Krebs et al., 1964). Increased concentrations of cyclic AMP, due to activated adenylate cyclase, activate a protein kinase (Walsh et al., 1968) which is able to phosphorylate inactive phosphorylase kinase. Thus, cyclic AMP, via protein kinase, is able to modulate enzymes and evoke the physiological response distinctive of the particular target cell, in this case the breakdown of glycogen to glucose-1-phosphate. Cyclic AMP dependent phosphorylation in glycogenolysis is one of the three phosphorylation processes which fulfill all of Krebs' (1973) criteria, which establish a

positive assessment of cyclic AMP dependent phosphorylation to a particular hormone regulated process. The criteria include: i) the presence of a cyclic AMP dependent protein kinase in the tissue under study, ii) a protein, functional in the regulated process, is phosphorylated, iii) *in vitro* phosphorylation of protein leads to a modified function of that protein, iv) a stoichiometric correlation exists between phosphorylation and the modified function, and v) the modified function can be demonstrated *in vivo*. Other systems that fit these criteria include cyclic AMP dependent protein kinase activation of triglyceride lipase (Corbin et al., 1970) and cyclic AMP mediated inhibition of glycogen synthetase (Schlender et al., 1969).

Further suggesting a role for cyclic AMP dependent protein kinases in hormone controlled systems, Kuo and Greengard (1969) have reported a widespread distribution of cyclic AMP dependent protein kinases. Although these protein kinases do not fit all the criteria mentioned, many fulfill two or three.

Cyclic AMP dependent protein kinases are composed of regulatory and catalytic subunits. When the two subunits are bound together, the enzyme is inactive (Erlichman et al., 1973). Cyclic AMP promotes the disassociation of the two subunits by binding to the regulatory protein, thus producing a free catalytic subunit which is enzymatically active (Erlichman et al., 1973; Sodering et al., 1973). The ability of the regulatory subunit to bind and inactivate the catalytic subunit is not tissue specific, i.e., the regulatory unit, purified from rabbit muscle protein kinase, will inhibit catalytic subunits from a wide variety of rat tissues (Walsh and Ashby, 1974).

Cyclic GMP Dependent Protein Kinases

Cyclic GMP is known to increase in mammalian tissues in response to certain hormones (see Tables 2 and 3). It therefore seems likely that a target enzyme for cyclic GMP exists in mammalian tissues at functionally significant levels. Kuo (1974) has reported the presence of a cyclic GMP dependent protein kinase in a wide variety of guinea pig and rat tissue. Also, Casnellie and Greengard (1974) have reported the presence of an endogenous cyclic GMP-dependent protein kinase activity and associated substrate proteins in smooth muscle membranes, suggesting that physiological actions of cyclic GMP in smooth muscle may be mediated through phosphorylation of membrane proteins. Miyamoto et al. (1973) and Van Leemput-Coutrez et al. (1973) have reported evidence suggesting that cyclic GMP-dependent protein kinases, like cyclic AMP-dependent kinase, are composed of catalytic and regulatory subunits.

Protein Kinases Associated with Secretory Granules

As in other tissues, cyclic nucleotide dependent protein kinases are present in a wide variety of secretory tissues, including the anterior pituitary, the pancreas, the parotid gland, gastric cells, the thyroid, and the adrenal medulla. It is of interest that a cyclic nucleotide is implicated in the secretory response of each of the above tissues (see Tables 2 and 3). Whether cyclic AMP or cyclic GMP initiate secretion through phosphorylation is open to question. However, circumstantial evidence has been accumulating that such a cyclic nucleotide process may be directly involved in stimulus-coupled secretion.

Whether cyclic AMP or cyclic GMP (or both) modulate hormone induction of pancreatic secretion is unknown. However, the machinery exists within the pancreas to carry out a cyclic nucleotide-mediated phosphorylation during the secretory response. The cytosol of the pancreas contains three protein kinases: a cyclic nucleotide independent protein kinase, and cyclic AMP and cyclic GMP dependent protein kinases (Van Leemput-Coutrez et al., 1973).

In addition to cytoplasmic protein kinases, the pancreas contains zymogen granule membrane protein kinase (cyclic nucleotide independent) capable of phosphorylating endogenous zymogen granule acceptors. Pure zymogen granules have been shown to phosphorylate one 130,000 molecular weight protein (MacDonald and Ronzio, 1974). Conflicting results obtained by Lambert et al. (1974) show the presence of more than 10 phosphorylated membrane polypeptides. In either case, it appears that the zymogen granule has potential substrates for its own endogenous kinase activity or a cytoplasmic protein kinase. The latter could be activated by intracellular elevation of either cyclic AMP or cyclic GMP. Using a crude fraction of rat pancreas protein kinase (including all three types), Lambert et al. (1974) demonstrated that exogenous protein kinase could phosphorylate endogenous zymogen granule membranes and this phosphorylation could be increased by 10^{-6} M cyclic AMP. Results obtained by Lambert et al. (1973) show that membrane proteins are phosphorylated during secretion induced by pancreozymin, carbachol and caerulein. Whether this phosphorylation is due to zymogen granule protein kinase or to the cytoplasmic enzyme is not known.

Cytoplasmic and secretory granule protein kinases have been implicated in the secretory response of other secretory systems.

Thyroid stimulating hormone activates a cyclic AMP dependent protein kinase in calf thyroid tissue (Spaulding and Burrow, 1974). It has also been reported that a cyclic AMP dependent protein kinase is activated and participates in parotid gland amylase secretion (Kanamori et al., 1974; Kanamori et al., 1976). Luteinizing hormone, which stimulates progesterone synthesis and release, also appears to activate a cyclic AMP dependent protein kinase (Darbon et al., 1976).

In addition to zymogen granules, secretory granules isolated from the anterior pituitary are able to utilize (γ-$^{32}PO_4$) ATP to phosphorylate endogenous protein components (Labrie et al., 1971). The activity of this granule protein kinase could be stimulated up to 80% by cyclic AMP. However, phosphorylation of endogenous granule membrane proteins was not effected by cyclic AMP. Chromaffin granules also possess an endogenous protein kinase capable of phosphorylating certain membrane proteins (Trifaro and Dworkind, 1970; Trifaro, 1972). Cyclic AMP mediated phosphorylation of membrane polypeptides also occurs in gastric cells (Ray and Forte, 1974), the cerebral cortex (Ehrlich and Routtenberg, 1974), and synaptic membranes (Johnson et al., 1972).

Possible Mechanisms for Protein Phosphorylation in Secretion Granule Exocytosis

Possible mechanisms of exocytosis utilizing the phosphorylation of membrane protein or proteins have largely been ignored in the literature. Presumably, the addition of phosphate to membrane protein(s) could increase the negative charge and/or alter the tertiary structure of the protein, thereby altering the protein's function.

In the rat pancreas, zymogen granules migrate towards and fuse with the plasmalemma, bordering the excretory duct. This fusion process is specific for the plasmalemma and it is possible that a zymogen granule membrane protein kinase recognizes a specific plasmalemma protein substrate. Isolated plasma membranes of the anterior pituitary are phosphorylated (Lemay et al., 1974), as are synaptic vesicles (Johnson et al., 1972). Whether this phosphorylation is due to secretory granule protein kinase is unclear. Kirchberger et al. (1972) have reported that cyclic AMP mediated phosphorylation of cardiac sarcoplasmic reticulum doubled calcium uptake. More recent results have shown that phosphorylation of a 22,000 molecular weight protein correlated closely with facilitated calcium transport (Kirchberger and Chu, 1976). Thus, there is evidence that phosphorylation of membrane proteins may enhance the permeability of membranes, thereby facilitating exocytosis. Cyclic AMP is known to alter cell membrane permeability (Handler, 1973); it is likely cyclic AMP dependent protein kinases may be directly involved in this process.

Ridderstap and Bonting (1969) proposed that cyclic AMP would activate a phospholipase present on the zymogen granule membrane. This would lead to the formation of lysophosphatides, which could cause membrane fusion through their lytic effect on membranes (Lucy, 1970). However, attempts to test this hypothesis (Rutten et al., 1975) have yielded negative results.

The role of calcium ions in pancreatic secretion is unknown, although calcium ions are an absolute requirement for secretion. By establishing a negative charge on zymogen granules (for example, phosphorylation of proteins), calcium may interact with the charged particle to form a calcium bridge, which possibly could bring the

granule within fusion range of the membrane. Since zymogen granules have a high concentration of calcium (Clemente and Meldolesi, 1971), phosphorylation of the zymogen granule membranes may release calcium, similarly to the increase of calcium flow due to phosphorylation of cardiac sarcoplasmic reticulum membrane proteins.

Statement of the Problem

The evidence for cyclic nucleotide mediation of secretory granule secretion through protein kinase is circumstantial. The mechanism of secretagogue action in the rat pancreas is controversial. Although cyclic AMP is believed to be involved in amylase release in parotid gland, as well as other secretory systems, both cyclic AMP and cyclic GMP are proposed second messengers in the pancreatic acinar cell.

Although the pancreatic cyclic nucleotide question is confusing, it is apparent that either cyclic nucleotide would probably act through an appropriate protein kinase. Studies have suggested that protein kinases are involved in many biological processes mediated by cyclic nucleotides. Evidence exists suggesting a protein kinase role in pancreatic protein secretion. These observations include: i) the presence of cyclic AMP and cyclic GMP dependent protein kinases in the pancreatic cytosol, ii) the presence of an endogenous zymogen granule membrane protein kinase, iii) the presence of potential protein kinase substrates on the zymogen granule membrane, iv) secretaoguge stimulation of pancreas tissue results in an increase of cell and zymogen granule membrane protein phosphorylation as reported by Van Leemput-Coutrez et al. (1973), and v) the involvement of protein kinases in other secretory systems mentioned.

The first objective of this research is to determine the role of the zymogen granule protein kinase and zymogen granule phosphorylation in pancreatic protein secretion. This research will shed light on the role of cyclic nucleotides in the acinar cell and perhaps uncover a mechanism for exocytosis in the pancreas.

The presence of a major glycoprotein (GP-2) on the zymogen granule membrane is potentially an excellent marker for studying the role and fate of the zymogen granule membrane during the secretory response. Figure A presents a model of exocytosis in the exocrine pancreas as it is thought to occur. It is obvious that the orientation of proteins within the zymogen granule membrane would be important in assessing roles for these proteins. GP-2 may either be localized on the interior (y) or the exterior (x) of the zymogen granule membrane (Figure A). If GP-2 is located on the exterior of the granule membrane, GP-2 may function by recognizing a secretion marker located on the interior of the plasmalemma. If GP-2 is located on the interior of the granule membrane, GP-2 may function as a stabilizer of the granule, or facilitate release of the secretory product following membrane fusion. The second objective of this research is to determine the surface topology of zymogen granule glycoproteins.

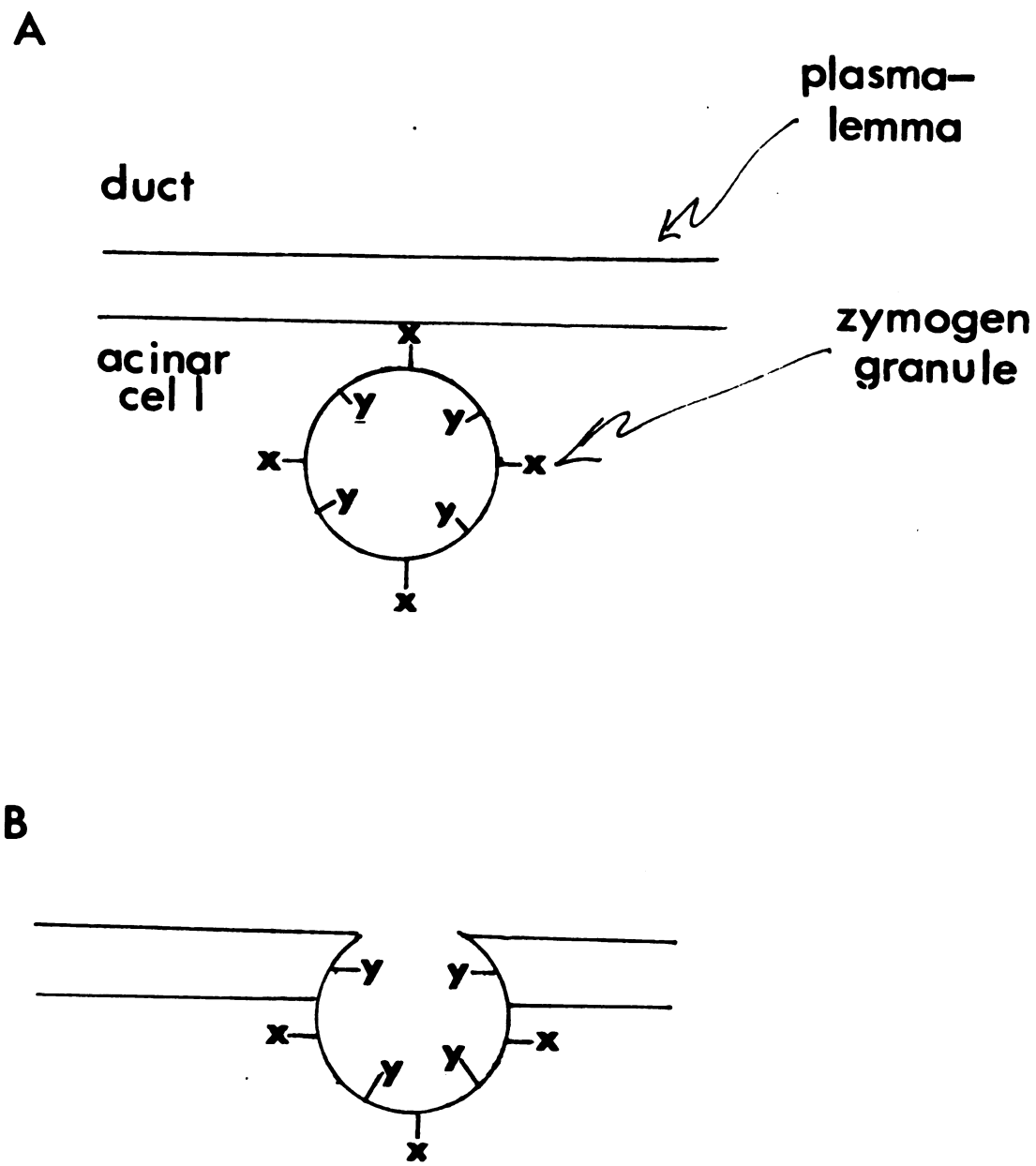

Figure A. Zymogen granule exocytosis.

MATERIALS

Electrophoresis Reagents

acrylamide, technical grade	Eastman Organic Chemicals, Rochester, NY
N,N'-methylenebisacrylamide	Canalco, Rockville, MA
Sodium dodecyl sulfate	Pierce Chem. Co., Rockford, IL
Ammonium sulfate	Canalco, Rockville, MA
N,N,N',N'-tetramethyl-ethyelenediamine (TEMED)	Bio-Rad Labs, Richmond, CA
Coomassie blue R	Sigma Chem. Co., St. Louis, MO
Coomassie blue G	K and K Labs, Plainview, NY
Pyronin B	Harleco, Philadelphia, PA
basic fuchsin	Harleco, Philadelphia, PA

Reagents for Analytical Procedures

Cyclic AMP and cyclic GMP	Sigma Chem. Co., St. Louis, MO
Dansyl chloride	Sigma Chem. Co., St. Louis, MO
Galactose oxidase	Sigma Chem. Co., St. Louis, MO
Glycogen	Sigma Chem. Co., St. Louis, MO
Polyamide sheets	Gallard-Schessinger Chem. Manf. Corp., NY
Silica gel G TLC plates	Analtech Inc., Newark, DE
Silica gel H TLC plates	EM Labs Inc., Elmsford, NY

Miscellaneous

(^{3}H)sodium borohydride, 120 mCi/mmol	New England Nuclear, Boston, MA
($^{32}PO_4$)orthophosphate carrier free	New England Nuclear, Boston, MA
($^{32}PO_4$)-3'5' cyclic AMP 6 Ci/mmol	ICN Pharmaceuticals, Inc., Irvine, CA
Soybean trypsin inhibitor	Sigma Chem. Co., St. Louis, MO
Phospholipid standards	Sigma Chem. Co., St. Louis, MO
Triton X-100	Sigma Chem. Co., St. Louis, MO

Tissue Sources

Sprague-Dawley rats were obtained from Spartan Research Animals, Haslett, MI. Fresh dog pancreases were obtained through the courtesy of Dr. J. Hook, Department of Pharmacology, Michigan State University, and Dr. D. F. Merkley of the Michigan State University College of Veterinary Medicine.

METHODS

Pancreas Tissue Fractionation

All procedures were performed at 4°C according to MacDonald and Ronzio (1972). Using a glass teflon Potter-Elvehjem homogenizer, freshly excised pancreases from decapitated Sprague-Dawley rats were homogenized in ten volumes 0.3 M sucrose containing 0.25 mg/ml soybean trypsin inhibitor (SBTI). To remove cell debris, the homogenate was filtered through several layers of cheesecloth. The filtered homogenate was then centrifuged for 10 minutes at 700 x g to remove cell nuclei, plasma membrane fragments, and other cell debris. The 700 x g pellet was discarded or it was used for the isolation of an enriched plasma membrane fraction.

Zymogen Granule Isolation

The 700 x g supernate was centrifuged at 1600 x g for 30 min in glass conical centrifuge tubes. The resulting pellet consisted of a white bottom layer of zymogen granules overlaid with a light brown fluffy layer of mitochondria. Mitochondria could easily be removed by gently washing them off with a Pasteur pipet and sucrose solution. The washed zymogen granule pellet was then suspended in sucrose-SBTI solution and centrifuged at 1600 x g for 30 min until no visible mitochondria were associated with the granule fraction.

Modifications for Isolation of Dog Zymogen Granules

Dog pancreases were homogenized in the same manner described for rat pancreases. However, the 500 x g supernate was centrifuged for 15 min at 1200 x g (MacDonald, 1974). The rest of the isolation procedure remained the same.

Mitochondria Isolation

Mitochondria, removed from the zymogen granule pellet, were recentrifuged at 8700 x g several times until the visible zymogen granule pellet was removed.

Isolation of Microsomes and the Postmicrosomal Supernatant

The 1600 x g supernate from the zymogen granule isolation was centrifuged at 8700 x g for 15 min. The resulting supernate was centrifuged at 195,000 x g for 1.5 h to pellet total microsomes. When assaying for protein kinase, the microsomes were suspended in Tris-HCl buffer, pH 7.5, and assayed soon after. Rough and smooth microsomes were separated on a 5 to 20% sucrose gradient centrifuged at 195,000 x g for 1.5 h as described by Ronzio (1973). The post-microsomal supernate was defined as the 195,000 x g supernate from which total microsomes were isolated.

Preparation of Subcellular Membranes

Zymogen Granule Membrane Purification

Zymogen granules and granule membranes were isolated and purified as described by Ronzio (1973) and MacDonald and Ronzio (1972). Zymogen granules from 4 g of rat pancreatic tissue (20 to 60 g of dog pancreas matter) were suspended in 1/4 volume of 0.17 M NaCl

containing 0.67 mg/ml SBTI. For every milliliter of zymogen granule suspension, 3 ml of 0.2 M fresh $NaHCO_3$, pH 8.2, was added in order to initiate granule lysis. This suspension was allowed to stand for 1 to 4 h at 4°C or until the solution had an absorbance less than 0.5 O.D. at 660 nm.

In a swinging bucket number 41 cellulose nitrate centrifuge tube, the lysed zymogen granules (7 ml) were layered over 0.3 M sucrose (2 ml), which was above 1.0 M sucrose (3 ml). Following centrifugation at 195,000 x g for 1 h (40,000 rpm in Beckman preparative ultracentrifuge), membranes were collected at the 0.3 M-1.0 M sucrose interface (Meldolesi et al., 1971). This zymogen granule membrane fraction was designated ZGM-2. Mitochondrial membranes as well as other debris were pelleted through the sucrose (MacDonald, 1974).

To remove adsorbed secretory proteins from the ZGM-2 fraction, 0.25 M NaBr (8 ml) was added and the entire solution was sonicated 10 seconds with a Biosonik sonic oscillator equipped with a micro-probe (Bronwill Scientific). More NaBr solution was added to make the total volume 12 ml and then the membranes were pelleted at 195,000 x g for 1.5 h. The pelleted membrane, designated ZGM-3, contained less than 6% of the secretory protein associated with intact zymogen granules. Mitochondrial contamination, as judged by the lack of cytochrome c oxidase activity (Ronzio et al., 1977), was less than 1%.

ZGM-3, when assayed for protein kinase activity, was utilized the same day it was purified. For other experiments, ZGM-3 could safely be stored at -80°C.

Mitochondrial Membrane Isolation

Mitochondria were lysed in 0.2 M $NaHCO_3$, pH 8.2, and centrifuged at 195,000 x g for 1 h. The membrane pellet was resuspended in 0.25 M NaBr, sonicated and then collected by centrifugation at 195,000 x g.

Preparation of an Enriched Plasma Membrane Fraction

An enriched plasma membrane fraction was isolated according to O'Donnell (1977). Pancreases from 6 to 9 rats were homogenized and the 700 x g pellet was obtained as described earlier. The 700 x g cell debris pellet was then resuspended in 6 ml of 0.3 M sucrose and again centrifuged at 700 x g for 10 min. This second pellet was resuspended in 12 ml 0.3 M sucrose using two hand strokes with a glass-glass homogenizer. The homogenate was then stirred at 40°C while 39 ml of 2.0 M sucrose, containing 0.1% dextran (t500, Pharmacia) and 25 mg/ml SBTI, was added dropwise. The final concentration of sucrose was 1.58 M. Nine 1 ml aliquots of the homogenate-sucrose-dextran solution were placed into 12 ml SW41 nitrose cellulose centrifuge tubes with 3 ml of cold 0.3 M sucrose carefully layered on top. After centrifugation at 195,000 x g for 1 h, plasma membrane particles were collected at the 0.3-1.58 M sucrose interface and combined with two other interfaces into an SW50.1 centrifuge tube. After cold double-distilled water was added, the membrane fraction was pelleted by centrifugation at 195,000 x g for 1 h. Following sonication in the presence of NaBr and subsequent collection, the plasma membrane could be stored at -80°C.

Pancreas Acinar Cell Isolation

All incubations were at 37°C under a 95% air-5% CO_2 atmosphere. Acinar cells were isolated from rat pancreas tissue with modifications of the procedure of Amsterdam and Jamieson (1974), as developed by O'Donnell and Mohrlok (unpublished results).

Pancreases from 2 or 3 rats were immediately placed into Solution 1, consisting of: 6 mg/ml NaCl, 0.35 mg/ml KCl, 0.15 mg/ml KH_2PO_4, 0.014 mg/ml $CaCl_2$-$2H_2O$, 0.14 mg/ml $MgSO_4$, Eagles essential amino acids, nonessential amino acids, 2.1 mg/ml $NaHCO_3$, 2.5 mg/ml glucose, and 0.29 mg/ml glutamine. Each pancreas was then injected with 5 ml of Solution 1 containing 0.8 mg/ml collagenase (100-200 U/mg, class II or class IV, Worthington), and 0.3 mg/ml of α-chymotrypsin (60 U/mg, Worthington). After incubating with the enzyme solution for 12 min, the pancreatic tissue was transferred to an EDTA solution, designated Solution 4. Solution 4 is similar to Solution 1 except that 0.74 mg/ml of EDTA replaced $CaCl_2$-$2H_2O$ and $MgCl_2$. EDTA helps to loosen tight junctions between acinar cells by chelating Ca^{2+}. Following 10 min incubation, Solution 4 was decanted and 5 ml of Solution 1 was added back to the tissue. This incubation allows the removal of most of the EDTA.

Next, another 5 ml of the collagenase-chymotrypsin solution was added and the incubation was continued for another 20 to 40 min. After the first 20 min of the final enzyme incubation, tissue samples were removed from the incubation flask and examined under an inverted phase microscope. At the time when freed cells were flowing from the tissue mass, the incubation period was terminated. A glass disposable pipet was used to gently shear the tissue fragments before the tissue was successively filtered through Nitex numbers 26 and 20. The

filtrate containing single cells was layered in a conical centrifuge tube over 5 ml of 4% bovine plasma albumin (BPA) in a high calcium solution designated Solution 8. Solution 8 is similar to Solution 1 except that a higher concentration of calcium is present (36 mg/ml). The higher calcium level increases cell viability.

The cell suspension was centrifuged through 4% BPA to remove cell debris. To the cell pellet, 1 ml of 4% BPA in Solution 8 was added. The viability of the cells was determined by the method of Phillips (1973), using trypan blue. This test does not guarantee the viability of cell processes, but it is an excellent marker for membrane integrity. However, isolated pancreatic acinar cells are able to incorporate radioactive leucine into protein (Mohrlok and Ronzio, unpublished observations) and to respond to hormonal stimulation by releasing amylase (see Results). When the viability of an acinar cell preparation dropped below 90%, the experiment was terminated.

Tissue Culture Incubations

Krebs-Ringer buffer (KRB), used in all the tissue incubations, contained: 95 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$-$7H_2O$, 20 ml of 50x Eagles essential amino acids (GIBCO), 10 ml of 100x nonessential amino acids (GIBCO), 25 mM $NaHCO_3$, 14 mM glucose, and 2 mM glutamine. All tissue incubations containing KRB were incubated at 37°C under 95% air-5% CO_2.

Pancreas Tissue Slices

Once pancreases were removed from the animal, they were placed into 1 ml of KRB and sliced with two scalpel blades into small pieces no bigger than 3 mm in any one dimension. These tissue slices were

then pre-incubated in KRB for 30 min, after which the buffer was replaced with fresh KRB containing either hormone or control saline. In experiments designed to determine *in vitro* zymogen granule phosphorylation patterns, the pre-incubation buffer contained $^{32}PO_4^{-3}$ (30 μCi/ml) and the incubation time was increased to 1 h. Following the pre-incubation, either hormone or saline was added in microliter amounts directly into the tissue media. When tissue was harvested from media containing $^{32}PO_4^{-3}$, it was washed 3 times with cold 0.3 M sucrose (SBTI). In all cases, tissue slices were homogenized in 0.3 M sucrose containing SBTI.

Pancreas Acinar Cell Incubations

Freshly prepared cells were incubated in KRB supplemented with 1% BPA at a concentration of 0.5-1.0 x 10^6 cells per ml of KRB media. Incubations were the same as for tissue slices. The cell viability was checked throughout the incubation.

Analytical Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed with modifications of the method of Fairbanks et al. (1971). Protein or membrane samples were solubilized by heating 5 min at 95°C in 10 mM Tris-HCl, pH 8.0, containing 1% SDS, 2% 2-mercaptoethanol and 5 mM EDTA. Trichloroacetic acid-precipitated protein sometimes remained insoluble under those conditions, and 0.01 N NH_4OH was added to improve solubility. A one-third volume of 20% sucrose-4 mg/ml pyronin B was added to the solubilized protein. Proteins were separated by electrophoresis on 9% polyacrylamide-1% SDS cylindrical gels (0.5 x 10 cm) for 4.5 h at 5 milliamps per gel. Chromic acid washed gel tubes were

dipped in dimethyldichlorosilane (Sigma) and rinsed with hot 1% SDS before use.

Assay for Radioactivity in Polyacrylamide Gels

Following electrophoresis, gels were carefully removed from glass gel tubes with a disposable syringe and needle. The gel was then either sliced into 2 mm pieces with a razor blade and specially fitted block or fractionated into 10 drop fractions (Auto Gel Divider, Savant Instruments). Gel fractions were heated for 1 h at 100°C or overnight at 75°C in sealed scintillation vials containing 0.9 ml of 0.1 N NaOH-1% SDS. The fractions were cooled, neutralized with 1.0 N HCl and mixed with 10 ml of Triton X-100 toluene based scintillation fluid (Mostafa et al., 1970). Radioactivity was assayed using a Packard liquid scintillation spectrometer.

Gel Staining Procedures

Protein Staining

After gels were removed from the gel tube following electrophoresis, they were rinsed with several changes of 10% trichloroacetic acid over 24 h. This step was necessary for the removal of most of the SDS present, which can interfere with the staining process. The tracking dye (pyronin B) was marked with black india ink before staining. Gels were stained overnight in the dark with a fresh solution of 0.4% Coomassie blue in a mixture of 10% trichloroacetic acid and 35% methanol. Gels were then destained in the acid-methanol solution for 7 to 9 h and then rinsed with 10% trichloroacetic acid at 37°C for 10 h or until the background stain was removed. Stained gels were stored at 4°C in the dark. Spectrophotometric scans of

the gels were performed on a linear transport Gilford spectrophotometer at 550 nm.

Periodic Acid-Schiff (PAS) Staining for Carbohydrate Residues

This staining procedure, specific for carbohydrate species, was a modification of the procedure of Fairbanks et al. (1971). As with the protein stain, it was imperative that the SDS was removed from the gel. Trichloroacetic acid washes with frequent changes for a minimum of 36 h were employed for that purpose. Once the SDS was removed, the gel was stained with PAS as described (Fairbanks et al., 1971).

Preparative Slab Gel Electrophoresis

The method employed was the procedure of MacDonald (1974). A slab polyacrylamide gel (11 x 15 x 0.6 cm), containing a 5 to 17% linear acrylamide gradient in 0.1% SDS and 0.1 M sodium phosphate, pH 6.65 (Maizel, 1971), was prepared using a Beckman Density Gradient Former. The ratio of bisacrylamide to acrylamide was 1.1 to 40.

Purification of Glycoprotein-2 from Dog Zymogen Granules

Dog zymogen granule membrane protein designated glycoprotein-2 (GP-2) was purified with modifications of the procedure of MacDonald (1974). Dog zymogen granule membranes (2 to 6 mg protein) were solubilized in 10 mM sodium phosphate, pH 6.65, containing 1% SDS, 5 mM EDTA, 5% sucrose, 2% 2-mercaptoethanol and 5 µg/ml (w/v) pyronin B. The solubilized membranes were then submitted to electrophoresis at 3.3 volts/cm for approximately 24 h on the preparative acrylamide gel described above.

Since the relative mobility of GP-2 in relation to the other zymogen granule membrane proteins is known (MacDonald, 1974) and the fact that GP-2 represents almost 40 to 50% of the total membrane protein in dog zymogen granules, the position of GP-2 on the slab preparative gel was defined. Reference sections, 3 mm wide, were removed (left side, center, right side) from the slab gel and rapidly stained with Coomassie blue G for 1 to 2 h (Malik and Berrie, 1972). The regions of the unstained gel corresponding to GP-2 were excised and electroeluted from the gel in the presence of 0.1 M sodium phosphate, pH 6.65, containing 0.1% SDS and 50 μM phenylmethylsulfonyl fluoride. The addition of phenylmethylsulfonyl fluoride to the latter steps of the GP-2 purification helped to eliminate breakdown of GP-2 to an uncharacterized crystalline product. The eluate was then dialyzed against 2 changes of distilled water containing 3 g AG1X anion exchange resin (Biorad), 2 ml toluene and 50 μM phenylmethylsulfonyl fluoride. The isolated GP-2 was lyophilized and stored at -80°C.

N-Terminal Amino Acid Analysis of GP-2

The N-terminal amino acid of GP-2 was labeled with dansyl chloride by modifications of the procedure of Gros and Labouesse (1969). Approximately 3 nmol of GP-2 in 300 μl of 1% SDS and 4 M urea were reacted with 150 μl of 0.4 M phosphate buffer, pH 9.0, 250 μl of dimethylformamide and 100 μl of 0.2 M dansyl chloride for 30 min. Ten volumes of 10% trichloroacetic acid was added and the precipitate was collected by centrifugation at 1000 x g for 10 min. The precipitate was washed with 1 N HCl and sealed in a glass tube in the presence of 1 N HCl. The protein was hydrolyzed for 20 h at

105°C. Following hydrolysis, the sample was transferred with acetone-glacial acetic acid (3:2, v/v) to a 15 x 15 cm polyamide sheet (Gallard-Schlessinger Chemical Manufacturing Corp.). The sample was spotted on a corner of the sheet, 1 cm from the edges, and then developed in one direction with water-90% formic acid (200:3). After the solvent front migrated 15 cm, the sheet was dried, turned 90° and run in the second direction with benzene-acetic acid (9:1, v/v). The polyamide sheet was examined under ultraviolet light to detect dansylated amino acids. Comparison with dansyl-amino acid standards, kindly provided by Dr. Willis A. Wood, provided the identification of the unknown dansylated amino acid.

Enzyme Assays

Protein Kinases

Protein kinase assays of various pancreatic cell fractions were based on the transfer of the γ-phosphate of ATP to a protein acceptor by formation of a phosphate ester to either serine or threonine residues. The assay incubation included 20 to 100 μg protein containing the protein kinase activity, 50 mM Tris-Cl, pH 7.5, or 50 mM sodium phosphate, pH 6.5, 10 mM $MgCl_2$, 10 mM NaF, 10 mM theophylline, 5 mM EDTA, 0.1 to 0.2 mM (γ-^{32}P)ATP and, where indicated, exogenous substrate such as histone or protamine sulfate.

Theophylline and NaF were present in the assay mix to inhibit phosphodiesterases and ATPases. The latter are known to exist on the zymogen granule membrane (MacDonald, 1974). Theophylline was present in all assays containing cyclic AMP or cyclic GMP. The concentration of the cyclic nucleotides was determined using the

appropriate millimolar absorbances, 14.7 (cyclic AMP) and 13.7 (cyclic GMP) at pH 7.0.

Protein kinase assays were incubated at 37°C at indicated times and then terminated by the addition of 5 ml of 10% trichloroacetic acid and 500 µg bovine serum albumin (BSA). The BSA served as a protein carrier. To obtain low background cpm, the following procedure was employed. The trichloroacetic acid precipitated mixtures were chilled at 4°C for 1 h and the precipitates collected by centrifugation. The precipitated protein was solubilized in 0.1 N NaOH at 4°C, then the protein was quickly reprecipitated. This procedure was repeated twice. Control experiments showed that under these conditions NaOH treatment did not hydrolyze measurable amounts of serine or threonine phosphate esters. The precipitated protein was collected on filters (HAWP, 0.4µ, Millipore Corp.). The filters were dried and counted in a Triton X-100 toluene based scintillation fluid as described before.

Amylase

The amylase assay was a modification of the procedure of Bernfeld (1955). Aliquots (50-100 µl) of cell or tissue incubation media were removed at indicated times and diluted 100-fold with 0.05 M histidine-HCl, pH 6.5. To 0.5 ml of the diluted enzyme, 0.5 ml of 1 mg/ml glycogen in 0.05 M histidine-HCl with 0.03 M NaCl, pH 6.9, was added. After 3 to 15 min at 37°C, the incubation was terminated with the addition of 1.0 ml of 3,5-dinitrosalicylic acid reagent, consisting of 1.0 g 3,5-dinitrosalicylic acid reagent, 0.4 moles NaOH, 30 g sodium potassium tartrate, and water to 100 ml. The terminated enzyme assay mixture was then heated for 5 min in a

boiling water bath, cooled and diluted with 10 ml of distilled water. One milliliter of the diluted enzyme assay was measured spectrophotometrically at 495 nm. A calibration curve of standard maltose solutions (0.1 to 1.0 mg/ml) was used to determine the amount of maltose liberated. Amylase activity was then expressed in terms of mg maltose liberated in 3 min at 37°C by 1 ml of enzyme solution.

Galactose Oxidase

Before using galactose oxidase to label glycoproteins, its specific activity was checked using the assay of Avigad et al. (1962). Galactose oxidase oxidation of galactose was measured using a coupled peroxidase-o-tolidine system. To calculate units of galactose oxidase activity per mg protein, the ratio of change of absorbance at 425 nm to mg enzyme protein used in the assay was determined.

Galactose Oxidase NaB^3H_4 Labeling of Membranes

The procedure used to label membrane glycoproteins by oxidation of galactose residues and their subsequent reduction with NaB^3H_4 was similar to the procedure of Gamberg and Hakomori (1973). Galactose oxidase (15 U/ml) was incubated with 50 to 300 µg membrane protein suspended in phosphate buffered saline, pH 7.2, containing 0.3 mg/ml SBTI and 2.0 mg/ml BSA. After 1 h at room temperature, 10 µCi NaB^3H_4 was added for every 100 µl galactose oxidase-membrane protein solution. After 10 min incubation with NaB^3H_4, the reaction was terminated either by collecting the membranes by centrifugation at 195,000 x g, or by trichloroacetic acid precipitating membranes in the presence of 1% phosphotungstic acid. ^3H-labeled glycoproteins

were then detected by analysis on polyacrylamide-SDS gel electrophoresis.

It should be noted that during incubation of the membranes with NaB^3H_4, the pH did not go above 9.0.

Isolated membranes were not the only substance labeled by the galactose oxidase-NaB^3H_4 method. This method was also used to label isolated pancreas acinar cells with the object to incorporate ^3H into galactose residues of glycoproteins located on the cell surface. It was obvious that the main problem associated with these experiments was to keep the label on the outside surface of the cell and not in the interior. Therefore, cell viability was extremely important in these experiments. To insure over 90% viable cells during the labeling procedure, Krebs-Ringer buffer (KRB) with 1% BPA was used as the labeling medium in place of phosphate buffered saline. Other modifications in the labeling procedure included a shorter incubation with galactose oxidase (45 min) and higher concentration of NaB^3H_4 (200 μCi/ml).

After the cells were labeled, they were layered over 8 ml of Solution 9 containing 4% BPA. By centrifuging the cells through the BPA, free NaB^3H_4 and ^3H were easily removed. The ^3H-labeled cells were then analyzed on polyacrylamide-SDS gels.

Phospholipid Analysis

Lipid Extraction

Phosphate labeled zymogen granules or zymogen granule membranes were extracted with 20 volumes of chloroform:methanol (2:1).

Silicic Acid Column Chromatography

A silicic acid column was prepared by adding a chloroform slurry of 7 g Unisil (200-325 mesh), activated at 135°C for 18 h, to a 1 cm diameter glass column. Extracted $^{32}PO_4$-labeled lipids were dissolved in 5 ml of chloroform:methanol (2:1) and loaded on the column. Once the lipid fraction was loaded on the column, a successive order of chloroform-methanol elution solvents were added: 90 ml of (19:1), 75 ml of (4:1), and 80 ml of (2:1), as described by Chang and Sweeley (1963). Fractions (10 ml) were collected and 1 ml aliquots removed, placed into scintillation vials and dried with a stream of nitrogen. Once dry, 10 ml of Triton X-100 toluene scintillation fluid was added and the samples were assayed for radioactivity. Another 1 ml aliquot was used for a phosphate assay. The remaining fractions, representing peaks of radioactivity, were pooled and evaporated.

Thin Layer Chromatography

Lipid fractions were analyzed using two-dimensional thin layer chromatography (Singh and Privett, 1970). Heat activated (3 h at over 90°C) silica gel G plates (250 microns, Analtech, Inc.) were spotted with 50 to 300 µl of chloroform:methanol (2:1) soluble lipid extract in the lower right-hand corner 2 cm from the edges. The plate was then developed in the first-dimensional solvent, chloroform-methanol-28% ammonia (65:35:5). Once the solvent front reached the top of the plate, the TLC plate was dried by evaporation in a hood. After turning the plate 90°, the second dimension in chloroform-methanol-acetic acid-water (25:15:4:2) was developed.

Once the two-dimensional plate was completed, phospholipids were detected and identified by several methods. Comparison of unknown relative mobility values to standard phospholipids (phosphatidylcholine, Sphingomyelin, phosphatidylserine, lysophosphatidylcholine, phosphatic acid, phosphatidylethanolamine, and phosphatidylinositol) enabled the identification of unknown phospholipids detected with iodine and phosphate reagent (molybdenum blue, Applied Science Lab., Inc.). Ninhydrin (Ninsol, Pierce Chemical Co.) spray was also employed to detect phospholipids containing $-NH_2$ groups.

To confirm the identification of phosphatidylinositol, a second TLC system was employed. The phosphatidylinositol spot on the two-dimensional TLC system was removed and extracted with chloroform: methanol (2:1). The extract was then spotted on a silica gel H plate 250 microns thick. The plate was developed in chloroform-methanol-4 N ammonia (9:7:2) according to the procedure of Gonzalez-Sastre and Folch-Pi (1968). Phosphate spray was used to identify phosphatidylinositol.

Purification of Protein Kinase Inhibitor

A heat stable protein fraction capable of inhibiting cyclic AMP dependent protein kinases was isolated from rat pancreas (Kuo, 1975) by the method of Ashby and Walsh (1974).

Preparation of (γ-$^{32}PO_4$)ATP

(γ-$^{32}PO_4$)ATP was prepared by the procedure of Richardson (1971) yielding 600 to 4000 cpm/pmol ATP (4 mCi/mmol). Analysis of the product on DEAE chromatography (Whatman microgranular DE 52) showed that over 90% of the incorporated $^{32}PO_4$ was associated with ATP.

Analytical Procedures

Protein Assay

Protein was measured by the method of Lowry (1951) using BSA as a standard. Samples containing sucrose were precipitated with 10% (w/v) trichloroacetic acid and solubilized in 0.1 N NaOH-1% SDS before analyzed.

Phosphate Analysis

This procedure, measuring inorganic phosphate, was a modification of the procedure of Chen et al. (1956). One volume of 10% ascorbic acid and 6 volumes of 0.42% ammonium molybdate in 1 N H_2SO_4 were mixed with the phosphate unknown at a ratio of 7 to 3. After 1 h incubation at 37°C, absorbance at 820 nm (0.01 μmole = 0.260 O.D. units) was read.

RESULTS

Studies on Zymogen Granule Protein Kinase

Subcellular Distribution of Pancreatic Protein Kinase

Protein kinase was assayed, using histone as an exogenous substrate, in various pancreatic subcellular fractions and was found to be widely distributed among those fractions (Table 4). The postmicrosomal supernate contained almost 35% of the total cell protein kinase activity, including activity dependent on cyclic AMP and cyclic GMP. In contrast, zymogen granules accounted for 4.5% of the total protein kinase activity. Smooth microsomes and zymogen granule membranes contained the highest protein kinase specific activity of all the fractions, 41.9 and 60 pmol $^{32}PO_4$ per mg protein per min, respectively. Although the smooth microsomal protein kinase could be stimulated 3-fold in the presence of 2 μM cyclic AMP and 2-fold in the presence of 2 μM cyclic GMP, protein kinase of zymogen granule membranes was non-responsive to either cyclic AMP or cyclic GMP. However, zymogen granules contained both cyclic AMP (5.5 pmol $^{32}PO_4$/mg) and cyclic GMP (3 pmol $^{32}PO_4$/mg) dependent kinase activities. Cyclic nucleotides were unable to activate a significant level of protein kinase activity in rough microsomes and mitochondria.

Table 4. Subcellular distribution of protein kinase in fractions isolated from rat pancreas

Pancreas tissue was homogenized and cell fractions were isolated as described in Methods. Protein kinase was assayed using histone as an exogenous substrate. All assays were performed as described in Methods.

Fraction	Cyclic nucleotide[a]	n	Net activity[b]	Cyclic nucleotide dependent activity[b]
Homogenate	none	6	56.8 ± 2.8	
	cyclic AMP	6	94.8 ± 3.7	38
	cyclic GMP	6	66.2 ± 8.0	8
Zymogen granules	none	6	11.0 ± 1.0	
	cyclic AMP	9	16.5 ± 0.5(50)	5.5
	cyclic GMP	9	14.5 ± 0.5(32)	3.5
Zymogen granule membranes	none	3	60.0 ± 5.0	
	cyclic AMP	3	56.0 ± 4.0	
	cyclic GMP	3	57.0 ± 6.0	
Smooth microsomes	none	2	41.9 ± 2.3	
	cyclic AMP	5	113.7 ± 3.6(171)	71.8
	cyclic GMP	3	89.5 ±10.4(114)	47.6
Rough microsomes	none	3	22.4 ± 1.3	
	cyclic AMP	4	19.4 ± 1.5	
	cyclic GMP	4	16.8 ± 1.6	
Postmicrosomal supernate	none	6	22.2 ± 1.2	
	cyclic AMP	6	34.2 ± 1.7(54)	12.0
	cyclic GMP	6	39.1 ± 3.6(76)	16.9
Mitochondria	none	6	9.0 ± 1.0	
	cyclic AMP	6	10.0 ± 0.5	
	cyclic GMP	6	8.5 ± 2.0	

[a]The concentration of cyclic AMP and cyclic GMP was 2 μM; n denotes number of experiments.

[b]pmol $^{32}PO_4$ transferred to histone per mg protein per min ± SEM. Numbers in parentheses represent percent stimulation ($P<0.05$).

Zymogen Granule Protein Kinases

Zymogen granules were used as an enzyme source, in place of granule membranes, for several reasons. One reason was the ease of availability of granules compared to membranes. The time required to isolate granules was 45 min compared to 8 h for the isolation of membranes. Second, at least 10 rats are required to isolate about 200 μg membrane protein, whereas 1 rat pancreas yielded milligram quantities of granules. With the use of zymogen granules, several precautions were made. Since zymogen granule membranes had 10-fold less mitochondrial contamination than granules (Ronzio et al., 1977), it was possible that a portion of the granule protein kinase activity was derived from mitochondria. The lack of cyclic nucleotide dependent protein kinase activity associated with the mitochondrial fraction, combined with a lower kinase specific activity in the mitochondrial fraction than in zymogen granules (Table 4), suggest that the amount of zymogen granule protein kinase due to mitochondrial contamination was minimal.

The zymogen granule membrane protein kinase was dependent on Mg^{2+} and phosphorylated serine and threonine residues to form phosphate esters (MacDonald, 1974). Tables 5 and 6 show similar properties of the zymogen granule protein kinase. Inhibition of kinase activity by excess EDTA is indicative of a requirement for Mg^{2+}. Stability of the phosphorylated product (histone) in sodium acetate, pH 5.4, and susceptibility to alkaline hydrolysis is indicative of peptidyl serine or threonine phosphate esters (Labrie et al., 1971). Trixon X-100 increased total cellular protein kinase activity (no effect on cyclic nucleotide dependent activity), but inhibited zymogen granule protein kinase. Triton X-100 has been used in some

Table 5a. Partial characterization of protein kinase activity

Zymogen granule and crude homogenate fractions were prepared as described in Methods. See Methods for details of protein kinase assay.

Condition Varied	% Protein Kinase Activity	
	Crude Homogenate	Zymogen Granules
None	100	100
Minus $MgCl_2$	40	45
Plus EDTA (100 mM)	25	50
Plus 0.1% Triton X-100	300	50
Boiled enzyme	7	6

Table 5b. Characterization of protein kinase product

Treatment	% cpm $^{32}PO_4$ Incorporated	
	Crude Homogenate	Zymogen Granules
None	100	100
Plus 1 N NaOH[a]	6	8
Plus 0.2 M sodium acetate[b] pH 5.4	95	98

[a] 5 min in boiling water bath.

[b] 10 min at room temperature.

systems to uncover latent membrane protein kinase activity (Korenman et al., 1974). This appears not to be the case with zymogen granules.

Figure 1 summarizes some kinetic data of zymogen granule-mediated phosphorylation of exogenous substrates. Freshly prepared zymogen granules were able to phosphorylate histone (2 mg/ml) at a linear rate for 2 min in the presence of 0.2 mM (γ-$^{32}PO_4$)ATP, 10 mM NaF, and 5 mM EDTA (Figure 1B). The pH optimum of zymogen granule kinase was found to be 7.5, slightly higher than the crude homogenate enzymes (Figure 1C). Also, Figure 1A illustrates linearity of $^{32}PO_4^{-3}$ incorporation versus µg of zymogen granules. Granules up to 100 µg protein were able to phosphorylate histone at a linear rate.

The zymogen granule protein kinase is capable of phosphorylating several protein substrates, including protamine sulfate and histone (Figure 2). Saturating levels of these substrates were around 1 mg/ml. Endogenous phosphorylation of zymogen granule polypeptides was considerably lower than phosphorylation of the exogenous substrates mentioned.

MacDonald and Ronzio (1974) showed that purified zymogen granule membranes selectively phosphorylated a single membrane component of 130,000 molecular weight. Lambert et al. (1974) claimed that endogenous phosphorylation of zymogen granule membranes resulted in phosphate incorporation into no less than 9 polypeptides. SDS-acrylamide gel electrophoretograms of zymogen granules and granule membrane phosphorylation patterns are presented in Figure 3. Under assay conditions described by Lambert et al. (1974), zymogen granule membranes phosphorylated a single high molecular weight membrane polypeptide. These results are similar to the data obtained by

Figure 1. Kinetic data of zymogen granule protein kinase.

Zymogen granules were isolated according to Methods. Protein kinase was assayed, using histone as an exogenous substrate. The assay incubation contained 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM NaF, 5 mM EDTA, and 0.1 to 0.2 mM (γ-$^{32}PO_4$)ATP. Histone (2 mg/ml) was added to provide an exogenous substrate. All incubations, except where indicated, were terminated after 1 min with 10% trichloroacetic acid and 500 µg BSA. Samples were counted as described in Methods.

A) Enzyme linearity. Varying amounts (15 to 100 µg) of zymogen granules were assayed as described above.

B) Time course. At indicated times, reactions were terminated as described above. About 50 µg protein were used in the kinase assays.

C) pH optimum curve. Different phosphate buffers were prepared and used in the protein kinase assays. Solid circles represent zymogen granules, open circles represent crude homogenate enzyme.

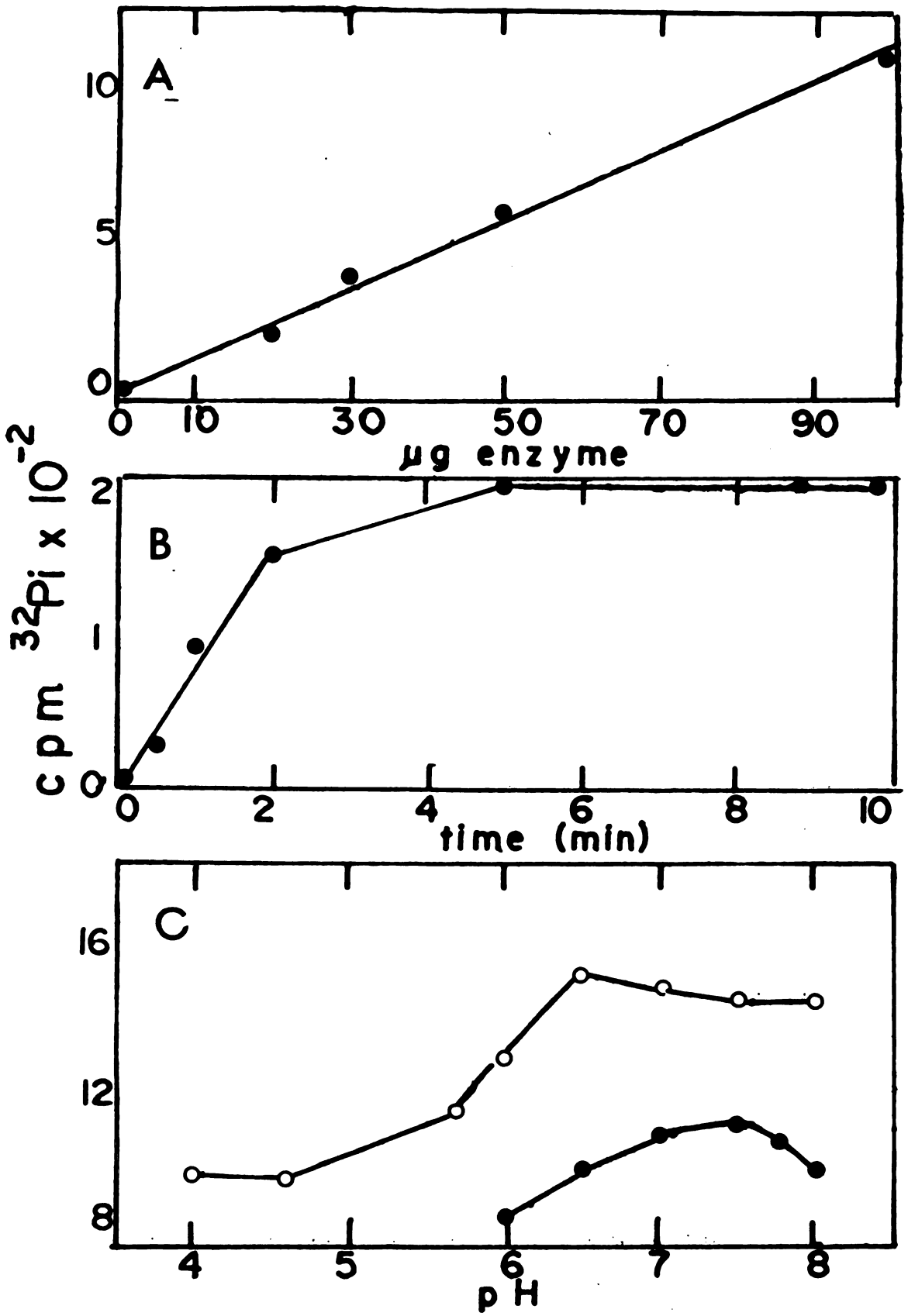
A
10
5
0
0
10
30
50
70
90
μg enzyme
B
cpm ^{32}Pi x 10^{-2}
2
1
0
0
2
4
6
8
10
time (min)
C
16
12
8
4
5
6
7
8
pH

Figure 1

Figure 2. Exogenous substrates for the zymogen granule protein kinase.

The ability of zymogen granules to phosphorylate exogenous substrates was studied. Varying amounts of histone or protamine sulfate were added to the protein kinase assay incubation containing zymogen granules. Enzyme assays were carried out as described in Figure 1. Endogenous represents endogenous phosphorylation of zymogen granule polypeptides.

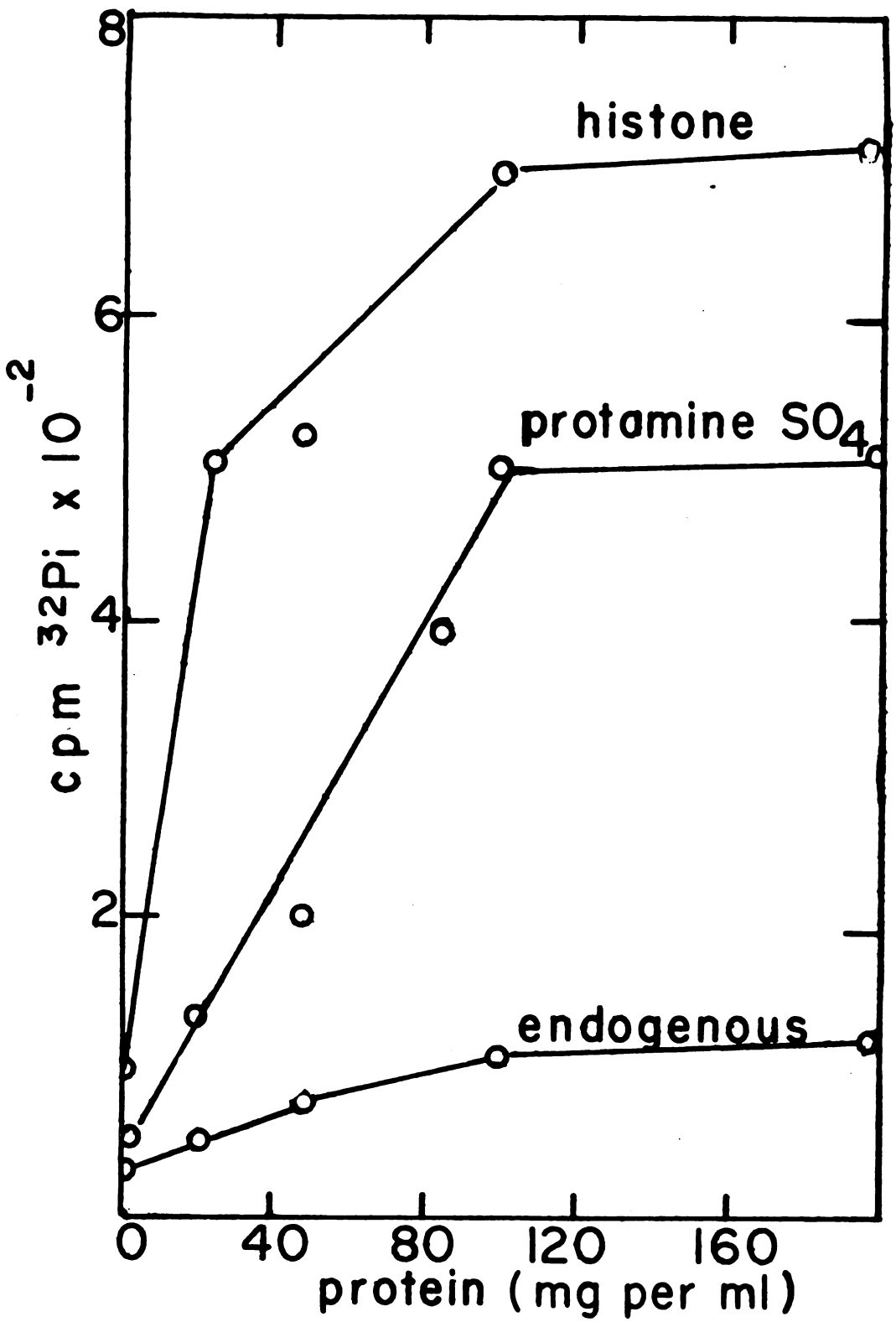
8
6
4
2
cpm 32Pi x 10-2
histone
protamine SO4
endogenous
0
40
80
120
160
protein (mg per ml)

Figure 2

Figure 3. Electrophoretic analysis of protein kinase substrates in zymogen granules and granule membranes.

Zymogen granules (open circles) and granule membranes (closed circles) were prepared as described in Methods. Granule membranes (150 µg) and intact zymogen granules (190 µg) were incubated with 1.2×10^{-4} (γ-$^{32}PO_4$)ATP, 10 mM $MgCl_2$, 10 mM NaF, 1 mM EDTA, and 50 mM Tris-HCl, pH 7.5, for 1 min at 37°C. Incubations were terminated by the addition of 300 µl of 0.3 N perchloric acid, 0.5 mM H^3PO_4, and 25 mM ATP to the 100 µl reaction mixtures. Precipitates were collected by centrifugation. The pellets were dissolved in 1% SDS, 5 mM EDTA, 0.01 M Tris-HCl, pH 8.0, and 2% β-mercaptoethanol and then applied to 9% polyacrylamide-1% SDS gels and analyzed as described in Methods.

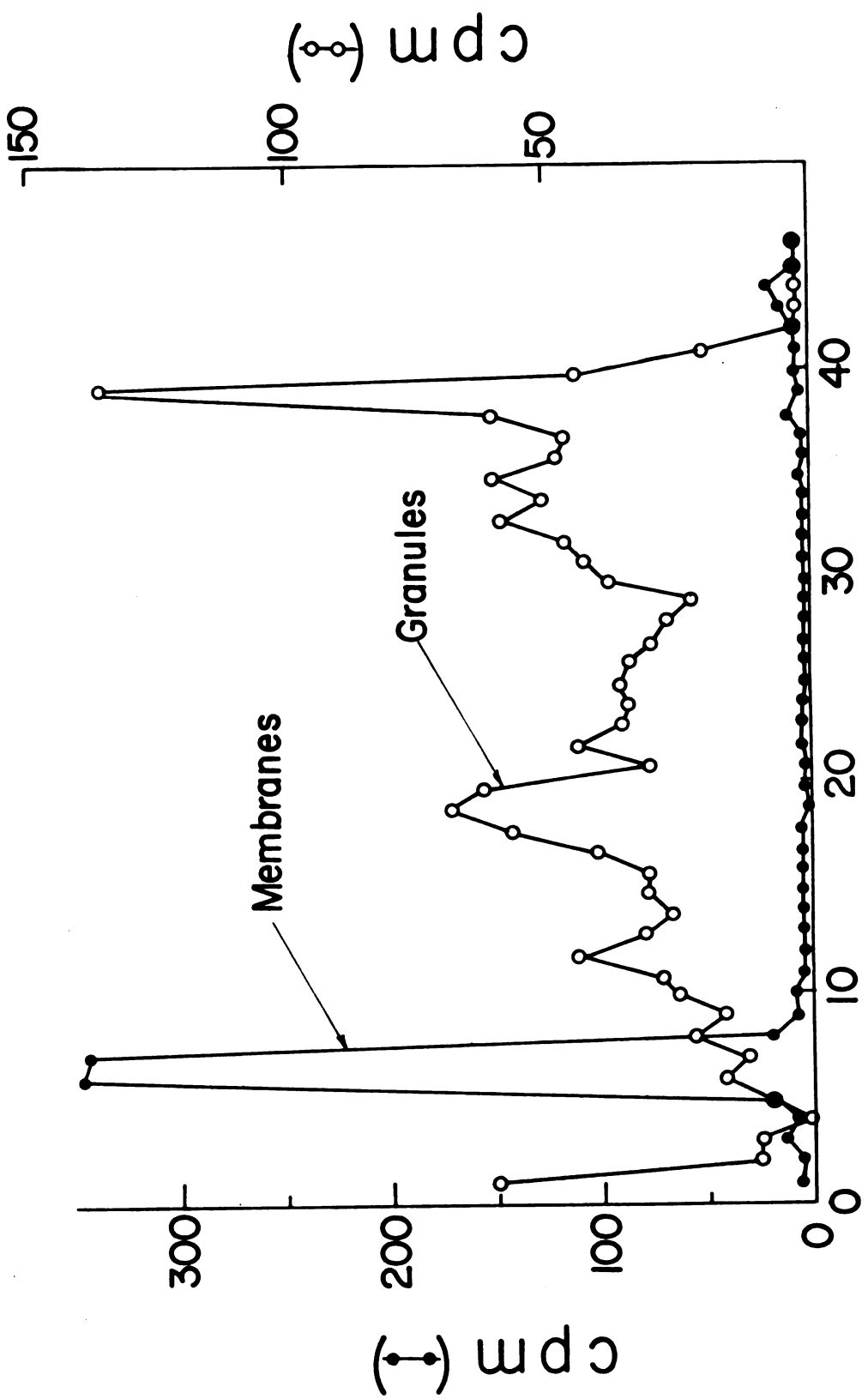
cpm (•)
300
200
100
0
cpm (○)
150
100
50
Membranes
Granules
0
10
20
30
40
FRACTION NUMBER

Figure 3

MacDonald and Ronzio. When granules were incubated with (γ-$^{32}PO_4$)ATP, more than 6 polypeptides of predominantly low molecular weight were phosphorylated (Figure 3). Due to the low counts of $^{32}PO_4^{-3}$ incorporated into zymogen granule acceptors, determination of whether these phosphorylated peptides were loosely associated with or intrinsic to the granule membrane was not possible. However, less than 20% of the counts associated with granules was associated with secretory proteins.

Zymogen granules, isolated from the rat pancreas, have 3 different protein kinase activities: cyclic nucleotide independent, cyclic AMP dependent, and cyclic GMP dependent. Data presented in Table 4 illustrate that the addition of cyclic AMP or cyclic GMP to zymogen granules produces a small increase in the phosphorylation of histone. The difference between basal protein kinase activity and activity in the presence of a cyclic nucleotide is defined as cyclic nucleotide dependent activity. Therefore, in zymogen granules, cyclic AMP stimulates protein kinase activity 5.5 pmol $^{32}PO_4$/mg protein/min, and cyclic GMP stimulates protein kinase 3 pmol $^{32}PO_4$/mg protein/min. Figure 4 illustrates the effect of cyclic AMP and cyclic GMP concentrations upon zymogen granule protein kinase. Cyclic AMP and cyclic GMP maximally stimulate separate granule kinase activities at 2 μM.

It has already been mentioned that granule membrane protein kinase is non-responsive to cyclic nucleotides. Upon purification of zymogen granule membranes, cyclic nucleotide dependent protein kinase activities were lost, apparently during NaBr wash of zymogen granule membrane fraction 1 (Table 6). The NaBr extracted membrane fraction (ZGM-2) contained less than 10% of the original cyclic nucleotide dependent protein kinase activities. In contrast, cyclic nucleotide

Figure 4. Effect of cyclic AMP and cyclic GMP concentrations on zymogen granule protein kinase.

One milligram of cyclic AMP or cyclic GMP was dissolved in 1 ml of phosphate buffer, pH 7.0. Concentration of each cyclic nucleotide was determined spectrophotometrically using 14.7 and 13.7 as the mM absorbancies of cyclic AMP and cyclic GMP, respectively. Zymogen granules were assayed for protein kinase activity in the presence of various concentrations of each cyclic nucleotide. Theophylline, 10 mM, was included in the assay mixes. Otherwise the protein kinase assay mix was identical to Figure 1. Results are expressed as a ratio of plus cyclic nucleotide activity to activity measured in the absence of cyclic nucleotides. Open circles refer to kinase activity in the presence of 2 μM cyclic GMP. Solid circles refer to kinase activity stimulated by 2 μM cyclic AMP.

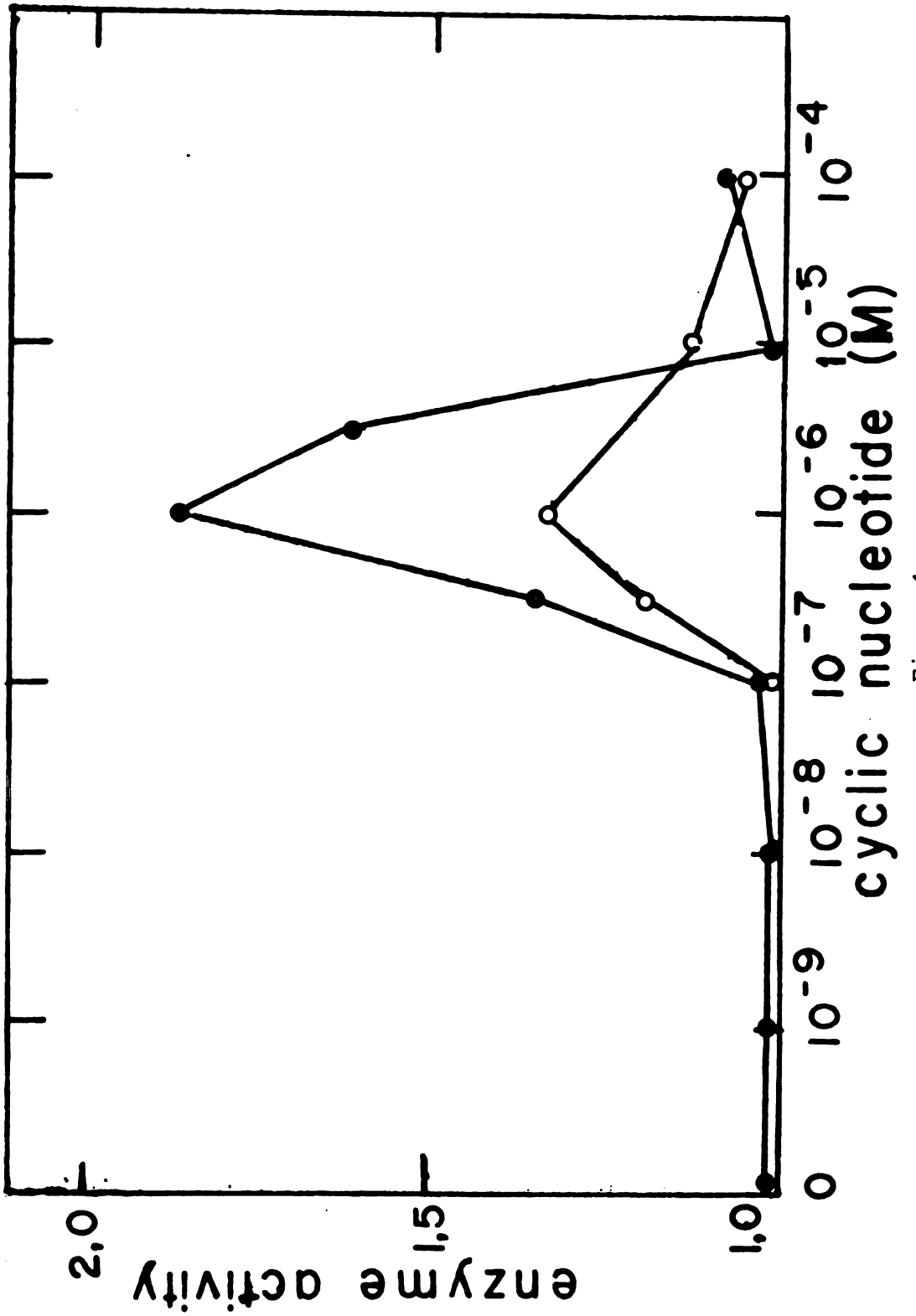
enzyme activity
2,0
1,5
1,0
0
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
cyclic nucleotide (M)

Figure 4

Table 6. Protein kinase activity in zymogen granule fractions

Zymogen granule fractions were isolated and assayed for protein kinase as described in Methods. Cyclic nucleotide concentrations were 2 μM, and theophylline (10 mM) was included in all assays. Granule contents refer to the soluble fraction following centrifugation of lysed granules. ZGM-2 is NaBr extracted ZGM-1.

Fraction	% Protein Kinase Activity		
	minus cyclic nucleotide	plus cyclic AMP	plus cyclic GMP
Intact granules	100	100	100
Granule content	0	0	0
ZGM-1[a]	80	75	70
ZGM-2	60	8	5

[a]ZGM = zymogen granule fraction. The number refers to the stage in the isolation procedure described in Methods.

independent kinase activity was reduced by only 40%. It is not clear whether just the regulatory subunit or the holoenzyme was removed from the granule membranes during NaBr extraction. No detectable protein kinase, cyclic nucleotide dependent or independent, was found in the NaBr wash, nor was there an increase of cyclic nucleotide independent kinase activity on the granule membrane. Such an increase would be expected if the regulatory subunit of the cyclic nucleotide dependent protein kinase was removed. The effect of ionic strength on the zymogen granule protein kinase is presented in Table 7. Washing zymogen granules in 150 mM KCl reduced all three protein kinases by about 40%. Cyclic AMP and cyclic GMP dependent protein kinases were still measurable. The activity remaining associated with salt washed granules will be considered to be the intrinsic protein kinase of zymogen granules.

Translocation of Protein Kinase Activity to Zymogen Granules

Korenman et al. (1974) first demonstrated a hormone controlled redistribution of cellular protein kinase in rat uterus. Isoproterenol and epinephrine induced uterine tissue to translocate cyclic AMP dependent protein kinase (catalytic subunit) from the cytoplasmic fraction to microsomes. Presumably this process occurs during a transient elevation of intracellular cyclic AMP, which dissociates the cyclic AMP dependent holoenzyme complex. Although the zymogen granule fraction contains 4.5% of the total pancreatic protein kinase, the amount of kinase activity stimulated by cyclic AMP or cyclic GMP is small compared to postmicrosomal supernate and smooth microsome protein kinases. It is possible that intracellular rises of cyclic GMP or cyclic AMP would entail a dissociation of cytoplasmic

Table 7. Stability of zymogen granule protein kinases

Zymogen granules, derived from 10 ml of pancreatic homogenate (see Methods), were resuspended in 5 ml of 0.2 M sucrose containing 150 mM KCl. Zymogen granules were collected by centrifugation at 1,000 x g for 30 min. Granules were then assayed as described in Methods. KCl wash reduced the yield of zymogen granules by 24%, equivalent to the yield following 0.3 M sucrose wash.

Addition	Zymogen Granule Protein Kinase Activity[a]		Activity recovered in KCl wash
	0.3 M sucrose wash	KCl wash	
None	11	6	3
Cyclic AMP (2 μM)	16	9	8
Cyclic GMP (2 μM)	14	8	5

[a] pmol $^{32}PO_4^{-3}$/mg protein/min.

protein kinases resulting in catalytic subunits being transferred to zymogen granules. Thus, phosphorylation of granule membrane polypeptides could result from hormone modulation of pancreatic cyclic nucleotides, i.e., during secretion (Lambert et al., 1974).

To determine whether zymogen granules could bind protein kinase in a reconstituted system, zymogen granules were incubated with cyclic nucleotides, theophylline and the postmicrosomal supernate. This fraction contains cytoplasmic cyclic nucleotide-dependent protein kinases (Table 4). In a separate experiment, theophylline was shown to completely inhibit the breakdown of cyclic AMP to AMP, presumably by inhibiting cyclic nucleotide phosphodiesterase activity (Robberecht et al., 1974b). Cyclic ($^{32}PO_4$)AMP was incubated with 10 ml of postmicrosomal supernate with and without theophylline. At indicated times aliquots of the incubation were analyzed on polyethyleneime-cellulose plates by the method of Bohme and Schultz (1974). After the plate was developed in 0.1 M KCl, AMP migrated with a relative mobility of 0.07 (literature value 0.06) and was easily separated from cyclic AMP, whose relative mobility was 0.24 (literature value of 0.3). Figure 5 demonstrates that postmicrosomal supernate, without theophylline, degraded cyclic AMP to AMP by 20% after 10 min (open circles). In the presence of theophylline, the postmicrosomal supernate had no effect on cyclic AMP stability (closed circles).

Following incubation of zymogen granules with postmicrosomal supernate and theophylline, granules were isolated and protein kinase activity measured. Table 8 summarizes these results. Addition of cyclic AMP (2 μM) to the postmicrosomal supernate-zymogen granule mixture increased the level of cyclic nucleotide independent

Figure 5. Stability of cyclic AMP in the postmicrosomal supernate fraction.

Postmicrosomal supernate was prepared from 2 rat pancreases and incubated with 10^{-6} M cyclic ($^{32}PO_4$)AMP, 3 x 10 cpm. At indicated intervals 200 µl aliquots were removed and acid precipitated. Small aliquots (25 to 50 µl) plus 5 nmol of cyclic AMP and AMP were spotted 2 cm from the edge of a polyethyleneimine cellulose plate. After developing in 0.1 M KCl, nucleotide spots were visualized with ultraviolet light. Areas of the plate corresponding to cyclic AMP and AMP were removed and counted using a Triton X-100 toluene based scintillation fluid. Results are expressed as percent of counts in cyclic AMP of the total counts in both cyclic AMP and AMP. Closed circles, theophylline added; open circles, theophylline omitted from the incubation.

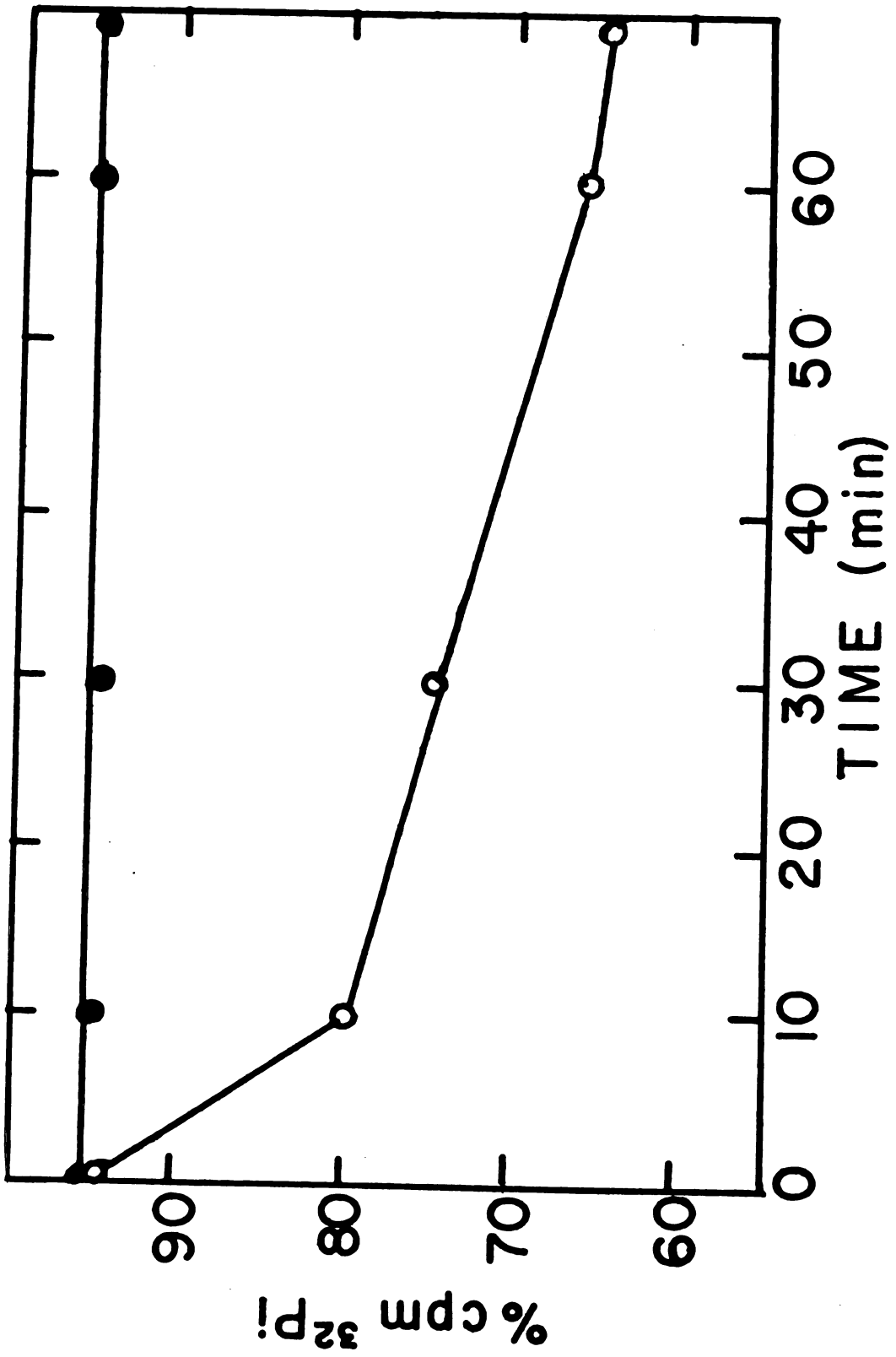

Figure 5

Table 8. Translocation of protein kinase from the postmicrosomal supernate to zymogen granules

Zymogen granules were suspended in the freshly prepared postmicrosomal supernate at a concentration equivalent to the original homogenate and incubated at 4°C for 10 min. Where indicated, additions were 10 mM theophylline, 2 μM cyclic AMP or 2 μM cyclic GMP. Zymogen granules were then collected by centrifugation and, where noted, resuspended at 4°C in 0.3 M sucrose containing 0.15 M KCl. The granules were recovered (75% yield) by recentrifugation, then assayed for protein kinase activity with histone as substrate.

Treatment	Specific Activity of Zymogen Granule Protein Kinase[a]	
No pre-incubation (control)	11.0 ± 1.0	(1.0)
No pre-incubation, single KCl wash	6.5 ± 0.5	(0.59)
Pre-incubation, plus theophylline	12.4 ± 0.9	(1.13)
Pre-incubation, plus 2 μM cyclic GMP, theophylline	12.4 ± 1.0	(1.13)
Pre-incubation, plus 2 μM cyclic AMP, theophylline	56.4 ± 4.2	(5.13)
Pre-incubation, plus cyclic AMP, theophylline, then single KCl wash	10.6 ± 0.5 [80%][b]	(0.96)

[a] pmol [^{32}P]phosphate transferred per mg protein per min ± SEM for n = 6. Numbers in parentheses designate the ratio of activity to control.

[b] The number in brackets refers to the percent recovery of kinase activity, which adsorbed to zymogen granules during pre-incubation with postmicrosomal supernate and cyclic AMP, found in the KCl wash supernatant fluid.

protein kinase activity bound to zymogen granules 5-fold. An increase in zymogen granule cyclic AMP dependent kinase activity was not detectable. This transfer of cytosol protein kinase to zymogen granules accounted for an 8% loss in total protein kinase (measured in the presence of cyclic AMP) present in the postmicrosomal supernate. These results suggest that cyclic AMP promotes the transfer of the catalytic subunit of the protein kinase holoenzyme complex *in vitro*.

Endogenous phosphorylation of zymogen granule polypeptides also increased following incubation of zymogen granules with the postmicrosomal supernate and cyclic AMP (Figure 6). This phorphorylation pattern is similar to results obtained by Lambert et al. (1974) when exogenous cyclic AMP dependent protein kinase (from rabbit muscle) was incubated with granules in the presence of cyclic AMP.

Cyclic GMP, at concentrations high enough to activate protein kinases contained in the postmicrosomal supernate, did not produce a translocation effect similar to cyclic AMP. Control experiments verified that cyclic GMP did activate a protein kinase during the incubation experiments outlined above.

A recent study of heart muscle protein kinase indicated that the soluble catalytic subunit of cyclic AMP dependent protein kinases adsorbed nonspecifically to particulate cell fractions. This activity could be released in the presence of 150 mM KCl (Keely et al., 1975). Keely et al. observed that addition of physiological salt to the homogenate prevented the adsorption of free catalytic subunits to cell particulate fractions. We examined the stability of the adsorbed granule protein kinase activity. Since high ionic strength conditions during homogenization reduce the yield of zymogen granules, experiments

Figure 6. Endogenous phosphorylation of zymogen granules incubated with cyclic AMP and postmicrosomal supernate.

Zymogen granules were incubated with 2 μM cyclic AMP, 10 mM theophylline, and the post-microsomal supernate fraction as described in Table 8. Following isolation from the post-microsomal supernate, zymogen granules were incubated with (γ-$^{32}PO_4$)ATP and the other elements of the protein kinase assay as described in Figure 1. After 10 min the granules were lysed and membrane particles were centrifuged 100,000 x g for 1 h. The pellet was then solubilized in protein solvent and submitted to electrophoresis on SDS-acrylamide gels. Gels were then fractionated into 2 mm slices and assayed for radioactivity as described in Methods. Open circles are granules without pre-incubation with cyclic AMP. Closed circles represent granules incubated with cyclic AMP.

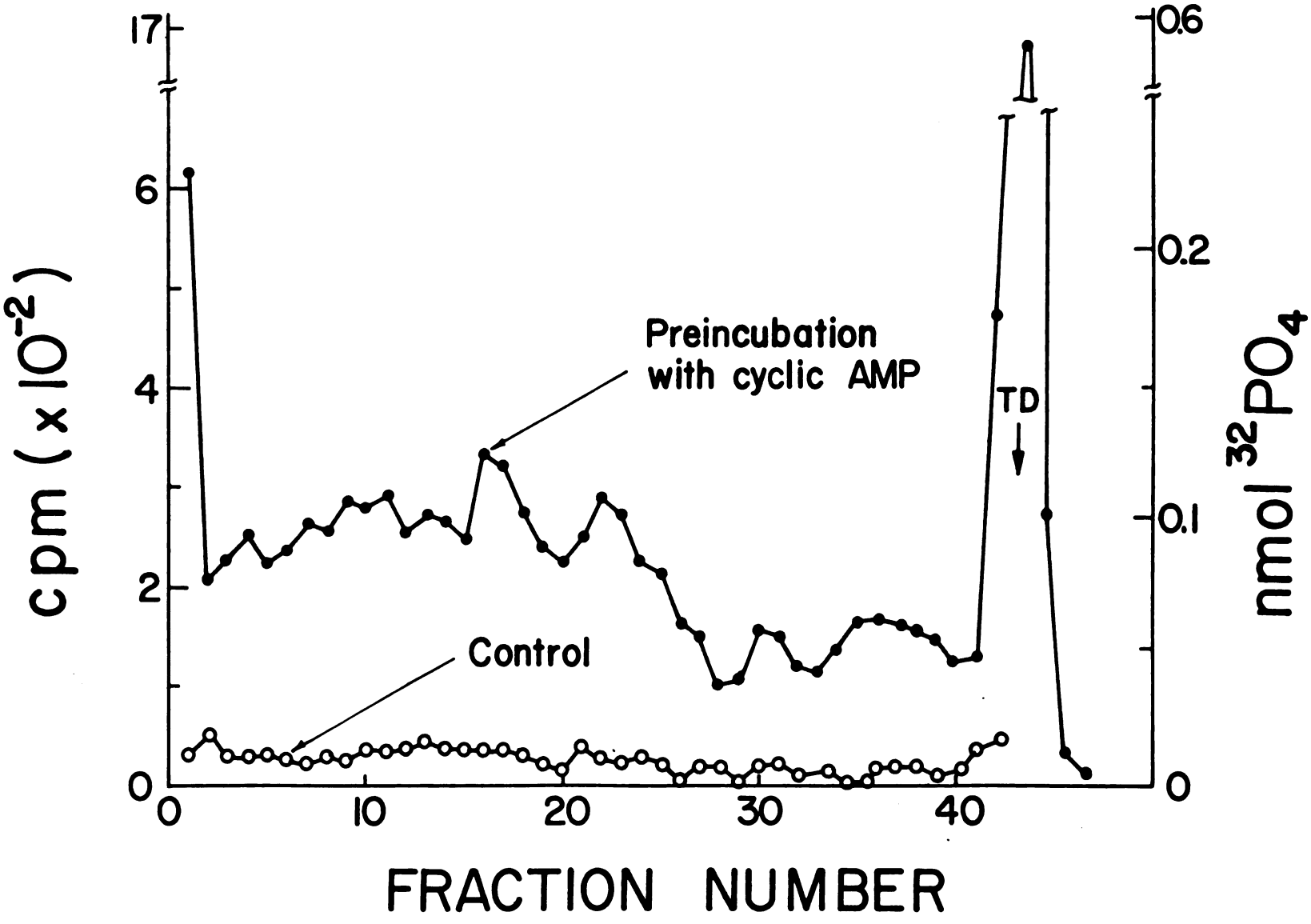
17
6
4
2
0
0
10
20
30
40
0.6
0.2
0.1
0
cpm (x 10⁻²)
nmol ³²PO₄
Preincubation
with cyclic AMP
Control
TD
FRACTION NUMBER

Figure 6

were limited to studying the stability of the granule kinase after isolation. Washing zymogen granules, previously incubated with cyclic AMP and postmicrosomal supernatant, with 150 mM KCl resulted in loss of most of the translocated protein kinase activity (Table 8). Over 50% of the translocated activity was recovered in the KCl wash.

To determine whether the adsorption of protein kinase to zymogen granules was a specific process, microsomes were evaluated in the same reconstituted system as zymogen granules. Table 9 illustrates the effect of cyclic AMP on microsome protein kinase when microsomes are incubated with the cytosol fraction. Similar to zymogen granules, microsomes adsorbed 5-fold protein kinase activity when cyclic AMP was added to the preincubation. As with zymogen granules, there was no translocation of cyclic AMP dependent protein kinases to the microsomes. Cyclic AMP dependent translocation of protein kinase activity from cytosol to particulate fractions appears not to be specific for any particulate fraction.

Levels of Protein Kinase Activity in Zymogen Granules During Secretagogue Stimulated Secretion

The preceding study opened the possibility that changes in intracellular cyclic nucleotides could modulate protein kinase activity associated with zymogen granules during secretion. Experiments were designed to illustrate a cyclic nucleotide dependent translocation of protein kinase to zymogen granules in an intact tissue system. Pancreas tissue slices were incubated with 3 classes of secretagogues: pancreozymin (0.1 unit/ml), carbachol (10^{-5} M), and dibutyryl cyclic AMP (10^{-4} M). As reviewed in Table 2, pancreozymin and carbachol have been shown to elevate cyclic GMP levels,

Table 9. Translocation of protein kinase activity from the cytosol to microsomes

Freshly prepared 15,000 x g pancreatic supernate was incubated with 2 x 10^{-6} M cyclic AMP and 10 mM theophylline for 10 min at 0°C. Microsomes were separated from the supernate by centrifugation at 100,000 x g for 1.5 h. Cyclic nucleotide independent microsomal protein kinase was then assayed according to Methods.

Fraction	Pretreatment	% Protein Kinase Activity
Microsomes	minus cyclic AMP	100
Postmicrosomal supernate	minus cyclic AMP	100
Microsomes	plus cyclic AMP	500
Postmicrosomal supernate	plus cyclic AMP	200

[a]Pretreatment of the 15,000 x g supernate.

pancreozymin and its derivatives have also been shown to activate adenylate cyclase, and dibutyryl cyclic AMP possesses secretagogue capabilities. The response of rat pancreas pieces to these secretagogues is summarized by Figure 7. Within 3 h pancreozymin and carbachol were most effective in eliciting release of amylase (44-55%), while dibutyryl cyclic AMP was somewhat less effective (39%). After 1 h of incubation, all 3 secretagogues produced a significant increase in amylase secretion. The ability of dibutyryl cyclic AMP to produce a response appears not to be the washout effect described by Case and Scratcherd (1972), as all incubations were preincubated prior to the addition of secretagogues.

Pancreatic tissue slices were next incubated in the presence or absence of secretagogues and then zymogen granules were isolated within the first hour of incubation. Protein kinase activity associated with zymogen granules was then assayed with histone as the exogenous substrate. Carbachol treatment produced no marked change in the level of that protein kinase bound to zymogen granules assayed in the presence of cyclic AMP (Figure 7). The specific activity of zymogen granule protein kinase did not increase when measured with endogenous acceptors. Figure 7 also summarizes the protein kinase levels in KCl-extracted zymogen granules isolated from tissue pieces after incubation in the presence or absence of dibutyryl cyclic AMP. Extraction with 0.15 M KCl reduced the protein kinase level by 50%, in agreement with Table 6. There was no significant increase in non-extractable granule kinase activity.

It was possible that secretagogues other than carbachol could alter cyclic nucleotide-independent protein kinase activity bound to zymogen granules. Alternatively, the level of cyclic GMP-dependent

Figure 7. Secretory response of pancreatic tissue to secretagogues.

Pancreatic tissue pieces (200 mg) were incubated at 37°C in 10 ml of medium containing Krebs-Ringer bicarbonate buffer, 14 mM glucose, 0.1 mg/ml soybean trypsin inhibitor, and Eagles' minimal essential amino acid mixture (controls). Other incubations contained pancreozymin, carbachol, or dibutyryl cyclic AMP as given in the figure. At intervals aliquots of the media were assayed for amylase activity as described in Methods. All incubations were pre-incubated for 20 min. Closed circles represent tissue slices incubated with 0.1 U/ml pancreozymin. Closed squares represent tissue slices incubated with 10^{-5} M carbachol. Open circles refer to tissue slices incubated with 10^{-4} M dibutyryl cyclic AMP. Open squares represent tissue slices incubated with control saline.

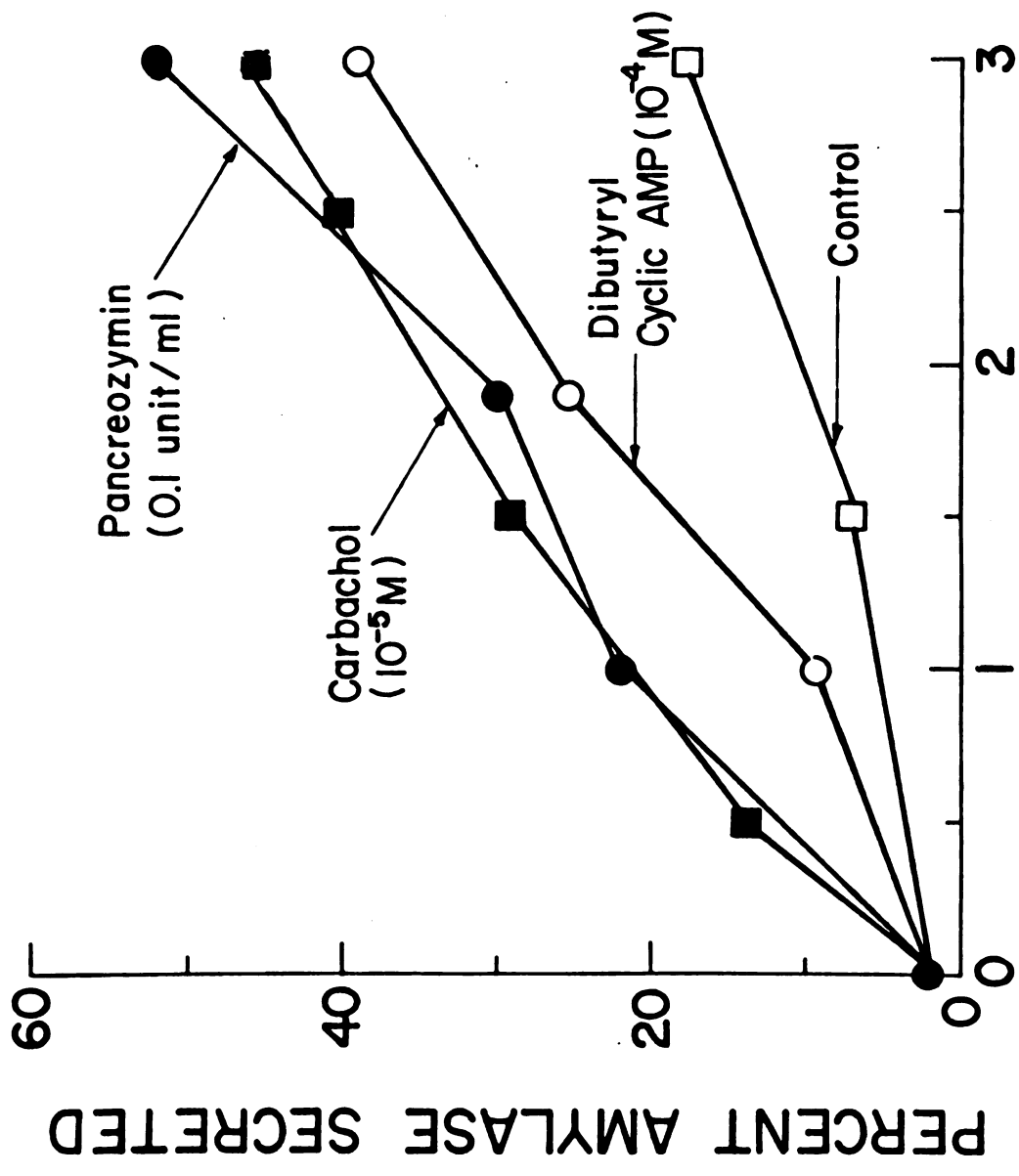
PERCENT AMYLASE SECRETED
60
40
20
0
0
1
2
3
INCUBATION TIME (HOURS)
Pancreozymin (0.1 unit/ml)
Carbachol (10^{-5}M)
Dibutyryl Cyclic AMP (10^{-4}M)
Control

Figure 7

Figure 8. Zymogen granule protein kinase levels during secretion *in vitro*.

Pancreatic tissue pieces (400 mg) were incubated in 20 ml of medium, as described in Figure 7, containing 10^{-5} M carbachol or 10^{-4} M dibutyryl cyclic AMP. No secretagogue was added to control incubation. At the indicated intervals, tissues were collected, washed and homogenized as described in Methods. Zymogen granules were isolated and washed in either 0.3 M sucrose or 0.15 M KCl. Zymogen granules were assayed for protein kinase activity in the presence of 2×10^{-6} M cyclic AMP. The substrate was histone (2 mg/ml). Due to the similarity of data obtained, only representative data are presented in Figure 8. Granules from both carbachol and dibutyryl cyclic AMP treated tissue were both washed with sucrose and KCl. However, only data for sucrose-washed granules (circles) from carbachol treated pancreatic tissue are presented. Likewise, only data for KCL washed granules (squares) from dibutyryl cyclic AMP treated tissue are presented. The open triangles represent endogenous protein kinase activity (histone omitted) from tissue treated with carbachol.

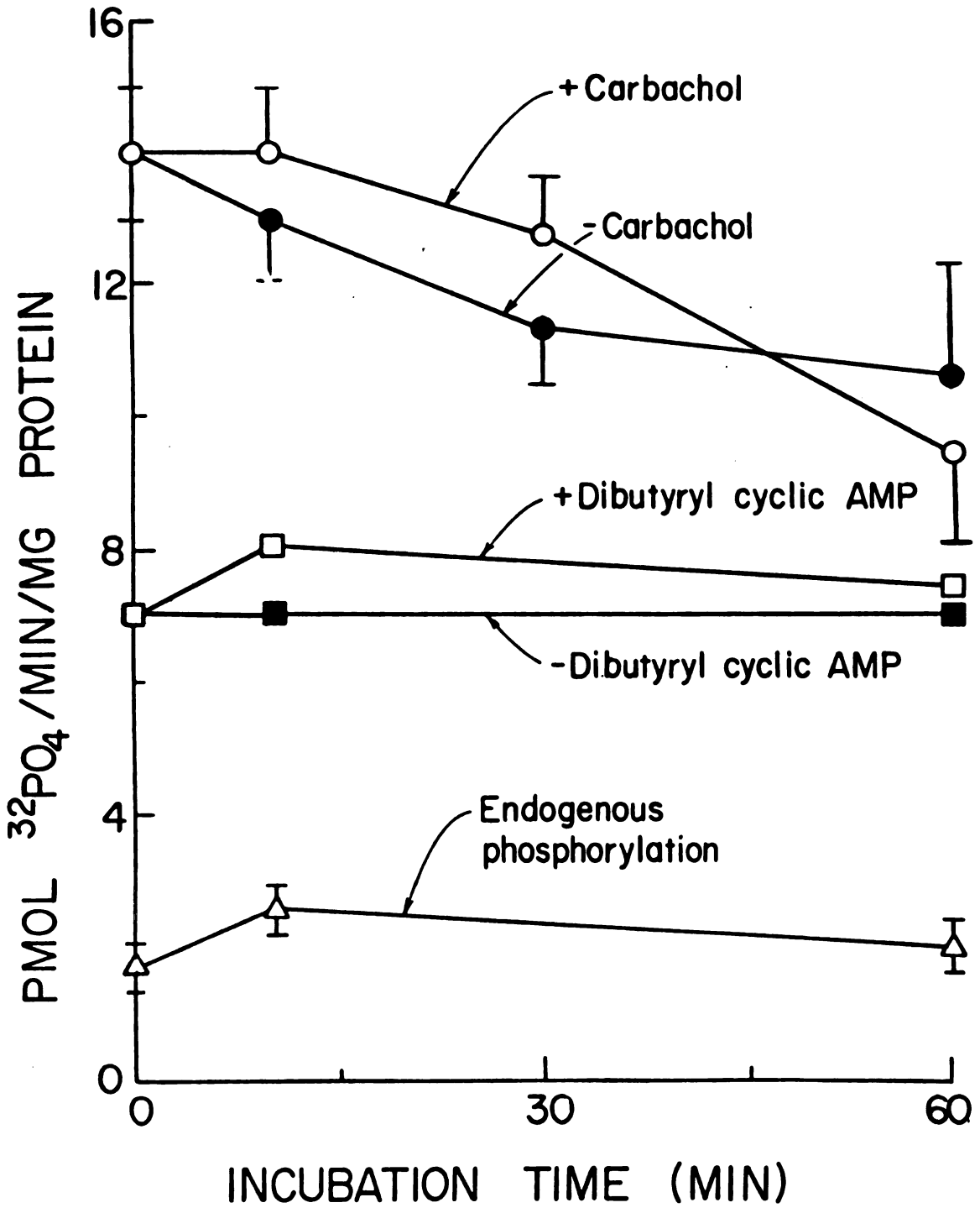
16
12
8
4
0
0
30
60
+Carbachol
-Carbachol
+Dibutyryl cyclic AMP
-Dibutyryl cyclic AMP
Endogenous
phosphorylation
PMOL $^{32}PO_4$/MIN/MG PROTEIN
INCUBATION TIME (MIN)

Figure 8

protein kinase associated with zymogen granules could vary in response to secretagogues. Table 10 illustrates the effect of pancreozymin upon zymogen granule protein kinase. Tissue slices were incubated with pancreozymin (0.1 unit/ml) and at appropriate times zymogen granules were isolated and assayed for protein kinase activity. It is apparent that pancreozymin treatment of tissue pieces had no effect on zymogen granule cyclic nucleotide independent, cyclic AMP or cyclic GMP dependent protein kinase activities. Haymovits and Scheele (1976) reported that secretagogue induced elevations of cyclic GMP occur within the first 5 min of stimulation. Table 10 also presents data on levels of granule protein kinase within the first 10 min after carbachol, pancreozymin, or caerulein administration to tissue slices. The results summarized in Table 10 indicate the granule kinase activity measured in assays containing either cyclic AMP or cyclic GMP increased at most 55% during the first hour of secretion in the presence of pancreozymin, caerulein or carbachol. There was no significant change in total protein kinase activity associated with zymogen granules, assayed with and without cyclic AMP or cyclic GMP, as judged by the Student's t-test.

Phosphorylation of Zymogen Granule Membranes During Secretagogue-Stimulated Secretion

To identify proteins that may be selectively phosphorylated during hormone stimulation, we analyzed zymogen granule fractions from tissue incubated 50 min with caerulein and $^{32}PO_4^{-3}$ by SDS-polyacrylamide gel electrophoresis. Zymogen granules were isolated from rat pancreatic pieces after incubation. The radioactivity profiles of polyacrylamide gels of intact granule, granule membrane

Table 10. Comparison of protein kinase activities in zymogen granules from secretagogue-treated and control tissues

Tissues were incubated in KRB medium in the presence of either 10^{-5} M carbachol or 0.1 unit/ml pancreozymin. Secretagogues were omitted from control incubations. At the indicated intervals, tissue pieces were harvested and KCl washed zymogen granules were isolated as given in Methods. For protein kinase assay conditions, refer to Methods. The results are expressed as the ratios of specific activities of protein kinase in zymogen granules from treated tissues to those in granules from untreated tissues.

	Relative Activity of Zymogen Granule Protein Kinase[a]					
	Carbachol-Treated Control			Pancreozymin-Treated Control		
Incubation time (min)	no cyclic nucleotides	+cAMP	+cGMP	no cyclic nucleotides	+cAMP	+cGMP
2.5	0.97	0.90	1.06	1.02 (1.0)[b]	0.95 (1.05)[b]	1.21 (0.95)[b]
5	0.90	0.90	1.10	0.90	0.90	0.86
10	1.05	0.93	1.02	0.97	0.80	1.10
30	1.27	0.89	1.15	1.29	ND[c]	1.55
60	0.80	1.16	1.03	0.80	1.25	0.65

[a]The specific activity of nucleotide independent activity in control granules was 5.7 ± 0.5 pmol $^{32}P_i$ per mg protein per min; the specific activity of cGMP dependent activity was 1.7 ± 0.5 pmol $^{32}P_i$ per mg protein per min.

[b]Ratio of protein kinase activity in zymogen granules from tissues incubated with 10^{-9} M caerulein to the activity in control granules.

[c]Not determined.

and mitochondrial fractions are shown in Figure 9. Intact granules isolated from secretagogue-treated tissues contained low and variable levels of $^{32}PO_4^{-3}$-labeled protein, relative to granules from untreated tissue. However, highly purified zymogen granule membranes from secreting tissue contained little $^{32}PO_4^{-3}$-labeled protein. Profiles of fractions from tissues treated with pancreozymin-C-octapeptide, carbachol and pancreozymin are similar to those shown (data not presented). During the preparation of membranes from zymogen granules, approximately 85% of the trichloroacetic acid-precipitable $^{32}PO_4^{-3}$ counts were recovered in the dense mitochondrial pellet after lysis (Table 11). It is likely that the preponderance of $^{32}PO_4^{-3}$-labeled proteins associated with the zymogen granule fraction was due to contaminating mitochondria and other soluble proteins. To assess adsorption of soluble phosphoproteins to granules, zymogen granules from non-labeled tissues were incubated with a postmicrosomal supernate from tissues incubated with $^{32}PO_4^{-3}$ and secretagogues. These isolated granules adsorbed variable amounts of soluble $^{32}PO_4^{-3}$-labeled protein. These results suggest that the apparent granule protein phosphorylation represents the adsorption by granules of $^{32}PO_4^{-3}$-labeled proteins from the cytosol and mitochondrial contamination in the granule fraction.

If zymogen granule membrane protein phosphorylation was an initiating event of stimulus-coupled secretion, phosphorylation of granule membrane proteins may occur within a few moments following administration of secretagogue. Granule membranes isolated from $^{32}PO_4^{-3}$-prelabeled pancreas tissue slices treated with caerulein, carbachol, or pancreozymin-C-octapeptide exhibited no protein

Figure 9. Electrophoretic analysis of phosphorylated products of zymogen granule fractions during secretion *in vitro*.

Pancreatic tissue pieces were preincubated for 1 h at 37°C in the medium described in Figure 1 containing $^{32}PO_4$ (30 μCi/ml). After 1 h, secretagogues were added and the incubations were continued for an additional hour. Tissues were then collected and washed with 0.3 M sucrose by repeated suspension and recentrifugation at 4°C. Zymogen granules, granule membranes and mitochondria were prepared from homogenized samples as described in Methods. During zymogen granule membrane isolation 80 mg unlabeled zymogen granules were added to $^{32}PO_4$-labeled granules to serve as a carrier. Mitochondria represent the fraction containing zymogen granule membranes after lysis in 0.2 M $NaHCO_3$ that sediments through 1.0 M sucrose in the discontinuous gradient (Table 4). Each fraction was solubilized for polyacrylamide-SDS gel electrophoresis as presented in Figure 3. The gels were fractionated and the distribution of radioactivity was determined. (O-O) fractions from tissues incubated in the presence of 10^{-5} M carbachol; (X-X) fractions from tissues incubated with 10^{-8} M caerulein; (●-●) fractions from tissues incubated in the absence of secretagogue. A) Intact zymogen granules; B) zymogen granule membranes; C) mitochondria. The top of the gel starts with fraction 1. Tracking dye migrates to fraction 43.

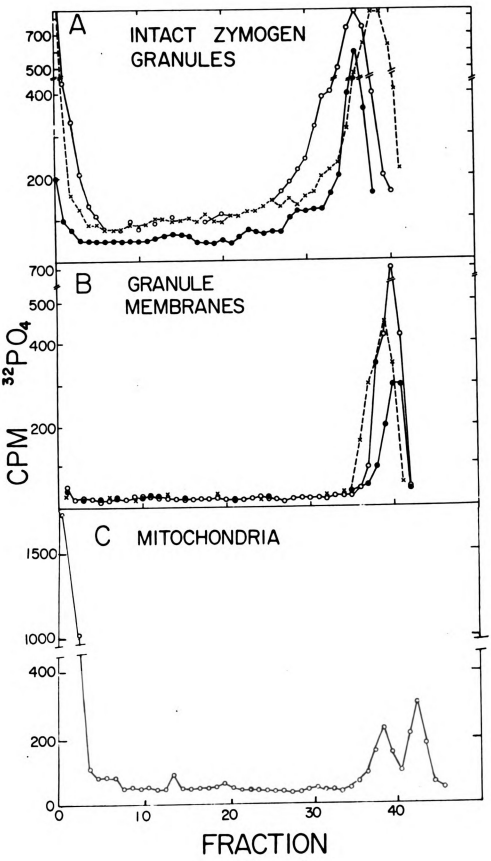
A
INTACT ZYMOGEN GRANULES
B
GRANULE MEMBRANES
C
MITOCHONDRIA
CPM $^{32}PO_4$
FRACTION
700
500
400
200
1500
1000
0
10
20
30
40

Figure 9

Table 11. Distribution of $^{32}PO_4$-labeled protein in zymogen granule subfractions

Tissue slices were pre-incubated 60 min in medium supplemented with $^{32}PO_4$ (30 μCi/ml) and incubated another 50 min with 10^{-5} M carbachol. Zymogen granules were isolated and granule membranes were purified as in Methods. Aliquots of each fraction were precipitated in 10% trichloroacetic acid. Protein precipitates were collected by centrifugation, then extracted twice with chloroform-methanol. The protein precipitates were collected on filters and counted. Results are expressed as total cpm per fraction. Mitochondria pellet refers to general contaminants, in addition to mitochondria, purified from granule membranes.

Fraction	Radioactivity (cpm)	Recovery (%)
Zymogen granules	4506	(100)
Zymogen granule content	686	15.2
Mitochondrial pellet	3615	80.2
0.2 M NaBr extracts of granule membranes	<5	<1
Zymogen granule membranes	80	1.8

Figure 10. $^{32}PO_4$ incorporation into granule membranes during early phase of stimulus-coupled secretion.

Pancreatic tissue was incubated with carbachol ($x10^{-5}$ M), caerulein ($x10^{-9}$ M), and pancreozymin-C-octapeptide ($x10^{-6}$ M) for 2 and 10 min according to Methods. Tissue was washed 3 times with 50 ml cold 0.3 M sucrose, then zymogen granules were isolated. Freshly prepared granules from 10 rats were then added to the $^{32}PO_4$-labeled granules. Zymogen granule membranes were isolated and analyzed by SDS-acrylamide gel electrophoresis as described in Methods. Gels were cut into 5 mm fractions and assayed for radioactivity. A) Open circles represent tissue plus pancreozymin-C-octapeptide, 2 min; closed circles represent tissue (-) secretagogue. B) Open circles represent tissue plus pancreozymin-C-octapeptide, 10 min. Due to the similarity of data, only results obtained with a single secretagogue are presented.

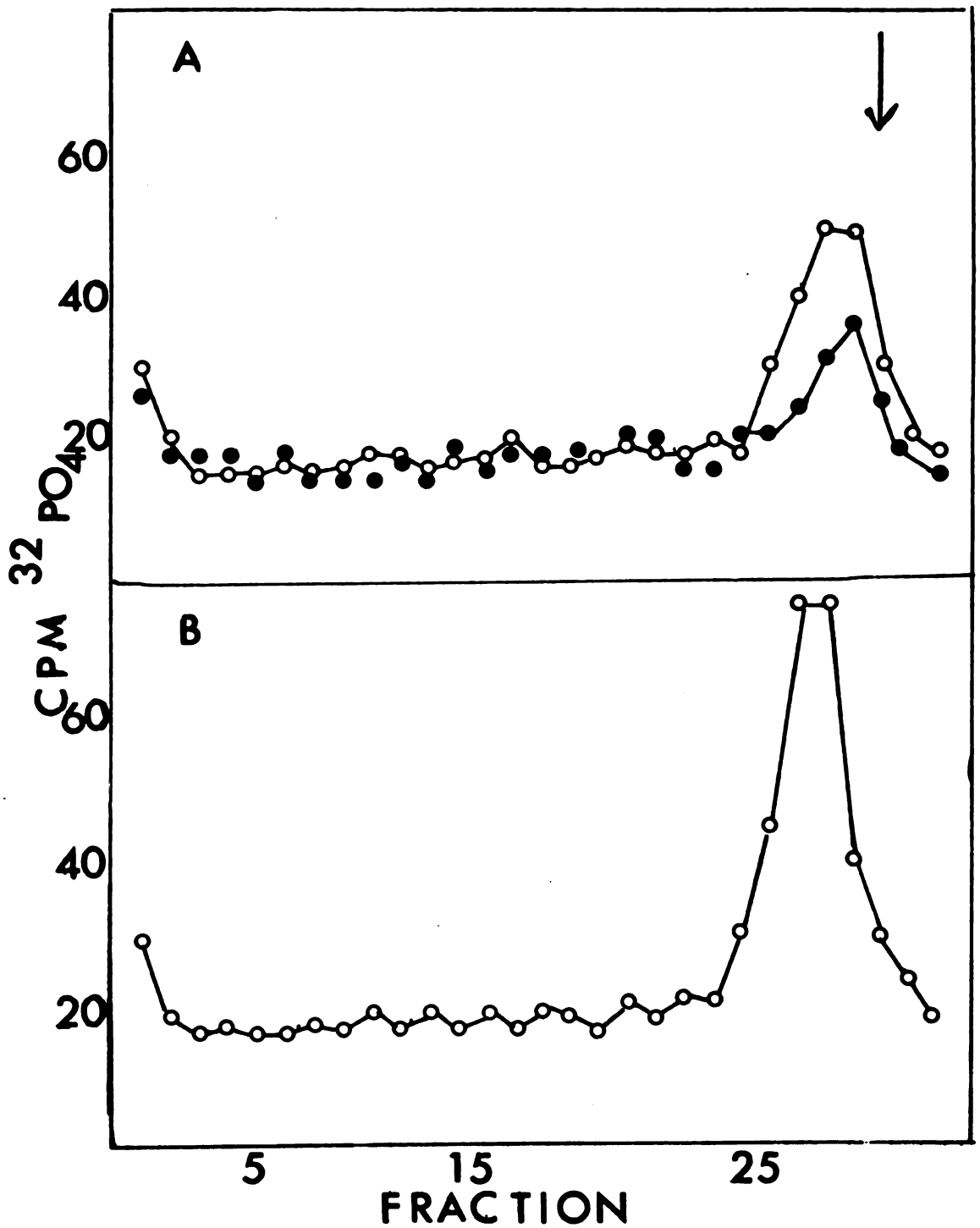

Figure 10

phosphorylation, when isolated at 2.5 or 10 min following addition of secretagogue (Figure 10).

Analysis of Zymogen Granule Phospholipids

Analysis of gel electrophoretograms in Figures 9 and 10 revealed an increase in $^{32}PO_4^{-3}$ incorporation into the phospholipid fraction of zymogen granules from secretagogue-treated tissue. Chloroform: methanol (2:1 v/v) extraction of $^{32}PO_4^{3}$-labeled granules removed over 90% of the $^{32}PO_4^{-3}$ migrating adjacent to the tracking dye. When the chloroform:methanol extract was analyzed by thin layer chromatography, essentially all of the $^{32}PO_4^{-3}$ cpm attributable to the secretagogue effect on phospholipid synthesis migrated at a rf corresponding to sphingomyelin, phosphatidylserine, or phosphatidylinositol (Figure 11).

Two-dimensional thin layer chromatography was employed to completely separate phosphatidylserine, phosphatidylinositol, and sphingomyelin (Singh and Privett, 1970; Burton, 1975). Figure 12 illustrates the degree of separation of the major phospholipids present in zymogen granule membranes (Rutter et al., 1975; Meldolesi et al., 1971). Comparison of rf values of the unknown lipids with rf values of standard phospholipids allowed identification of zymogen granule membrane phospholipids. Due to the difficulty of separation of phosphatidylinositol and phosphatidylserine (Sweeley, 1969), ninhydrin and phosphate spray were used to identify NH_2-containing lipids and those without NH_2-groups.

Evidence exists that inorganic $^{32}PO_4^{-3}$ incorporation into total cellular phosphatidylinositol occurs in the pancreas during secretagogue stimulated secretion (Hokin and Hokin, 1952; Bauduin et al., 1971; Bauduin and Cantraine, 1972). Acetylcholine treatment decreased

Figure 11. Thin layer chromatographic analysis of $^{32}PO_4^{-3}$-labeled granule phospholipids.

Zymogen granules were isolated from 400 mg pancreatic tissue pre-incubated with $^{32}PO_4$ (30 µCi/ml) for 1 h and plus or minus carbachol ($x10^{-5}$ M) for an additional 50 min. Granules were washed twice with 12 ml cold 0.3 M sucrose-0.15 mg/ml SBTI. At a ratio of 20:1 volumes, granules were extracted with chloroform-methanol (2:1 v/v). This extract was then analyzed on a Silica Gel G thin layer plate developed in chloroform-methanol-acetic acid-water (170-25-25-6). Phospholipid spots were visualized with I_2 and then removed and analyzed for radioactivity. Open circles refer to granule isolated from carbachol treated tissue.

x = {phosphatidylinositol, phosphatidylserine, sphingomyelin} x

PC = phosphatidylcholine
PE = phosphatidylethanolamine
O = origin
= direction of migration

Closed circles represent granules isolajed from non-stimulated tissue.

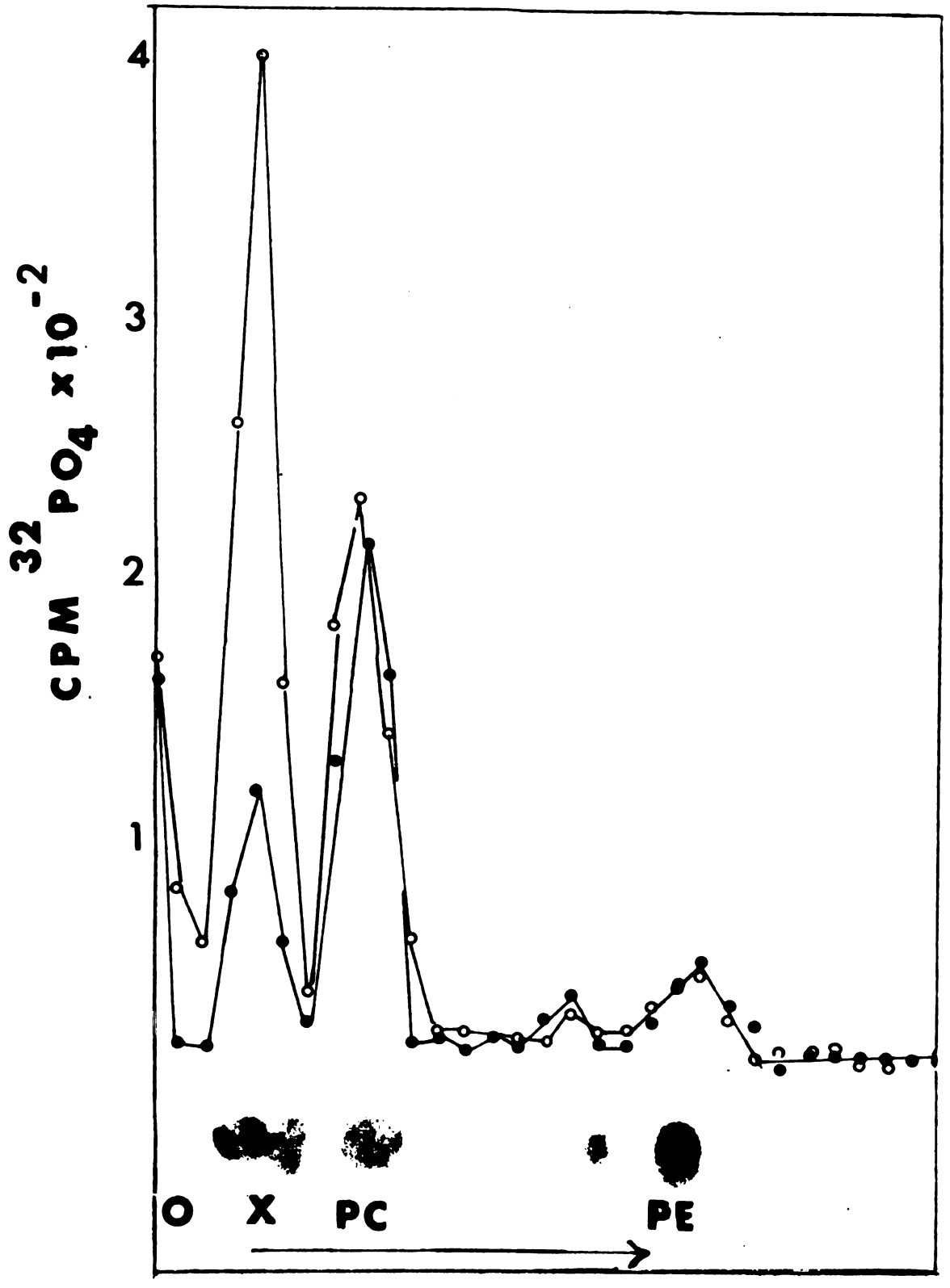

Figure 11

Figure 12. Two-dimensional thin layer chromatographic separation of phospholipids.

Phospholipids, either standards from Sigma or extracted from zymogen granules, were spotted 2 cm from the edges in the lower right-hand corner of a Silica Gel G thin layer plate. The first dimension was developed in chloroform-methanol-28% ammonia (65:35:5 v/v). After drying in a hood, the second dimension was developed in chloroform-methanol-acetic acid-water (25:15:4:2 v/v). The plate was then sprayed with 50% sulfuric acid and heated at 110°C for 10 to 20 min.

PA = phosphatic acid
PC = phosphatidylcholine
PE = phosphatidylethanolamine
PI = phosphatidylinositol
PS = phosphatidylserine
S = sphingomyelin
LPC = lysophosphatidylcholine

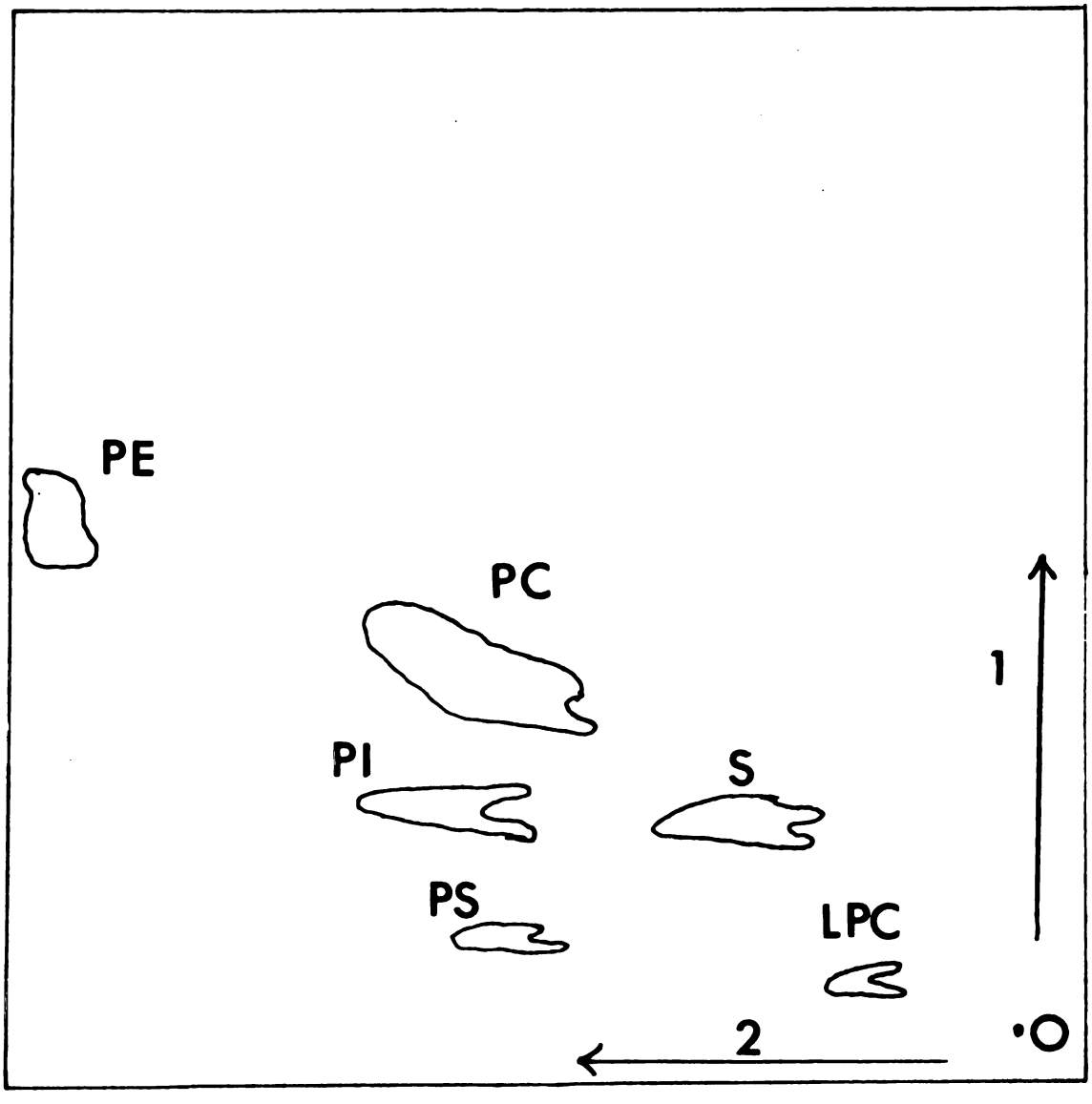

Figure 12

total cellular phosphatidylinositol (Hokin-Weaverson, 1974), but appeared to increase phosphatidylinositol synthesis in pancreatic microsomes (Hokin and Huebner, 1967). Thus, the incorporation of $^{32}PO_4^{-3}$ into phosphatidylinositol was investigated in further detail.

Pancreatic tissue slices were pre-incubated 20 min in KRB media, then 1 h in KRB supplemented with $^{32}PO_4^{-3}$ (30-50 μCi/ml media). The results are presented in Table 12. Caerulein ($x10^{-9}$ M) and carbachol ($x10^{-5}$ M) treatment of pancreatic tissue slices resulted in a 5-fold linear increase of $^{32}PO_4^{-3}$ incorporation into zymogen granule phosphatidylinositol (Figure 13). When zymogen granule $^{32}PO_4^{-3}$-labeled phospholipids were fractionated on a silicic acid column, a single large radioactive peak (designated A) was eluted (Figure 14). This peak was separated on two-dimensional thin layer chromatography into phosphatidylserine (4% of $^{32}PO_4^{-3}$ cpm), phosphatidylethanolamine (6% of $^{32}PO_4^{-3}$ cpm) and phosphatidylinositol (70% of $^{32}PO_4$ cpm). Total phospholipid yield from the silicic acid column was close to 50%. $^{32}PO_4^{-3}$ in phosphatidylinositol is not due to increased uptake of $^{32}PO_4^{-3}$ into tissue slices. Uptake of $^{32}PO_4^{-3}$ into pancreatic tissue slices equilibrated after about 35 min and remained unaltered throughout the incubation period (Figure 15).

Increased synthesis of granule phosphatidylinositol appears not to account for the increase of phosphatidylinositol $^{32}PO_4^{-3}$. Zymogen granules isolated from 1 g pancreas tissue slices and incubated in the presence and absence of secretagogue contained equivalent amounts of phosphatidylinositol, approximately 5% of the total phospholipids. This value is in agreement with published reports (Meldolesi et al., 1971; Rutten et al., 1975).

Figure 13. Incorporation of $^{32}PO_4^{-3}$ into zymogen granule membrane phosphatidylinositol.

Pancreatic tissue slices were pre-incubated with $^{32}PO_4^{-3}$ and then incubated with caerulein ($x10^{-9}$ M). At indicated times, tissue was removed from incubation and zymogen granules isolated. Purified granule membranes were then extracted as described in the text. $^{32}PO_4^{-3}$ incorporation into phosphatidylinositol was determined by two-dimensional thin layer chromatography. Solid circles represent zymogen granule membrane phosphatidylinositol from tissue incubated with caerulein. Open circles refer to control, i.e., tissue incubated in the absence of caerulein. Time refers to the time following the administration of caerulein to the tissue incubations.

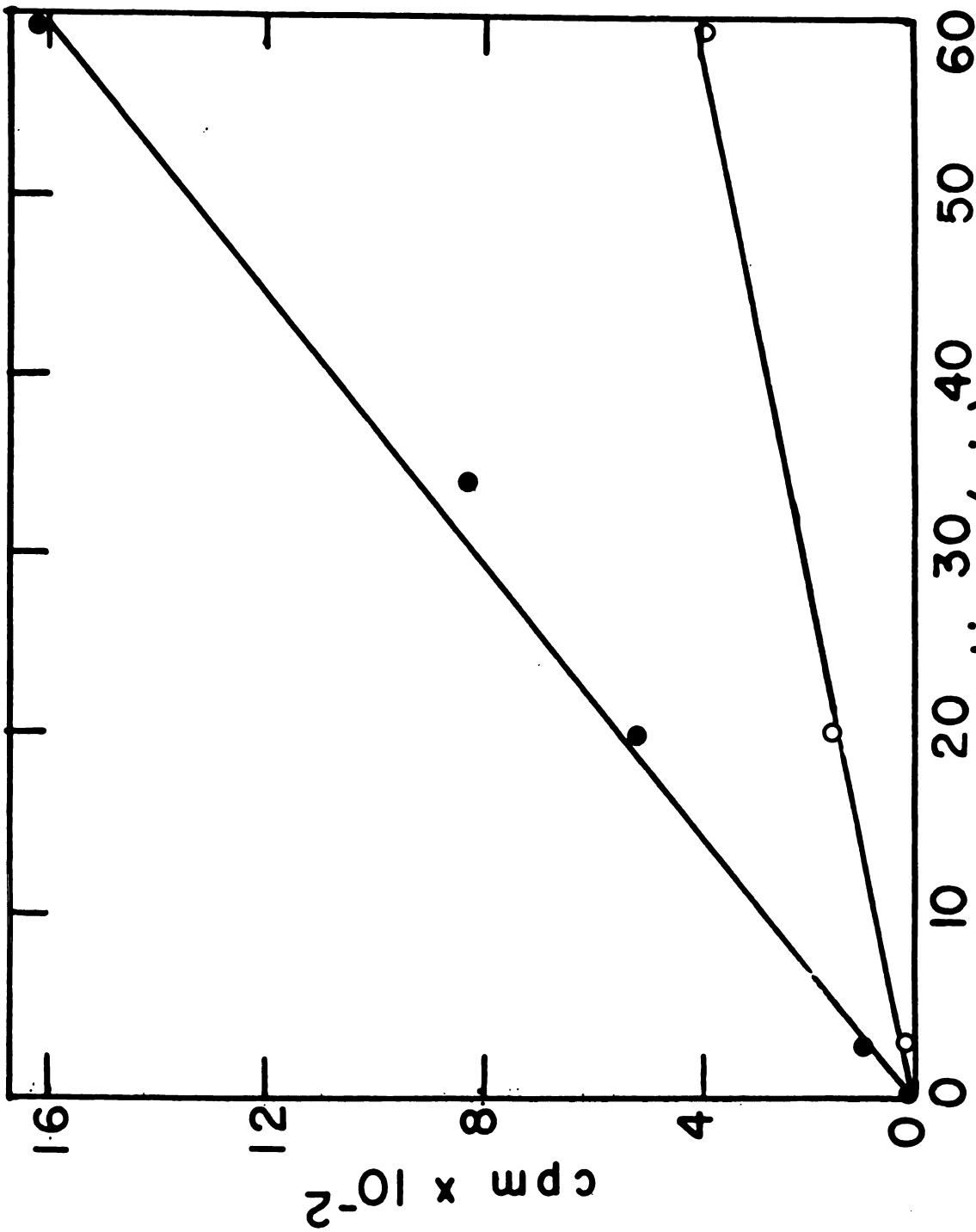

Figure 13

Figure 14. Silicic acid chromatography of $^{32}PO_4^{-3}$-labeled zymogen granule membrane phospholipids.

Chloroform-methanol (2:1 v/v) extract from $^{32}PO_4^{-3}$-labeled zymogen granules, derived from 400 mg pancreatic tissue prelabeled with $^{32}PO_4^{-3}$ for 1 h and incubated 50 min with carbachol ($x10^{-5}$ M), was loaded on a silicic acid column and eluted as described in Methods. Ten milliliter fractions were collected and assayed for $^{32}PO_4^{-3}$. Numbers across the top refer to chloroform-methanol solutions at 19:1, 4:1, 2:1, and 3:2 ratios.

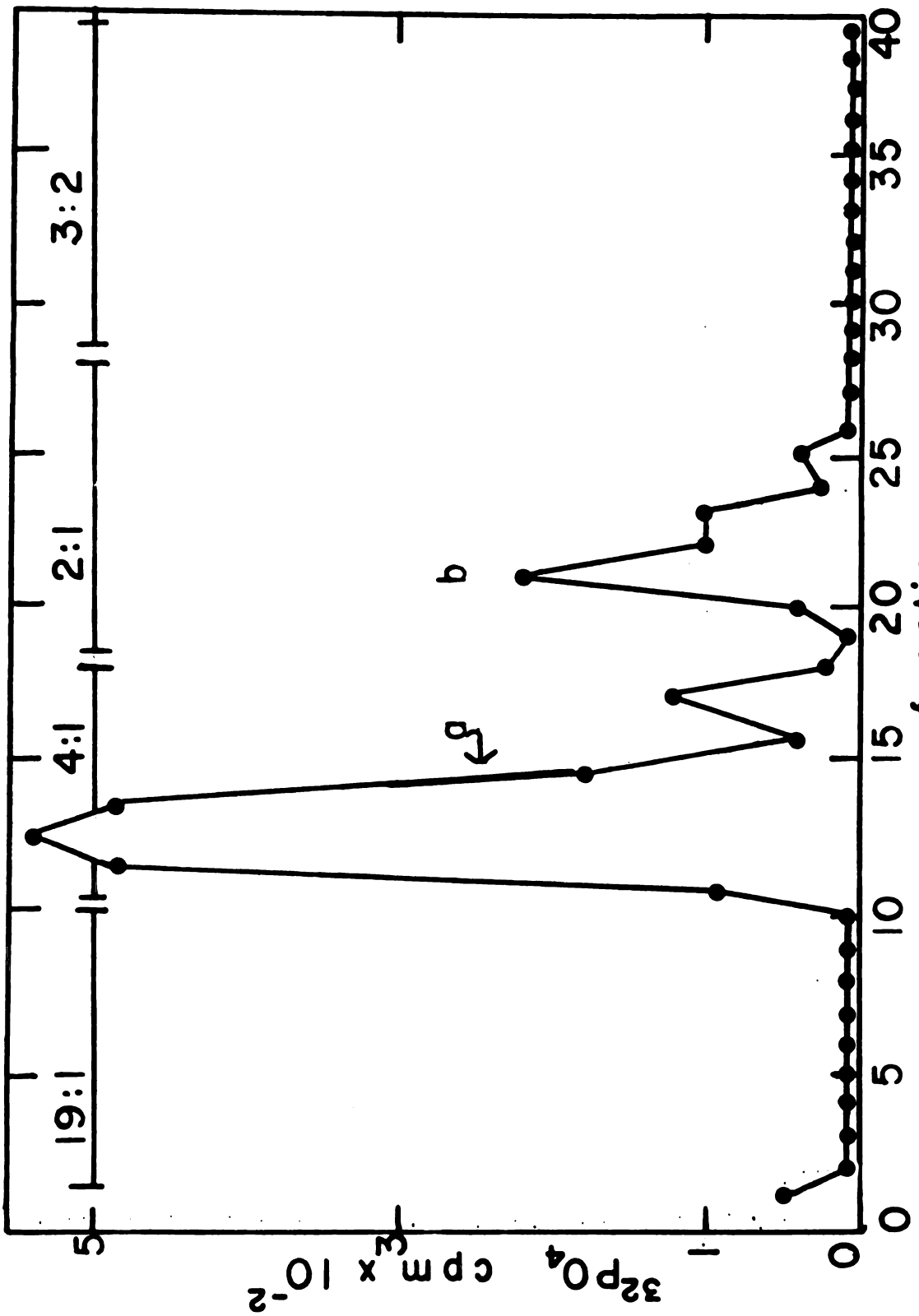
19:1
4:1
2:1
3:2
a
b
fraction
0
5
10
15
20
25
30
35
40
$^{32}PO_4$ cpm x 10^{-2}
0
1
5

Figure 14

Table 12. $^{32}PO_4^{-3}$ incorporation into zymogen granule phospholipids from secreting pancreatic tissue

Pancreatic tissue slices were incubated with $^{32}PO_4^{-3}$ (30 μCi/ml) and secretagogues as noted for 50 min at 37°C. Zymogen granules were isolated and granule membranes were prepared. Lipid standards were added as carrier and lipids were extracted with chloroform-methanol, 2:1. The lipid extract was fractionated by two-dimensional thin layer chromatography. The positions of the phospholipids were determined by co-chromatography with standards. Spots visualized with ninhydrin and/or iodine were removed and counted for radioactivity.

Phospholipid	Control (cpm)	Plus 10^{-5} M Carbachol (cpm)	Plus 10^{-9} M Caerulein (cpm)
Phosphatidylethanolamine	42	29	41
Phosphatidylcholine	475	260	405
Sphingomyelin	23	20	23
Phosphatidylserine	50	48	41
Phosphatidylinositol	173(38)[a]	988(69)	1205(82)

[a]Values in parentheses indicate cpm incorporated after 2.5 min incubation with/without secretagogue.

Figure 15. Effects of carbachol on $^{32}PO_4^{-3}$ uptake in pancreatic tissue slices.

Pancreas tissue slices (400 mg) were incubated with 30 µCi $^{32}PO_4^{-3}$ per ml media for 1 h followed by the addition of carbachol ($x10^{-5}$ M) for an additional hour. At various times, slices were removed from the medium, washed 3 times in 50 ml 0.3 M sucrose, homogenized, and assayed for $^{32}PO_4^{-3}$ as described in Methods. Results are expressed as cpm $^{32}PO_4^{-3}$ incorporated per µmol PO_4 present in the total cell homogenate. Closed circles represent tissue slices treated with $x10^{-5}$ M carbachol at t = 0. Open circles represent non-stimulated tissue.

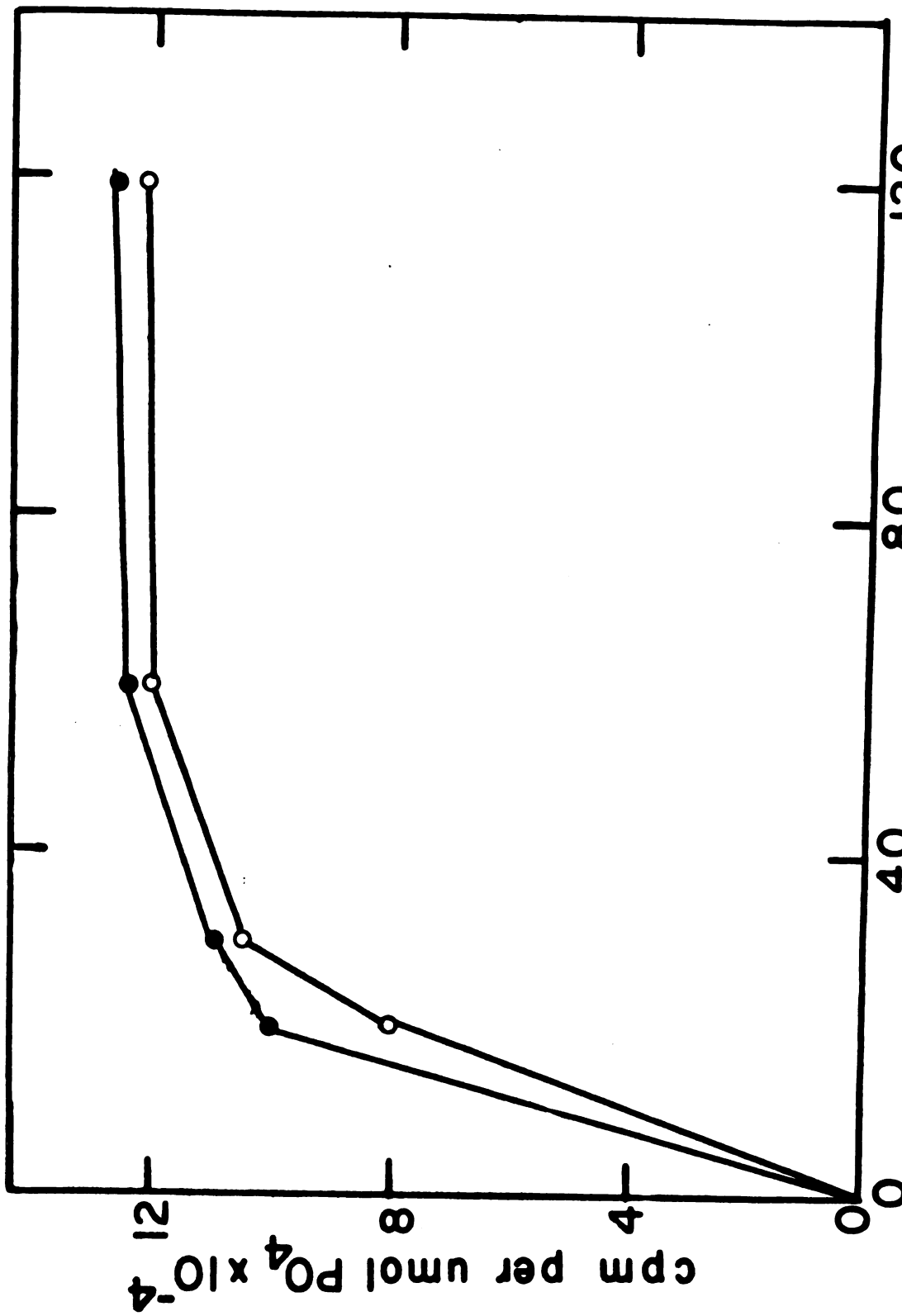

Figure 15

To determine whether phosphatidylinositol was selectively phosphorylated during stimulus-coupled secretion, zymogen granule phosphatidylinositol extract was analyzed on calcium free Silica Gel H thin layer plates. This method has been shown to effectively separate mono-, di-, and triphosphoinositides (Gonzalez-Sastre and Folch-Pi, 1968). The $^{32}PO_4$-labeled product migrated in one spot with a rf equivalent to phosphatidylinositol. Also, under similar assay conditions used for chromaffin granule phosphatidylinositol kinase (Sharoni et al., 1975; Trifaro and Dworkind, 1975), zymogen granules failed to incorporate $^{32}PO_4^{-3}$ into phosphatidylinositol. These results tend to suggest that phosphorylation of phosphatidylinositol does not account for the observed increase noted during secretion.

Studies on Zymogen Granule Membrane Glycoprotein Topology

Isolation and Partial Characterization of GP-2

Dog pancreas was used as the source for GP-2 because GP-2 represented an unusually large percentage of the protein of dog zymogen granule membranes (MacDonald and Ronzio, 1972). Following isolation of GP-2 on a 5% to 20% acrylamide 0.1% SDS preparative slab gel, GP-2 was analyzed on cylindrical SDS-acrylamide gels. Figure 16 compares electrophoretograms of zymogen granule membranes, granule content (secretory proteins) and GP-2 stained for polypeptides or carbohydrates. The major membrane glycoprotein (GP-2) represented 40% of the protein-stain intensity (Figure 16A) and 52% of the Schiff-periodic acid stained polypeptides (Figure 16D). Stained material migrating adjacent to the tracking dye represents lipid (Ray and Marinetti, 1971). Purified GP-2 yielded a single band that

Figure 16. Electrophoretic analysis of dog zymogen granule polypeptides.

Zymogen granule membranes, content, and GP-2 were electrophoresed in 9% acrylamide gels containing 1% SDS as described in Methods. For gels stained for protein (Coomassie blue), 40 µg protein were applied; for gels stained for carbohydrate (PAS), 80 µg protein were used. The following molecular weight standards were employed: a) *E. coli* β-galactosidase (130,000), b) bovine liver catalase (60,000), c) yeast alchol dehydrogenase (37,000), d) hen lysozyme (14,300). The arrows indicate the position of the tracking dye.

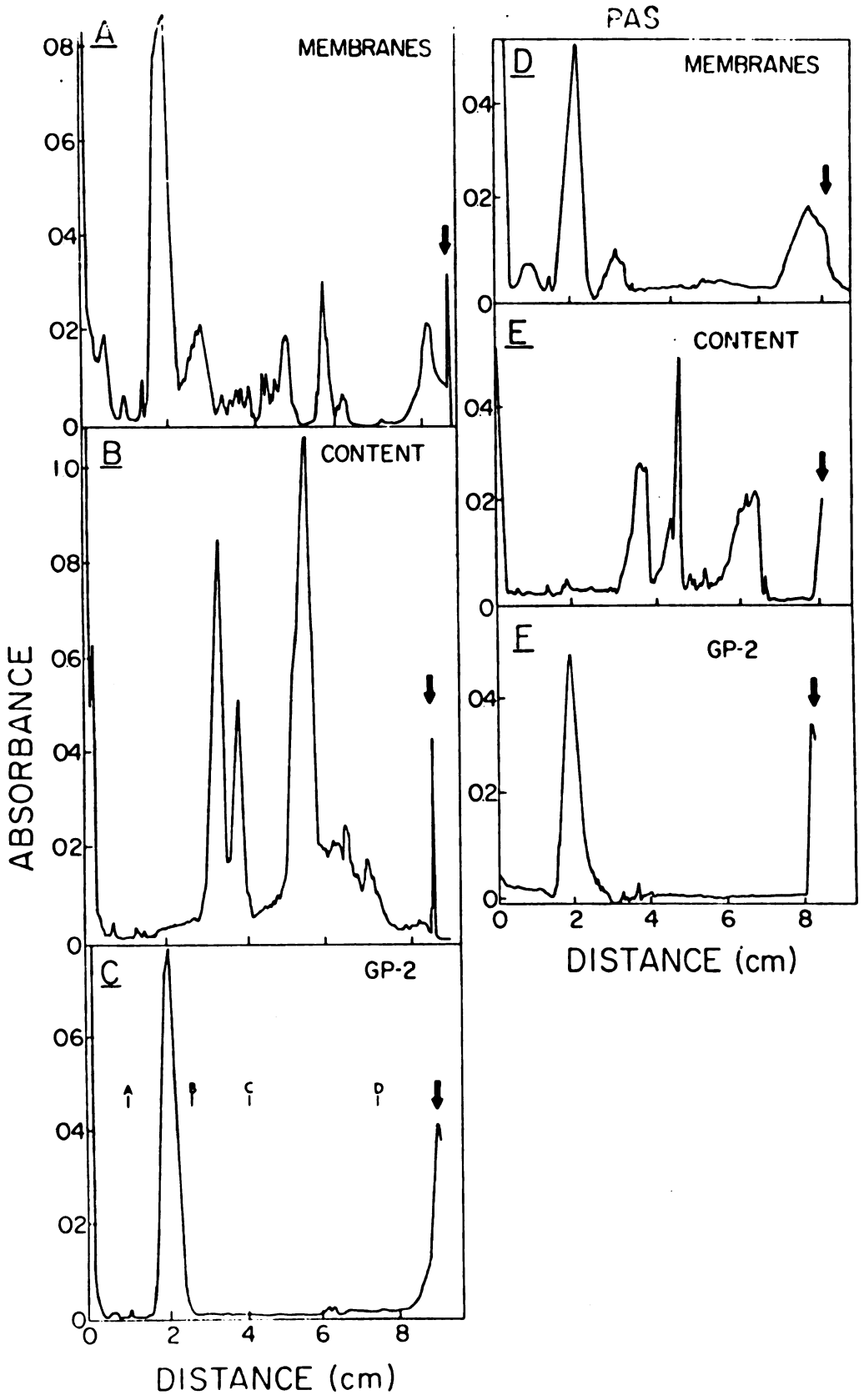
PAS
A
MEMBRANES
B
CONTENT
C
GP-2
D
MEMBRANES
E
CONTENT
E
GP-2
ABSORBANCE
DISTANCE (cm)
DISTANCE (cm)

Figure 16

corresponded to the major membrane polypeptide (Figures 16C and 16F). An additional criterion of purity of GP-2 was the detection of a single N-terminal amino acid, valine, in GP-2 preparations (see Methods). MacDonald (1974) reported that GP-2, in the presence of SDS, migrated as a single band with an apparent molecular weight of 74,000, as judged by the mobilities of protein standards. This molecular weight was confirmed on 5% and 12% acrylamide gels (Figure 16D).

Galactose-Oxidase NaB^3H_4 Labeling of GP-2 and Zymogen Granule Membranes

The orientation of GP-2 in the zymogen granule membrane is an important consideration in assessing the possible roles of GP-2 in exocytosis. To study the orientation of GP-2 in zymogen granules we utilized galactose oxidase, which can oxidize exposed galactosyl moieties of glycoproteins on membrane surfaces (Gahmberg and Hakomori, 1973). Subsequent reduction of oxidized galactose with NaB^3H_4 results in the incorporation of tritium into galactose.

Due to the insoluble nature of GP-2 (MacDonald, 1974), enzymatic labeling was ineffective. However, zymogen granule membranes could be labeled with galactose oxidase/NaB^3H_4 treatment. Zymogen granules incubated with various concentrations of galactose oxidase showed dependence of ^3H incorporation on galactose oxidase (Figure 17).

Electrophoretic analysis of dog ^3H-labeled zymogen granules, granule content, and membranes is presented in Figure 18. Galactose oxidase/NaB^3H_4 treatment of granules resulted in only two slowly moving glycoprotein species, not GP-2, being labeled (Figure 18A). Several secretory proteins of the granule content were labeled by this procedure (Figure 17B). These proteins were not accessible to

Figure 17. Dependence of ^{3}H incorporation into zymogen granule membranes on galactose oxidase concentration.

Dog zymogen granule membranes (30 μg protein) were incubated for 1 h with varying concentrations (units per 0.1 ml) of galactose oxidase. A unit of galactose oxidase activity will produce a change in absorbance at 425 nm of 1.0 per min at pH 6.0 at room temperature. Following a 1 h incubation of membranes and galactose oxidase, 0.1 mCi/ml $NaB^{3}H_{4}$ was added and the incubation continued for 15 min. Zymogen granule membranes were recovered by centrifugation at 100,000 x g for 1 h. Samples were then assayed for radioactivity.

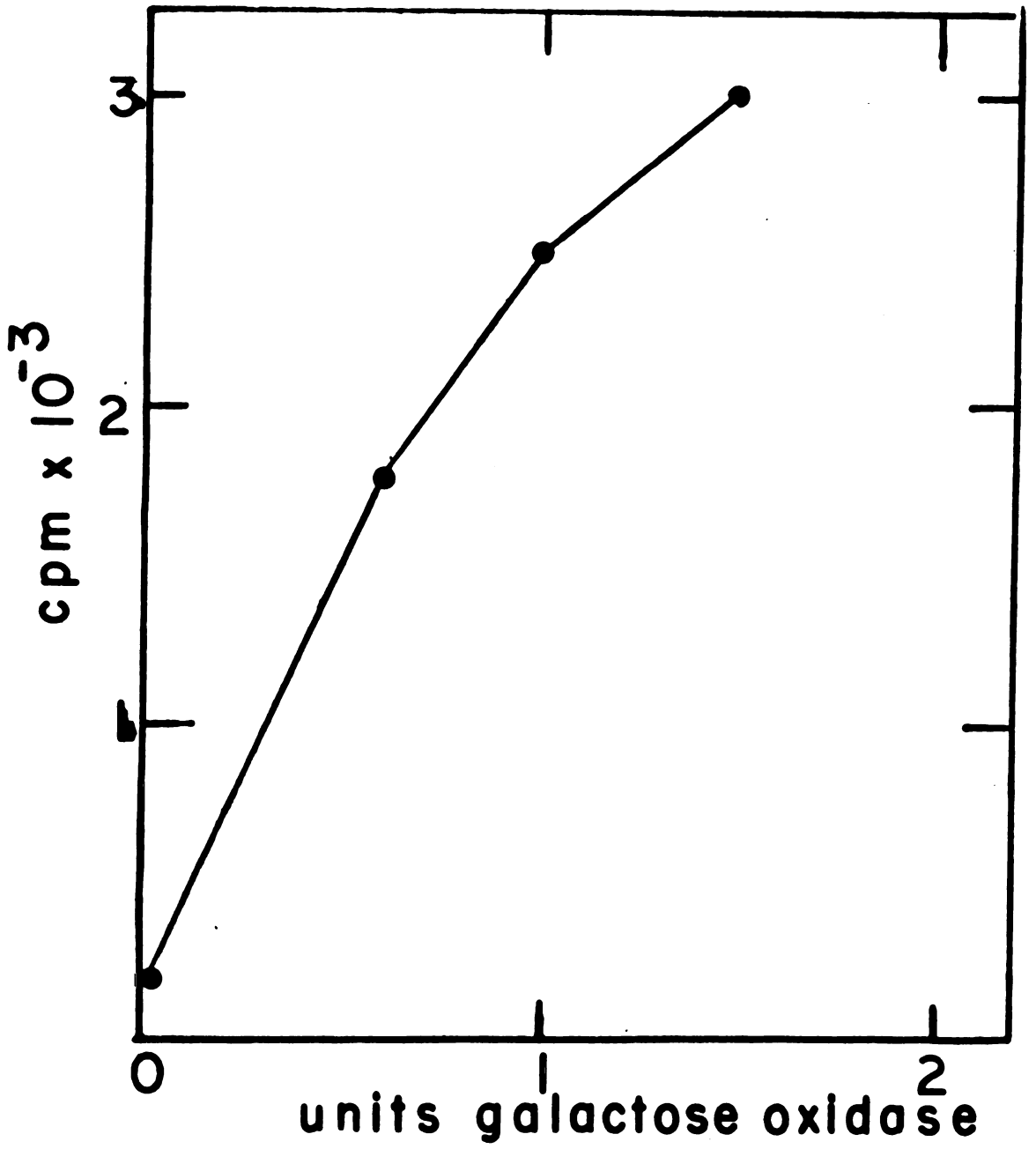

Figure 17

Figure 18. Electrophoretic analysis of intact zymogen granules and granule subfractions labeled with NaB^3H_4 following galactose oxidase treatment.

Dog zymogen granule fractions (30-50 µg protein) were incubated in phosphate-buffered saline, pH 7.2, containing galactose oxidase (15 units/ml) and soybean trypsin inhibitor (0.25 mg/ml). For intact zymogen granules, the incubation medium contained, in addition, 0.3 M sucrose. After incubation for 60 min, NaB^3H_4 (0.1 mCi/ml) was added and incubations were continued another 15 min. Intact zymogen granules were recovered by centrifugation at 1,000 x g and were washed several times in 0.3 M sucrose. Zymogen granule content was precipitated with 10% trichloroacetic acid. Zymogen granule membranes were recovered by centrifugation at 100,000 x g for 1 h. Samples were electrophoresed in polyacrylamide gels containing 1% SDS. Gels were fractionated and counted as described in Methods. Closed circles represent radioactivity from gels of fractions treated with both galactose oxidase and NaB^3H_4. Open circles represent radioactivity in control samples treated with NaB^3H_4 alone.

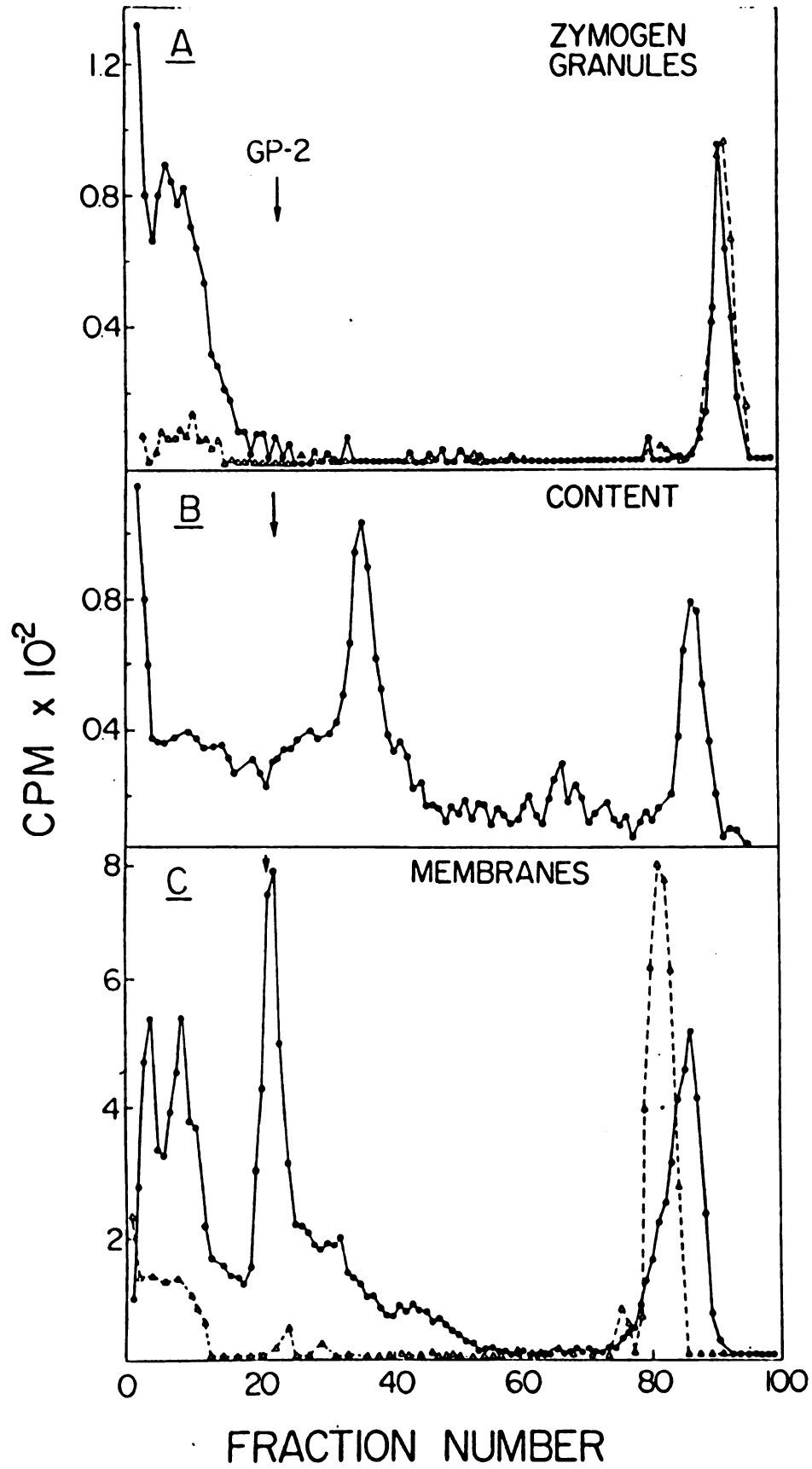
A
ZYMOGEN
GRANULES
GP-2
1.2
0.8
0.4
B
CONTENT
0.8
0.4
C
MEMBRANES
8
6
4
2
CPM x 10²
0
20
40
60
80
100
FRACTION NUMBER

Figure 18

galactose oxidase/NaB^3H_4, four glycoprotein species, including GP-2, were labeled (Figure 18C). Membranes or granule content reduced with NaB^3H_4 without prior incubation with galactose oxidase incorporated radioactivity only in glycolipids. When intact granules were treated with galactose oxidase and NaB^3H_4, about 30% of the granules lysed. Intact and lysed granules could easily be separated by centrifugation at 1000 x g.. The pellet, consisting of intact granules, incorporated ^3H into glycoproteins as shown in Figure 18A. However, the soluble fraction, containing granules that lysed during galactose oxidase/NaB^3H_4 treatment, incorporated ^3H into glycoproteins similar to a composite of Figures 18B and 18C. When zymogen granules, content, and membranes from rat pancreas are labeled, similar results are obtained (Figure 19). These data suggest that GP-2 is located on the interior of the zymogen granule membrane from at least 2 mammalian species.

The appearance of GP-2 on the interior of granule membranes allows the possibility of detecting the presence of GP-2 on the pancreatic acinar cell surface during secretion. To test this hypothesis, the following experiments were employed.

An enriched plasma membrane fraction (Meldolesi et al., 1971) of pancreatic acinar cells was isolated by the method of O'Donnell (1977). Similar to zymogen granule membranes, this plasma membrane fraction contained high molecular weight glycoproteins, which could be labeled with galactose oxidase (Figure 20). Membranes of this 700 x g pellet exhibited a distinctive glycoprotein profile compared to other fractions (Ronzio et al., 1977). The appearance of galactose containing glycoproteins within the enriched plasma membrane suggests that labeling of intact acinar cells was possible.

Figure 19. Electrophoretic analysis of intact rat zymogen granules and granule subfractions labeled with NaB^3H_4 following galactose oxidase treatment.

Granule fractions (30-50 µg) were incubated in 0.17 M NaCl containing 1 mM sodium phosphate, pH 7.2, galactose oxidase (15 units/ml) and soybean trypsin inhibitor (0.25 mg/ml). Intact granules were incubated in a medium containing, in addition, 0.3 M sucrose. Intact zymogen granules were recovered by centrifugation at 1,000 x g and were washed several times in 0.3 M sucrose. The zymogen granule content was precipitated with 10% trichloroacetic acid. Zymogen granule membranes were recovered by centrifugation at 100,000 x g for 1 h. Samples were electrophoresed in polyacrylamide gels containing 1% SDS. Gels were fractionated and counted as described in Methods. A) Zymogen granule membranes. The positions of GP-1, GP-2, GP-3 and the tracking dye (TD) are indicated by arrows. B) Intact granules (closed circles); zymogen granule content (open circles).

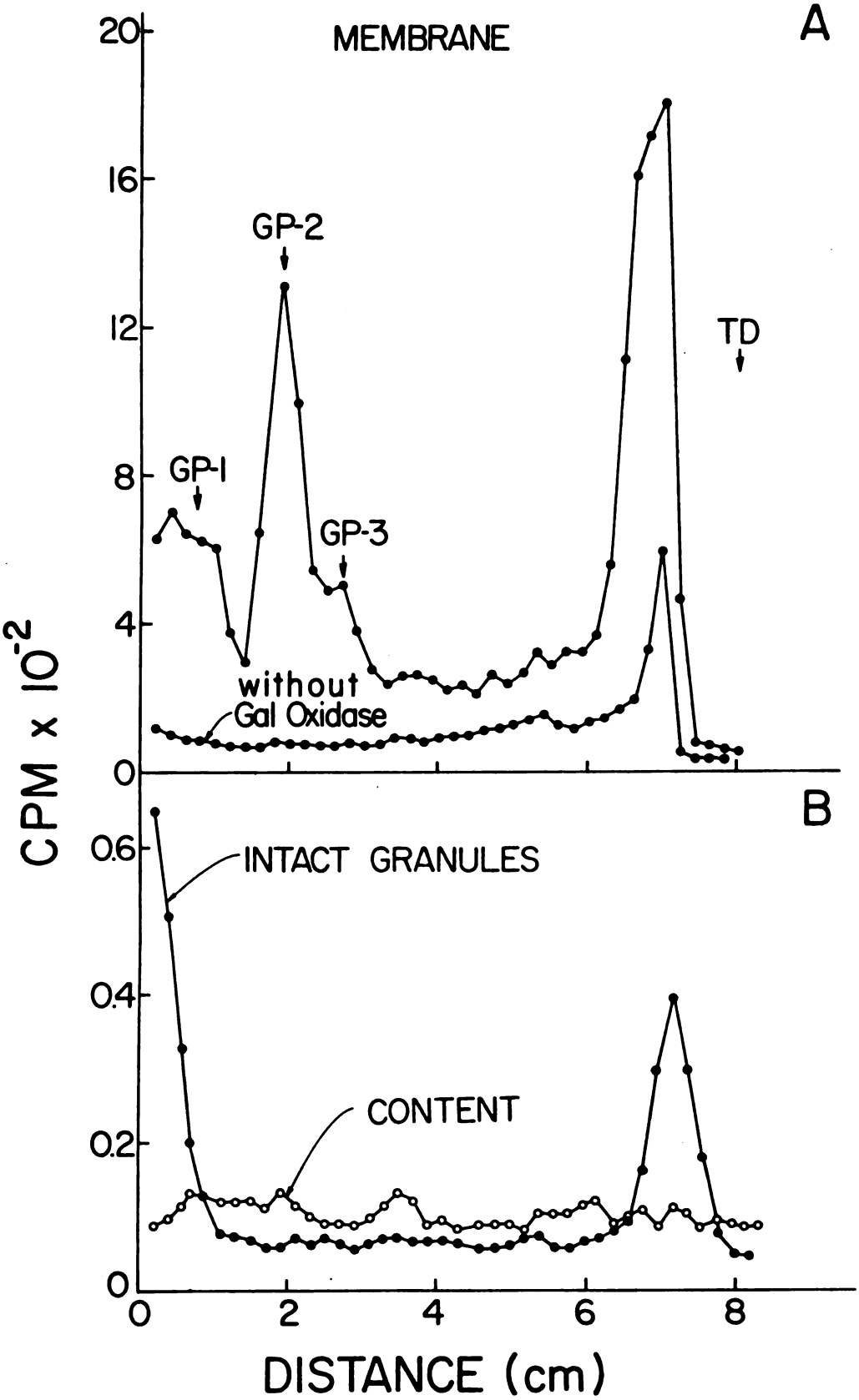
MEMBRANE
A
B
GP-1
GP-2
GP-3
TD
without
Gal Oxidase
INTACT GRANULES
CONTENT
CPM x 10-2
DISTANCE (cm)
20
16
12
8
4
0
0.6
0.4
0.2
0
2
4
6
8

Figure 19

Figure 20. Electrophoretic analysis of enriched plasma membrane glycoproteins.

Procedures for the preparation of the membranes are given in Methods. A) Approximately 250 µg of enriched plasma membrane was submitted to electrophoresis on 1% SDS-9% acrylamide gels as described in Methods. Gels were developed by the PAS stain procedure and scanned at 560 nm. B) Approximately 250 µg intact pancreatic acinar cells were incubated with 15 U/ml galactose oxidase and 0.1 mCi/ml NaB^3H_4 as described in Methods. Following electrophoresis, gels were fractionated into 2 mm sections and assayed for tritium according to Methods (closed circles). In a second experiment, 10^6 cells were homogenized at pH 7.0 and about 300 µg of cell homogenate were labeled and assayed as described in (B) above (open circles). GP-2 denotes the position of granule membrane glycoprotein-2; TD denotes migration of tracking dye.

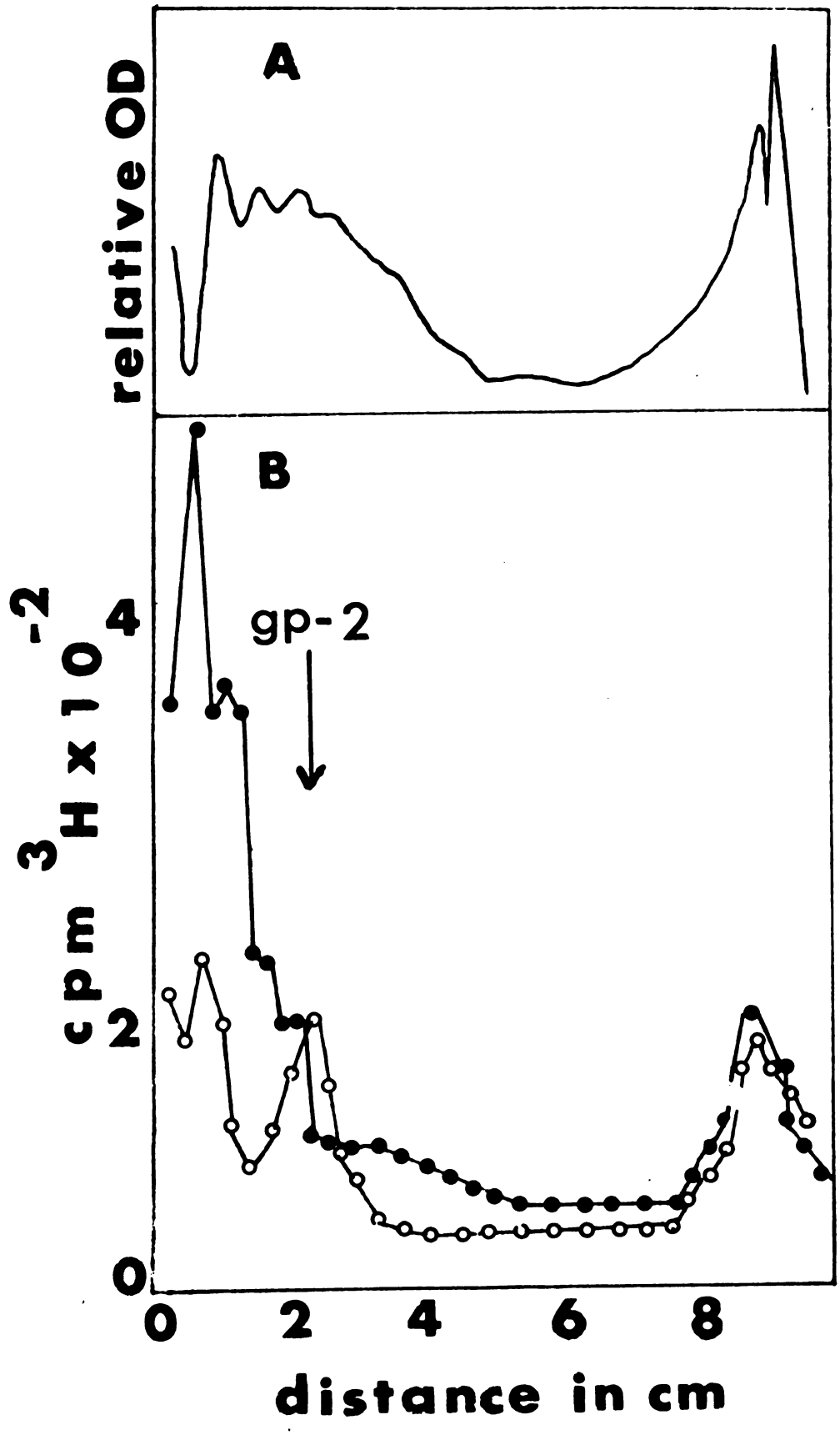

Figure 20

By galactose oxidase/NaB$^3H_4$ labeling of intact acinar cells before and during secretion, the appearance of GP-2 on the cell surface could be recognized. To insure that galactose oxidase/NaB$^3H_4$ treatment did not readily label proteins within the cell, cell viability was maintained over 90%.

When intact acinar cells and lysed acinar cells were labeled with NaB$^3H_4$, lysed cells incorporated almost one-third of the total ^3H cpm incorporated into a low molecular protein (Figure 20B). Fractionation of the cell lysate showed that this species was associated with the postmicrosomal supernate (data not shown). The absence of label in this low molecular weight protein, coupled with the high viability of labeled cells, suggests that only glycoproteins exposed to the outer cell surface were labeled.

Pancreatic acinar cells exposed to 10^{-5} M carbachol secrete similarly to tissue slices, as shown by Figure 21. Acinar cells were incubated in KRB supplemented with 10^{-5} M carbachol. At 1 and 2 h cells were harvested and labeled with galactose oxidase/NaB$^3H_4$ as in Figure 19. The results are summarized in Figure 22. After 1 h, control cells and carbachol treated cells incorporated ^3H into high molecular weight glycoproteins at the same rate. Cells, induced to secrete by carbachol for 2 h, incorporated 155% more ^3H into cell surface glycoproteins than control cells (Figure 22). This apparent lag in the appearance of zymogen granule membrane glycoproteins into the cell surface is similar to results obtained by O'Donnell (1977). Identification of the protein responsible for the increase in acinar cell surface label was attempted on SDS-acrylamide gels (Figure 22). Migration of the labeled product(s) corresponded to a region of the gel between GP-1 and GP-2 markers. However, in order to determine

Figure 21. Secretory response of pancreatic acinar cells.

Acinar cells (10^6) were isolated and incubated in the presence of 10^{-5} M carbachol as described in Methods. At indicated times aliquots of media were removed and assayed for amylase by the method of Bernfield. Control was acinar cells incubated at 4°C in the absence of carbachol (solid circles). Open circles represent cells plus carbachol.

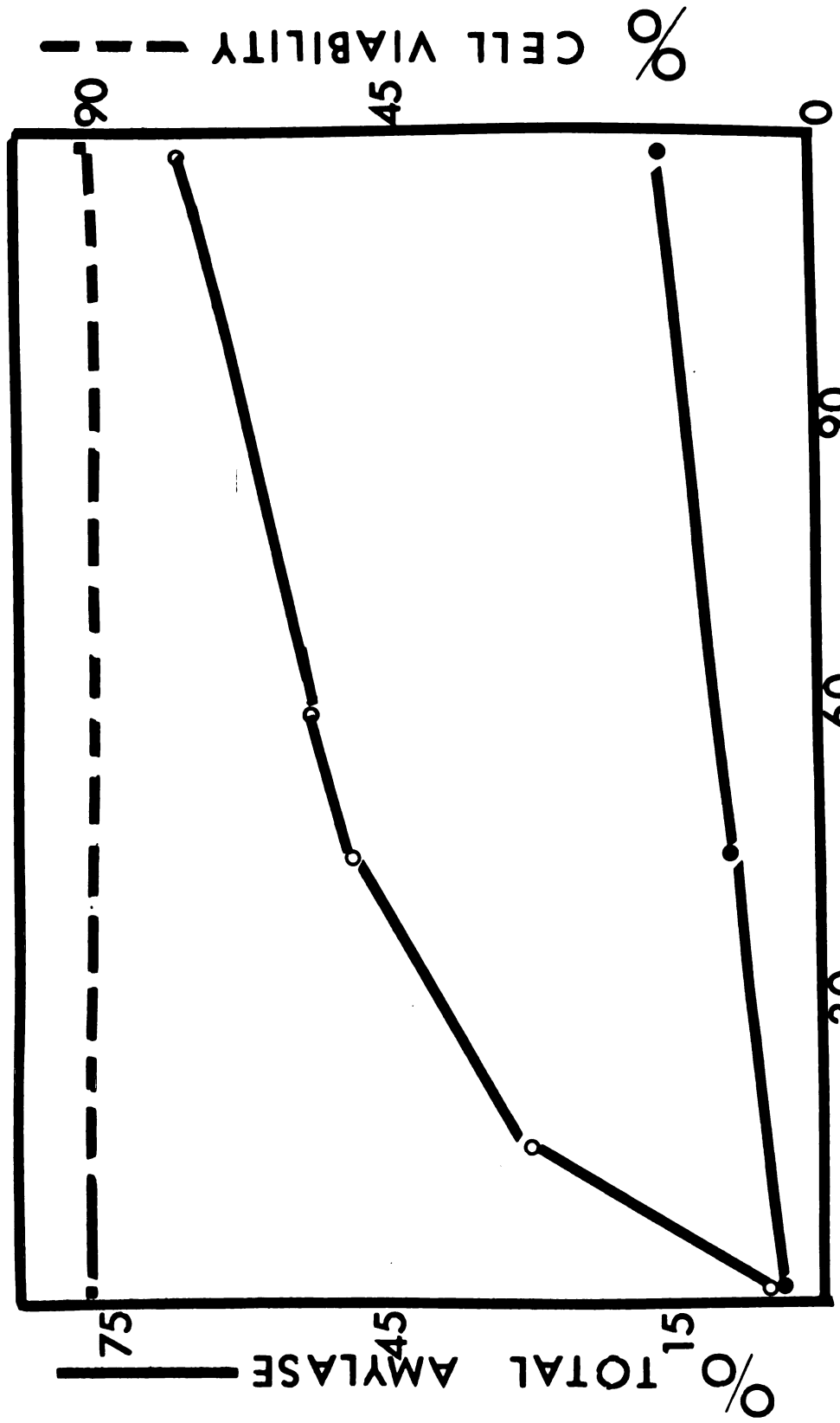

Figure 21

Figure 22. Electrophoretic analysis of galactose oxidase-NaB^3H_4-labeled secreting acinar cells.

Acinar cells were prepared and incubated as described in Figure 21. Following 2 h in the presence of carbachol, cells were removed from media and treated with galactose oxidase (15 U/ml) and NaB^3H_4 (0.1 mCi/ml) as described in Figure 20. After electrophoresis, gels were analyzed for radioactivity. A) Open circles represent cells pre-incubated with carbachol; solid circles represent cells pre-incubated without carbachol. B) Solid circles represent the difference between the electrophoretograms presented in (A).

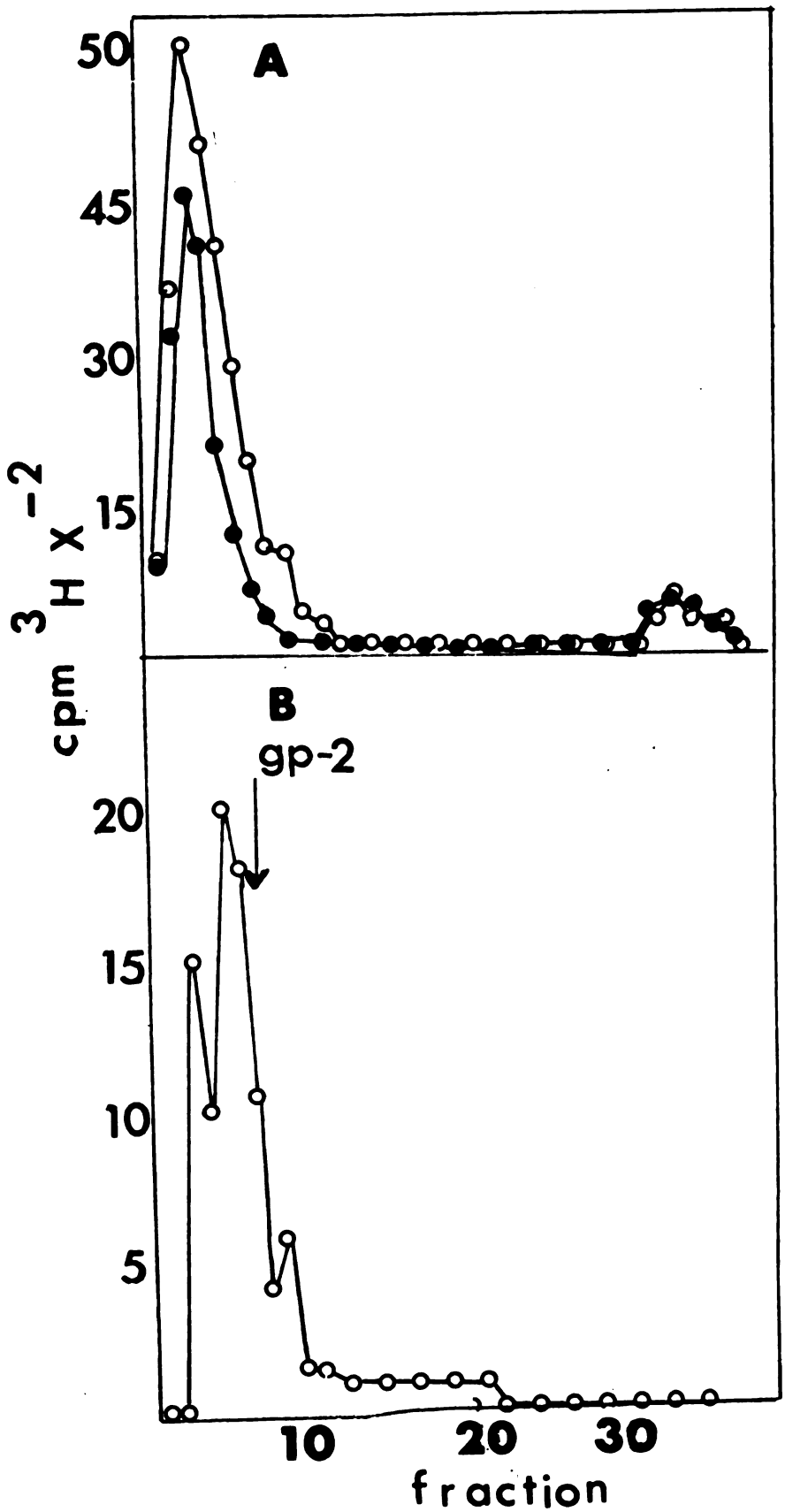

Figure 22

whether secreting acinar cells did incorporate label, presumably by incorporating granule membranes into the cell surface, large amounts (600-800 µg) of protein had to be applied to the gels. An additional experiment illustrated that GP-2 in the presence of large quantities of protein (overload) migrated slower on SDS-polyacrylamide gels.

DISCUSSION

Zymogen Granule Membrane Protein Kinase and Pancreatic Secretion

Amplification of the response to several hormones occurs via increased levels of intracellular mediators, for example, cyclic nucleotides. A plausible model for the involvement of cyclic nucleotides in exocrine pancreatic secretion could entail protein phosphorylation via cyclic nucleotide dependent protein kinases. One possible mechanism entails the following steps: A) Hormonal stimulus causes a rapid, transient increase in cyclic AMP (Kempen et al., 1975; Kempen et al., 1977; Klaveman et al., 1975; Gardner et al., 1976) or cyclic GMP levels (Kapour and Krishna, 1977; Haymovits and Scheele, 1976; Albano et al., 1976; Robberecht et al., 1974). B) Elevated concentrations of cyclic nucleotides could promote the dissociation of regulatory and catalytic subunits of cytoplasmic cyclic nucleotide dependent protein kinases (Lambert et al., 1974) or promote the dissociation of an intrinsic secretory granule protein kinase holoenzyme complex. C) The resulting free catalytic subunit is transferred to a specific phosphorylation site, i.e., zymogen granules (Lambert et al., 1974). This model predicts that a protein kinase is associated with zymogen granules and that the level of phosphorylation of zymogen granule membrane polypeptides increases during secretion (Lambert et al., 1973).

The present study demonstrates that zymogen granules from the rat pancreas contain a protein kinase capable of phosphorylating endogenous and exogenous substrates. Previous reports have described a cyclic nucleotide independent membrane bound protein kinase associated with zymogen granule membranes from the rat pancreas (MacDonald and Ronxio, 1974; Lambert et al., 1974). When granule membranes were isolated, 60% of the protein kinase associated with the zymogen granule preparation was recovered in the membrane fraction (Table 6). However, the membrane protein kinase was no longer responsive to cyclic nucleotides. Elsewhere it has been shown that purified zymogen granule membranes contain less than 1% mitochondrial membrane or microsomal membranes as judged by marker enzymic activities and analyses of membrane polypeptides (MacDonald and Ronzio, 1974; Ronzio, 1973). Hence, it is highly unlikely that the zymogen granule protein kinase represents a contamination by these sources. Since the majority of protein kinase activity associated with granules cannot be extracted by procedures that remove adsorbed soluble proteins (see Table 6), the granule protein kinase is not likely to be a soluble contaminant.

Aside from containing protein kinase activities, zymogen granules and granule membranes also appear to contain endogenous phosphoproteins. Reports by MacDonald and Ronzio (1974) and Lambert et al. (1974) have illustrated the presence of granule membrane polypeptides capable of being phosphorylated by an endogenous protein kinase. MacDonald and Ronzio have claimed that a single high molecular weight (130,000) membrane protein incorporates $^{32}PO_4^{-3}$ when zymogen granules are incubated with (γ-$^{32}PO_4$)ATP. In contrast, Lambert et al. revealed multiple phosphorylation of 9 to 10 zymogen granule membrane

polypeptides when membranes were incubated with (γ-$^{32}PO_4$)ATP. Using reaction conditions described by Lambert et al., we found that pure zymogen granule membranes phosphorylated a single high molecular weight membrane protein, similar to data obtained by MacDonald and Ronzio. However, the zymogen granule fraction, consisting of whole zymogen granules, was able to incorporate $^{32}PO_4^{-3}$ into several membrane polypeptides in addition to several soluble proteins. These data are similar to those obtained by Lambert et al. As judged by cytochrome c oxidase activity, Lambert et al. (1973) reported that their granule fraction contained one-third of the total cytochrome c oxidase activity present in the mitochondria fraction. When compared to less than 1% mitochondria contamination for MacDonald and Ronzio's membrane preparation, the granule membrane purity of Lambert et al. is in doubt. It is likely that pure granule membranes possess an intrinsic polypeptide of high molecular weight capable of being phosphorylated. In contrast, whole granules contain mitochondrial contaminants and loosely bound soluble proteins capable of being phosphorylated.

Zymogen granules and granule membranes contain relatively low specific activities of cyclic nucleotide dependent and independent protein kinases. However, several mechanisms are possible in which zymogen granule protein kinases can be modulated in response to secretagogues. Cyclic AMP and/or cyclic GMP may activate intrinsic granule cyclic nucleotide dependent protein kinases or intracellular rises of cyclic nucleotides could promote the dissociation of cytoplasmic cyclic nucleotide dependent protein kinases, followed by subsequent translocation of the catalytic subunit to zymogen granules.

Translocation of the catalytic subunit from the cytoplasmic fraction to particulate fractions has been observed in several tissues. Protein kinase translocation from the cytoplasmic fraction to microsomes has been described as an early event in the hormonal control of uterine contraction (Korenman et al., 1974). The kinase activity translocated was primarily in a cyclic nucleotide independent form. Other tissues, reported to respond to hormonal stimuli in a similar translocation phenomenon, include rat prostate (Tsang and Singhal, 1976), rat liver (Castagna et al., 1975), corpus luteum (Darbon et al., 1976), adrenal medulla (Kurosawa et al., 1976), and the anterior pituitary (Lemarie et al., 1971). In a reconstituted model system of postmicrosomal supernate and zymogen granules, a soluble cyclic nucleotide dependent catalytic subunit translocated to zymogen granules in the presence of cyclic AMP (Table 8). Cyclic AMP, but not cyclic GMP, at concentrations (10^{-6} M) high enough to stimulate cytoplasmic protein kinase was able to induce a 5-fold translocation of kinase to zymogen granules. However, the physiological significance of this observation is questionable for several reasons. First, the adsorbed activity was readily extracted under physiological ionic strength conditions, similar to results obtained by Keely et al. (1975) when studying protein kinase translocation in heart muscle. Secondly, only cyclic AMP promoted the binding of kinase activity to zymogen granules. Cyclic GMP, at a concentration capable of activating soluble cyclic GMP dependent protein kinases, failed to produce a translocation of kinase to zymogen granules. Since pancreatic cyclic nucleotide dependent protein kinases appear to have catalytic and regulatory subunits (Van Leemput-Coutrez et al., 1973), both cyclic AMP and cyclic GMP would likely promote

dissociation of the holoenzyme complex resulting in a free catalytic subunit. Apparently only the catalytic subunit from cyclic AMP dependent kinases binds to zymogen granules, in the reconstituted system described in Table 8. This can be explained by several possibilities. One explanation is that there is a fundamental difference between the two catalytic subunits. Perhaps substrate recognition is important in the translocation of protein kinase in the pancreatic reconstituted system. Another explanation concerns the stability of the cyclic GMP dependent protein kinase. According to Van Leemput-Coutrez et al. (1973), the pancreatic cyclic GMP dependent protein kinase is rather unstable. In either case, experiments using an intact tissue system were included in the analysis of cyclic GMP effects on zymogen granule protein kinase activity.

The significance of the cyclic AMP dependent translocation of protein kinase activity to zymogen granules is also lessened by the nonspecificity of the process. In an experiment similar to that described above, in the presence of cyclic AMP, microsomes bound protein kinase activity as readily as zymogen granules (Table 9).

Finally, in intact tissue systems treated with secretagogues, thought to elevate either cyclic AMP or cyclic GMP, an increase in zymogen granule protein kinase activity was not detectable. In these experiments protein kinase was measured in both sucrose and 0.15 M KCl washed granules, using both endogenous acceptors and exogenous substrates. The zymogen granules prepared from control tissues were compared with those from tissues incubated with carbachol, dibutyryl cyclic AMP, pancreozymin, pancreozymin-C-octapeptide, and caerulein for periods ranging from 2.5 min to 60 min (Table 10). Furthermore, cyclic AMP and cyclic GMP dependent kinase activities

were compared with cyclic nucleotide independent activities. Results summarized in Table 10 illustrate the stability of zymogen granule protein kinase activity (cyclic nucleotide dependent and independent) during the initiation of secretion by carbachol and pancreozymin. However, these negative results do not rule out the possibility that a very rapid (<2.5 min) transient interaction (binding) of protein kinase to zymogen granules occurs during stimulus-coupled secretion.

As indicated in Table 4, protein kinase activity was present in high levels in smooth microsomes, consisting primarily of fragments of the Golgi complex (Ronzio, 1973). Sinze zymogen granule membranes apparently arise from Golgi membranes (Palade, 1975), it is likely that the zymogen granule membrane protein kinase represents a plasma membrane precursor, inserted into the granule membrane at the Golgi complex and transported to the apical cell surface during exocytosis.

Another prediction of the model for the role of protein kinase in pancreatic secretion is that zymogen granule polypeptides, presumably membrane proteins, are phosphorylated during stimulus-coupled secretion. We were unable to detect reproducible granule membrane protein phosphorylation in secreting tissue that was significantly greater than control values (Figures 9 and 10). The $^{32}PO_4^{-3}$ associated with zymogen granule preparations probably represented a variable amount of adsorbed soluble phosphoprotein. It is also possible that other loosely bound cellular constituents, for example, microtubules, may be phosphorylated (Goodman et al., 1970; Sheterline and Schofield, 1975) and bound to secretory granules (Burridge and Phillips, 1970; Ostlund et al., 1977; Sherline et al., 1977). However, no single polypeptide species was apparent in the radioactivity profiles of intact granules after polyacrylamide gel electrophoresis. Furthermore,

no proteins in highly purified zymogen granule membranes were phosphorylated *in vitro*.

In summary, we have studied aspects of secretion regulation of the exocrine pancreas at the level of the zymogen granule, most notably protein kinase levels. The presence of a granule protein kinase, plus evidence implicating cyclic nucleotides, in the mechanism of action of pancreatic secretagogues suggested a possible role for phosphorylation at the zymogen granule membrane level. These results suggest that the much talked about role of cyclic nucleotides in pancreatic protein secretion may not be as important as other cell mediators, i.e., calcium ions may be of greater importance.

Zymogen Granule Phosphatidylinositol and Pancreatic Secretion

The increased incorporation of $^{32}PO_4^{-3}$ into total cellular phospholipids in response to hormone stimuli in secreting pancreas acinar tissue was first discovered by Hokin and Hokin (1953, 1956). Hokin and Hokin also were able to show that these changes were largely confined to the metabolism of phosphatidylinositol. More recent data have solidified the findings of Hokin and Hokin. In guinea pig pancreas, acetylcholine and pancreozymin were found to increase the specific activity of cellular ($^{32}PO_4$)-labeled phosphatidylinositol (White and Hawthorne, 1970; Hokin and Huebner, 1967). However, in mouse tissue, acetylcholine apparently decreases cellular phosphatidylinositol (Hokin-Neaverson, 1974). Carbachol was also found to increase $^{32}PO_4^{-3}$ incorporation into pancreatic phosphatidylinositol (Bauduin et al., 1971). More recently, Slaby and Bryan (1976) illustrated that high concentrations of myo-inositol were able to elicit an *in vitro* secretory response in rat pancreatic tissue.

These results tend to suggest that myo-inositol selectively stimulates the release of secretory protein from newly forming zymogen granules.

With this background it was interesting to explore the possibility of the direct involvement of granule membrane phospholipids in protein secretion. Although zymogen granule membrane protein phosphorylation was not affected by stimulus-coupled secretion, an increase in $^{32}PO_4^{-3}$ incorporation into the phospholipid fraction was readily discernible. One phospholipid, phosphatidylinositol, was responsible for the increase of $^{32}PO_4^{-3}$ incorporation into granule membrane phospholipids. Following separation of the granule membrane lipid extract on two-dimensional thin layer chromatography, phosphatidylinositol was separated from phosphatidylserine and the other phospholipids present in the zymogen granule membrane. The amount of $^{32}PO_4^{-3}$ incorporated into granule membrane phosphatidylinositol increased linearly throughout the first 60 min of secretagogue stimulated secretion. Two classes of secretagogues, cholinergic and polypeptide, were able to stimulate $^{32}PO_4^{-3}$ incorporation into phosphatidylinositol. Several explanations exist as to how $^{32}PO_4^{-3}$ is incorporated into phosphatidylinositol: i) *de novo* synthesis, ii) increased turnover, and iii) phosphorylation of the inositol moiety.

Increased *de novo* synthesis of zymogen granule membrane phosphatidylinositol during stimulus-coupled secretion was not detected in rat pancreas tissue, although phosphatidylinositol synthesis has been shown to increase during secretion in rough endoplasmic reticulum and in Golgi membranes of guinea pig pancreas (Hokin and Huebner, 1967). Hokin and Huebner suggest that the increased synthesis of phosphatidylinositol may be concerned with the formation of new

endoplasmic reticulum and Golgi membranes to replace that which is presumably converted to granule membranes. It is possible that the observed increase of zymogen granule $^{32}PO_4$-labeled phosphatidylinositol is due to the appearance of newly synthesized (immature) zymogen granules in the acinar cell. Presumably this process reloads the acinar cell with secretory granules. Whether this synthesis process is stimulated by secretagogues or begins early in the secretory phase is unknown. However, the reappearance of zymogen granules in cells that have secreted exhaustively has been observed to occur generally after 3 to 4 h following the initiation of secretion by secretagogues (Kramer and Geuze, 1974). Therefore, it would appear likely that the increased $^{32}PO_4$-labeled zymogen granules (labeled within the first hour) are not derived from newly formed granules.

A second possible source for phosphatidylinositol-$^{32}PO_4^{-3}$ would be phosphorylation of the inositol moiety by a phosphatidylinositol kinase. Such an enzyme exists in chromaffin granules (Sharoni et al., 1975; Trifaro and Dworkind, 1975). Under conditions which induce the release of catecholamines, ATP and soluble proteins from chromaffin granules phosphatidylinositol was phosphorylated to form diphosphatidylinositol (Trifaro and Dworkind, 1975). Trifaro and Dworkind also illustrated the synthesis of diphosphatidylinositol in chromaffin granules *in situ* during stimulation of the adrenal medulla by acetylcholine. Analysis of the zymogen granule phospholipid fraction revealed no detectable polyphosphatidylinositols. Furthermore, when granules were incubated with (γ-$^{32}PO_4$)ATP for long periods of time, there was no detectable phosphorylation of phosphatidylinositol. Therefore, the probable effect of secretion on zymogen granule membrane

phosphatidylinositol is increased turnover. However, the mechanisms of this proposed turnover remain to be studied.

Whether phosphatidylinositol turnover helps to initiate secretion upon hormonal stimulation or is part of the exocytosis process is unknown. The lipid metabolism of secretory granule membranes may play an important role in secretion. Recent evidence has shown that in the exocytosis process in rat mast cells (Lawson et al., 1977) and in the process of encystment in *Phytophthora palmivora* zoospores (Pinto da Silva and Nogueira, 1977), fusing membranes become particle free at the fusion site. That is, as membranes approach each other, membrane particles, i.e., membrane proteins, move laterally from the fusion site, resulting in interaction of two particle free membranes. Such a process would likely implicate lipid metabolism, i.e., breakdown, synthesis or turnover, in the secretion process.

In conclusion, zymogen granule membrane phosphatidylinositol incorporates $^{32}PO_4^{-3}$ at a linear rate for 1 h following the administration of carbachol or caerulein. It is possible that this hormone induced phosphate incorporation is derived from increased rate of phosphatidylinositol turnover. The importance of this observed process is unclear at this time. But one can conclude that phosphatidylinositol may be an important component of granule membranes involved in secretion.

Topology of Zymogen Granule Glycoproteins

The relatively simple protein composition of zymogen granule membranes, especially concerning glycoprotein content, suggests that these proteins may have a single function. For example, proteins located on the exterior of zymogen granule membranes may function as a specific receptor capable of recognizing the specific region of the

plasmalemma, where zymogen granules have been observed to fuse in exocytosis. A possible role for proteins located on the granule interior could be stabilization of the life-span of such granules.

MacDonald and Ronzio (1972) illustrated by comparative analysis of zymogen granule membrane polypeptides that a glycoprotein, designated GP-2, characterizes granule membranes of the mammalian pancreas. When purified, GP-2 migrates on SDS-polyacrylamide gels at a relative mobility equivalent to 74,000 molecular weight (MacDonald, 1974). Pure preparations of GP-2 contained 15% carbohydrate, a single N-terminal amino acid and GP-2 represented approximately 45% of the firmly bound granule membrane protein (Lewis et al., 1977).

Galactose oxidase-NaB^3H_4 labeling of granule membranes resulted in 3 glycoproteins being labeled (Figure 18C). However, labeling of intact granules resulted in only the high molecular weight glycoprotein, GP-1, being labeled. GP-2 was apparently protected from galactose oxidase by presumably being located on the interior of granule membranes (Figure 18B). However, it is possible that under conditions in which zymogen granules readily lyse, the glyco region of GP-2 could become unmasked (whether located on the exterior or interior side of the granule membrane). The conclusion that GP-2 is located on the interior of granule membranes is supported by data obtained by Kronquist (1975). GP-2 contained almost 90% of the total membrane sialic acid (Lewis et al., 1977). Experiments with neuraminidase treatment of intact granules and granule membranes showed that neuraminidase was effective in releasing appreciable amounts of sialic acid when incubated with membranes. Neuraminidase was ineffective in releasing sialic acid from intact zymogen granules. The presence of GP-2 on the interior of granule membranes allows the

opportunity to establish the appearance of GP-2 in the plasma membrane. Fusion of vesicle membranes with the plasma membrane would provide a mechanism for the insertion of plasma membrane components (Bennett et al., 1974; Morré, 1975). With GP-2 located internally within the granule and according to the exocytosis model, GP-2 should become accessible on the outer surface of the cell membrane. Data presented in Figure 20 illustrate the appearance of a high molecular weight glycoprotein associated with intact pancreatic acinar cells following 2 h stimulation by either carbachol or caerulein. This appearance was assayed using galactose oxidase-NaB^3H_4 labeling of acinar cells. Several problems were immediately raised with these results: 1) the apparent lag in the appearance of ^3H-labeled proteins during the secretory cycle (there was no lag in the release of amylase from isolated cells; see Figure 21), and 2) the mobility of the increased ^3H-labeled product migrated slower than GP-2.

The lag in appearance of label can be explained in several ways. First, it should be noted that in experiments using zymogen granule membrane antibodies, antibodies bound to acinar cells only after a lag period of about 100 min (O'Donnell, 1977). This lag period could be the result of a critical concentration of the substrate appearance on the cell surface. In the other case, due to the low amount of counts incorporated into acinar cell surface, large quantities of protein, upwards to 800 µg, were analyzed on cylindrical SDS-polyacrylamide gels. Such large quantities of protein apparently interfered with the mobility of GP-2 by reducing the distance migrated. In an experiment where granule membranes plus excess BSA (700 µg) were electrophoresed on SDS-polyacrylamide gels, the mobility of GP-2

was considerably reduced. However, it is not possible to determine whether the increase in glycoprotein on the acinar cell surface during stimulus-coupled secretion is due to either GP-1 or GP-2. However, GP-2 is the most likely candidate due to the topology studies conducted in Figures 18 and 19.

The evidence supporting the exocytosis theory (Scheme 1) is based almost exclusively on electron microscopic data. These morphological studies have observed increases in plasma membrane corresponding to loss in the number of secretory granules. Biochemical evidence illustrating the incorporation of secretion vesicle membranes into the plasma membrane is practically nonexistent. Viveros et al. (1971) have suggested an apparent incorporation of dopamine-β-hydroxylase activity (associated with chromaffin granules) into plasma membranes of the adrenal medulla. However, their data are weak and do not allow a conclusion about how much of the secretory membrane entered into the plasma membrane. It is obvious from this background that the data presented concerning the appearance of zymogen granule membrane glycoproteins into the cell surface is important, especially when combined with the antibody experiments of O'Donnell (1977). These data represent biochemical evidence for the incorporation of zymogen granule membrane into the plasmalemma of secreting pancreatic cells.

In summary, GP-2 is an intrinsic glycoprotein marker for zymogen granule membranes. GP-2 appears to be sequestered within granules, where its sialyl moieties could interact with basic secretory proteins or calcium ions. During secretagogue induced secretion, GP-2 appears to be incorporated into the acinar cell surface, along with the zymogen granule membrane, where it can be labeled with galactose

oxidase-NaB^3H_4 labeling techniques. The role of GP-2 as well as GP-2's fate following insertion into the plasmalemma are unknown.

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