SULFATED GLYCOPOLYPEPTIDES OF THE EXOCRINE RAT PANCREAS

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IN THE EXOCRINE RAT PANCREAS

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ABSTRACT

SULFATED GLYCOPOLYPEPTIDES OF THE
EXOCRINE RAT PANCREAS

By

Kathryn E. Kronquist

Sulfated macromolecules identified as mucopolysaccharides, have been shown to be present in secretion granules from several cell types. Electronmicrographic evidence has indicated that sulfated macromolecules are also present in the zymogen granules of acinar pancreas. The present study describes the characterization and subcellular distribution of sulfate-containing macromolecules from rat pancreas.

The major classes of high molecular weight, sulfated compounds synthesized from $H_2\ ^{35}SO_4$ by pancreas tissue in vitro were analyzed. Sulfated glycolipids accounted for only 1% to 3% of the total non-dializable $^{35}SO_4^{-2}$ counts incorporated. The remainder of macromolecule-bond ^{35}S-sulfate from tissue extracts was resistant to complete degradation by proteases and was tentatively identified as a heterogeneous family of glycopolypeptides or proteoglycans.

The subcellular distribution and specific radioactivity of ^{35}S-sulfate in major organelle subfractions of labeled pancreas tissue was determined. The zymogen granule and mitochondrial fraction each contained 14% of the total bound sulfate of the homogenate. Microsomes

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and the soluble cytoplasmic fraction (post microsomal supernatant) contained respectively 30% and 25% of the total sulfate. The remainder of ^{35}S-sulfate was accounted for in the cellular debris.

The specific radioactivity, defined as $^{35}SO_4^{-2}$ counts per minute per microgram of protein, was highest in purified zymogen granule membranes. The specific radioactivity of mitochondrial and microsomal membranes was 12-fold lower than that of zymogen granule membranes. Smooth microsomal membranes, which in pancreas predominantly contain elements of the Golgi complex, had a specific radioactivity three-fold lower than zymogen granule membranes.

Purified membranes from sulfate-labeled zymogen granules, mitochondria, and microsomes, and also the soluble zymogen granule contents and the cytoplasmic fraction were analyzed by electrophoresis on 9% acrylamide gels in 1% sodium dodecylsulfate to determine if sulfate label was coincident with major polypeptide components in these fractions. In all samples, the sulfated macromolecules migrated in the high molecular weight range of the gels but sulfate was not coincident with any major Coomassie blue-staining polypeptide. In the soluble fractions, in addition to high molecular weight material, minor sulfated components of lower molecular weight were also present.

To further characterize the zymogen granule membrane and zymogen granule lysate (contents), sulfate-labeled fractions were mixed with ^{3}H- or ^{14}C-leucine-labeled or ^{3}H-glucosamine-labeled fractions and were analyzed on acrylamide gels. In zymogen granule membranes, sulfate co-electrophoresed with leucine and glucosamine, indicating that sulfate was associated with protein and carbohydrate. In zymogen

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granule lysate, sulfate was found associated with glucosamine but no co-migrating leucine could be detected.

After treatment of zymogen granule membranes with proteolytic enzymes, 50% of the ^{3}H-leucine counts present before enzyme treatment in high molecular weight material migrated off of the gel, indicating extensive proteolytic degredation, while 100% of the sulfate counts similarly digested could still be recovered in the high molecular weight region of the gel. This finding indicated that sulfate was not covalently bound to amino acid residues. Sulfate counts did co-migrate on gels with ^{3}H-glucosamine counts in labeled zymogen granule membranes both before and after proteolysis, which was consistent with the interpretation that sulfate was covalently linked to carbohydrate.

The stability of sulfated macromolecules in zymogen granule membranes, mitochondrial membranes, microsomal membranes and zymogen granule lysate to weak acid and the resistance of these fractions to digestion by Chondroitinase-ABC demonstrated that hyaluronic acid, dermatan sulfate and chondroitin-4- and 6-sulfate were not present. This finding was confirmed in zymogen granule membranes and lysate when carbohydrate analysis by gas-liquid chromatography did not detect the presence of glucuronic acid, a constituent of acid mucopolysaccharides. In the soluble cytoplasmic fraction, approximately 40% of the bound sulfate counts were hydrolyzed by weak acid, suggesting the presence of heparin or heparan sulfate. Preliminary carbohydrate analysis revealed 5 to 10 nmoles of glucuronic acid per milligram of protein in this fraction.

While the presence of mucopolysaccharides was indicated in the soluble cytoplasmic fraction, the sulfated macromolecule bound to

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intracellular membranes and found in the zymogen granule lysate displayed the properties of a sulfated glycoprotein or of the mucopolysaccharide keratin sulfate. Keratin sulfate, however is unlikely since it is usually of mesodermal origin, and pancreas is an epithelial tissue.

A detailed analysis of the carbohydrate composition of the rat zymogen granule and zymogen granule subfractions showed that the total protein of the granule contents was 0.23% carbohydrate by weight. In contrast, the zymogen granule membrane protein was heavily glycosylated, containing 22% carbohydrate by weight. All fractions contained, in varying amounts, the carbohydrates fucose, mannose, galactose, N-acetylglucosamine, and sialic acid.

Studies of the action of Neuraminidase on intact and lysed zymogen granules indicated that sialic acid was localized on the internal surface of the zymogen granule. This type of study can be extended to determine the localization of other sugars and of sulfate on the zymogen granule membrane.

The present study has demonstrated that sulfated mucopolysaccharides are not present in rat zymogen granules. The nature of the sulfated macromolecules in pancreatic acinar cells is quite different. While some mucopolysaccharide is present in the cytoplasmic fraction, the intracellular membranes contain a tightly bound sulfated glycoprotein and a similar sulfated glycoprotein is present in the zymogen granule lysate.

SULFATED GLYCOPOLYPEPTIDES OF THE EXOCRINE RAT PANCREAS

By

Kathryn E. Kronquist

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
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DOCTOR OF PHILOSOPHY

Department of Biochemistry

1975

DEDICATION

To my mother, Norma Kronquist, with gratitude for her support and assistance.

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ACKNOWLEDGMENTS

I wish to gratefully acknowledge the assistance and support so readily given to me by my thesis advisor, Dr. Robert A. Ronzio. Also, I wish to thank Dr. Charles C. Sweeley and his entire laboratory group, especially Dr. Robin E. Chambers, for the help which they gave me with carbohydrate analysis techniques.

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MEM	M
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PAP	
PAPS	
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TCA	
TEMED	
TMCS	

LIST OF ABBREVIATIONS

APS	adenosine-5'-phosphosulfate
BHT	butyrated hydroxytoluene
BSA	bovine serum albumin
DMDS	dimethyldichlorosilane
EDTA	(ethylenedinitrilo)-tetraacetic acid
Gal	galactose
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
GlcUA	glucuronic acid
HMDS	hexamethyldisilazane
IdUA	iduronic acid
KRB	Krebs-Ringer buffer
MEM	minimal-essential medium
NANA	N-acetyl neuraminic acid
PAP	adenosine 3',5'-diphosphate
PAPS	3'phosphoadenosine-5'-phosphosulfate
PTA	phosphotungstic acid
SBTI	soybean trypsin inhibitor
SDS	sodium dodecylsulfate
TCA	trichloroacetic acid
TEMED	N,N,N,', N'-tetra methylethylenediamine
TMCS	Trimethylchlorosilane

LITERATURE REVIEW

Statement of the Problem

Electromicrographic studies have shown that sulfated macromolecules are present in zymogen granules of exocrine pancreas. It has been hypothesized that the sulfated species is a mucopolysaccharide which functions in the packaging or release of secretory proteins. A chondroitin-sulfate-like mucopolysaccharide has been characterized in isolated chromaffin granules and in secretions from the adrenal medulla, however, the pancreatic macromolecule has never been studied.

In a unique type of study, we determined the subcellular distribution of sulfated macromolecules in rat pancreas. The only other such localization study in the literature not involving chondrocytes reported the discovery of membrane-bound and soluble forms of heparan sulfate in cultured animal cells.

Characterization of the sulfated macromolecules in pancreatic subcellular fractions revealed compounds tightly bound to the purified membranes of zymogen granules, mitochondria, and microsomes and in soluble fractions which have the properties of sulfated glycoproteins or keratan-sulfate-like molecules. Keratan-sulfate, however, is usually of mesodermal origin and pancreas is an epithelial tissue.

It is known that a wide variety of epithelial tissues such as salivary gland, stomach, and intestinal mucosa contain sulfated

glycopeptides. Unfortunately, in these tissues the glycopeptides were isolated after proteolytic digestion and no information is available on the native sulfated glycoprotein or its subcellular distribution. Our experimental rational was that determination of the distribution and characterization of the sulfated compound or compounds would lead to an understanding of their physiological role and their relationship, in zymogen granules, to secretion.

Secretion Granules

Many tissues synthesize and sequester in membrane bound vesicles products destined for secretion. The general release mechanism for such products from cells involves fusion of storage granules with the plasma membrane (Poste and Allson, 1974). It is possible to isolate such secretion granules from many tissues. Following rupture of the granule, the limiting membrane can be obtained in pure form, uncontaminated by other intracellular membranes. Isolated secretion granule membranes can be used as model systems for the general study of membranes. In addition, investigation of such granules and their membranes is useful in understanding the process of secretion. We feel that the answers to such questions as how are cells stimulated to secrete; why do secretion granules fuse with specified portions of the plasma membrane but not with other intracellular membranes; and what is the rate limiting step of membrane fusion and its reverse process, pinocytosis, will be found in the physical properties and enzymatic activities associated with the secretion granule itself.

The polypeptide composition of the membrane of the secretion granule from exocrine rat pancreas, the zymogen granule, has been studied (MacDonald and Ronzio, 1972; MacDonald, 1974). These membranes contain a major component that is a sialoglycopeptide. A protein kinase and a Mg^{+2}-ATPase were also found to be intrinsic membrane components.

Little is known about the properties of secretion granule surfaces. The granules of adrenal medulla (Mathews et al., 1972) and the neurohypophysis (Poisner and Douglas, 1968) possess a net negative charge. Mathews et al. (1974) have postulated that a significant fraction of this charge is due to external sialic acid residues. Data will be presented in this thesis which suggest that sulfate bound to membrane glycoproteins may also contribute to the anionic properties of storage granule membranes in rat pancreas.

Sulfated Macromolecules in Zymogen Granules and Other Secretion Vesicles

An autoradiographic experiment by Berg and Young (1971) investigated the uptake of inorganic ^{35}S-sulfate in pancreatic acinar cells of mice injected with $H_2{}^{35}SO_4$. They found that labeled sulfate was concentrated at the Golgi complex within 10 minutes after injection. In 30 minutes, much of the radioactive material was present in condensing vacuoles on the periphery of the Golgi complex. Four hours after injection, radioactivity was associated with zymogen granules and labeled material was present in the pancreatic duct system, presumably having been secreted. Because perfusion, prolonged aqueous fixation and subsequent extraction procedures removed free sulfate from the tissues, the authors concluded that the sulfate in the Golgi

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complex and zymogen granules was macromolecule-bound and speculated, in the absence of any chemical evidence, that it was a mucopolysaccharide and aided in the packaging or release of digestive enzymes

Other secretory granules have been shown to contain macromolecules which label with ^{35}S-sulfate. The presence of chondroitin-4- and chondroitin-6-sulfate and of sialoglycoproteins in purified chromaffin granules of bovine adrenal medulla has been demonstrated (Margolis and Margolis, 1973). A sulfated mucopolysaccharide-protein complex with the ability to bind 5-hydroxytryptamine and histamine was found in rat thrombocyte granules (Åborg and Uvnäs, 1971), in the catecholamine-containing vesicles of adrenal medulla in cats, dogs, and cows (Fillion et al., 1971), and in adrenergic vesicle fractions from sympathetically-innervated tissues, peripheral sympathetic nerves, and brain from dog and cat tissues (Åborg et al., 1972). Also a sulfated component in the soluble contents of chromaffin granules from adrenal medulla was found to bind to acidic granule proteins (chromogranins) in rat and ox, but this compound has not been characterized (Baumgartner et al., 1974).

There is controversy over the original hypothesis that mucopolysaccharides were present in these storage granules to bind catecholamines, since data has been presented that the molar ratios of uronic acids to monoamines is too low to account for the binding of major amounts of endogenous monoamines to the anionic glycosoaminoglycans (DaPrada et al., 1972). Therefore, the physiological role of such sulfated mucopolysaccharides in storage granules is uncertain and its presence in zymogen granules from pancreas has never been directly demonstrated.

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Sulfate Metabolism in Mammals

Uptake

The mechanism of intestinal absorption of ingested SO_4^{2-}ions is unknown. Deyrup (1963) showed that in rat it occurs only in the lower illium and that it can be inhibited by dinitrophenol, cyanide, and fluoride, indicating that it is an energy-requiring process.

Active Transport of Sulfate Ions

Sulfate ions are resorbed by an unknown mechanism as the glomerular filtrate passes down the kidney tubules (Smith, 1951). Deyrup (1956) showed that slices of kidney cortex can concentrate sulfate ions *in vitro* by a process which is independent of added ATP or substrate and, more recently (Deyrup, 1964), that uptake of SO_4^{2-}ions is inhibited by $S_2O_3^{2-}$ions, suggesting that both sulfate and thiosulfate bind to common sites in the kidney.

Within cells, isolated mitochondria from rat kidney cortex take up sulfate ions from their environment, effectuating a several hundred fold concentration (Winters et al., 1962).

Mammalian Incorporation of Sulfate into Proteins

The utilization of inorganic sulfate for synthesis of sulfur-containing amino acids is negligible or absent in mammals (Boström and Avquist, 1952; Dziewiatkowski, 1954). There is one exception: Rambaut and Miller (1965) have shown that adult cats maintained on diets lacking sulfate-containing amino acids can use SO_4^{-2}ions extensively for the synthesis of cystein and methionine in blood proteins. The cat is unique among monogastric animals in that it does not require

exogenous cystein or methionine. Ruminants can also utilize inorganic forms of sulfate for the synthesis of methionine and cystein but this process is a function of the micro-organisms of the rumen and not of the mammal itself (Block and Stekol, 1950; Block et al., 1951).

Mammalian Synthesis of Sulfate Esters and Sulphamates

Sulfate esters have the general formula $R.OSO_3^-M^+$, where R may be many compounds. Sulphamates are compounds, analogous to sulfate esters, of the general formula $R.NH.SO_3\text{-}M^+$. Sulphamates are not widespread in nature. The only macromolecule possessing a sulphamate linkage is heparin and its related compound, heparan sulfate, both of which contain the sulphamate of D-glucosamine, 2-deoxy-2-sulphoamino-D-glucose (Jorpes et al., 1950). Sulfate esters and sulphamates are formed *in vivo* by the transfer of sulfate from PAPS, 3'-phosphoadenosine-5'-phosphosulfate.

Chemical Stability of Sulfate Esters, Sulphamates, and Sulfatophosphates

All sulfate esters are readily hydrolyzed by acid. The sulphamino linkage in carbohydrates is much more acid-labile than the O-sulfonate ester linkage. The two types of ester can be readily differentiated by the much easier hydrolysis of the former (Foster and Huggard, 1955).

The sulphatephosphate bond of PAPS is highly acid-labile. Its half life in 0.1 M HCl at 37°C is only 6 minutes (Robbins and Lippman, 1957). It is, however, relatively stable to alkali. It is not hydrolyzed in 0.1 N NaOH after one hour and is only partially hydrolyzed

by treatment with 1N NaOH at 100° for 2 hours (Baddiley et al., 1959).

Biosynthesis of PAPS

Work by Wilson and Bandurski (1956) and by Robbins and Lipmann (1956, 1958) established that PAPS (3'-phosphoadenosine-5'-phosphosulfate) is the active sulfate in all biological sulfation reactions. The synthesis of PAPS involves two enzymes. The first, ATP-sulfurylase, catalyzes the reaction:

$$ATP + SO_4^{-2} \rightleftarrows APS + PPi$$

The equilibrium for the reaction favors ATP. The equilibrium constant is 10^{-8} (Robbins and Lippman, 1958) so that the standard free energy of the reaction is approximately +11 Kcal. The reaction proceeds because the pyrophosphate is broken down to inorganic phosphate by a pyrophosphatase and the APS participates in the second step of sulfate activation:

$$APS + ATP \rightarrow PAPS + ADP$$

This reaction, catalyzed by APS-kinase, is essentially irreversible with a standard free energy change of -5 Kcal. The overall reaction is:

$$2ATP + SO_4^{-2} \rightarrow ADP + PAPS + PPi$$

The transfer of the sulfuryl group from PAPS to acceptors is catalyzed by a group of enzymes, the sulfotransferase, with the formation of sulfate esters and PAP, adenosine 3',5'-diphosphate. The

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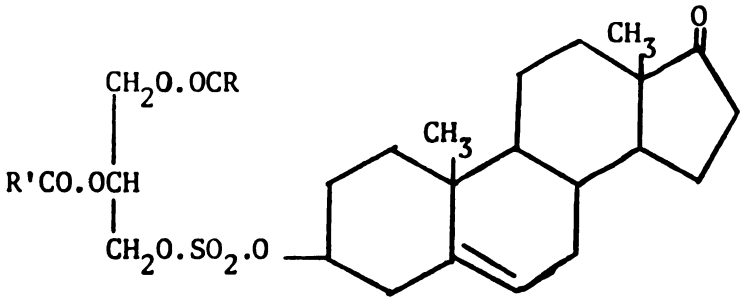
sulfotransferases will be discussed in the next section under the types of macromolecules which they sulfurylate.

Major Classes of Sulfated Macromolecules

In mammals there are three major classes of macromolecules which contain sulfate incorporated via PAPS. These include lipids, mucopolysaccharides, and sulfated glycoproteins. Occurrences, structural information and data on enzymatic synthesis of each of these classes will be briefly discussed.

Lipids

Among the lipids, there are two classes of sulfated compounds: steroid sulfatides and sulfated cerebrosides. Little is known about the steroid sulfates. They have been studied only by Oertel (1961). They are derived from diglycerides and have been assigned the structure shown below.



Androstenolone sulfatide

There appear to be at least three separate steriod sulfotransferases, specific for androstenolone, testosterone and desoxycorticosterone (Banerjee and Boy, 1966 and 1967). The steroid

sulfotransferases are of limited distribution in the adult, occurring in significant amounts only in liver, the adrenal, and the jejunal mucosa in human (Boström and Wengle, 1967) and in the ox (Holcenberg and Rosen, 1965).

The second class of sulfate containing lipids, the sulfatides, are the sulfate esters of cerebrosides. Sulfate is esterified to the carbon-3 of the carbohydrate moiety (Yamakawa et al., 1962; Stoffyn et al., 1963). Sulfatides occur in highest concentration in adult brain white matter (Norton and Autilio, 1966). Next to neural tissue they occur in highest concentration in kidney and, at lower levels in spleen (Svennerholm, 1963), liver (Green and Robinson, 1960), and plasma (Austin and Maxwell, 1961).

Sulfatides are synthesized mainly within the microsomes (Cumar et al., 1968; Herschkowitz, et al., 1968). McKhann et al. (1965) demonstrated sulfation of galactocerebroside in the 100,000 x g sediment, i.e., the microsomal fraction, of young rat brains. Fleischer and Zambrano (1973) reported the isolation of a Golgi-rich fraction from rat kidney which contained PAPS:cerebroside sulfotransferase activity which was enriched 80-fold over the homogenate. This is the first evidence that the Golgi apparatus functions in the modification of a lipid component.

Mucopolysaccharides

Mucopolysaccharides can be considered as a class of glycoproteins possessing especially extensive carbohydrate side chains. In higher animals, the mucopolysaccharides; hyaluronic acid, the chondroitin sulfates, dermatan sulfate, and the keratan sulfates, are

components of the extracellular ground substance which surrounds collagen and elastin fibers, and the cells of connective tissue. Heparin, while it is not usually found in connective tissue, has a chemical structure similar to that of the other mucopolysaccharides. Table 1 lists certain structural features of the known acid mucopolysaccharides.

The carbohydrate portion of these molecules consists of alternating units of amino sugar and uronic acid in a long sequence whose reducing end is attached to protein through a trisaccharide linkage sequence (Rodiń, 1968). Hyaluronic acid is a polymer of glucuronic acid and N-acetylglucosamine. Its molecular weight may vary from 10^6 to as low as 8×10^4.

Chondroitin-4-SO_4 and chondroitin-6-SO_4 differ only in the position of the sulfate ester. Recent evidence indiates that the chondroitin sulfates do not contain precisely 1 sulfate group per disaccharide but that, rather, there is considerable heterogeneity in their extent of sulfaton (Suzuki, 1960). Some disaccharide units lack sulfate, others contain sulfate on both the 4 and 6 position of the hexosamine or on the 2 or 3 carbon of glucuronic acid (Suzuki et al., 1968). There is also much variation in the chain length in chondroitin sulfate (Suzuki, 1960).

Dermatan sulfate differs from chondroitins in that its predominant uronic acid is L-iduronic acid, although some D-glucuronic acid is present (Fransoon and Rodén, 1967). Sulfate in dermatan sulfate may be linked to uronic acid as well as to N-acetylgalactosamine (Suzuki et al., 1968).

Table 1.--Structural features of acid mucopolysaccharides.

Table 1.--Structural features of acid mucopolysaccharides.

Structural features of the known classes of acid mucopolysaccharides are presented. Abbreviations used: D-GlcNAc, D-N-acetylglucosamine; D-GalNac, D-N-acetylgalastosamine; D-GlcUA, D-glucuronic acid; L-IdUA, L-idurmic acid; Gal, galactose.

Mucopolysaccharide	Amino Sugar	Uronic Acid	Sulfate Linkage	Susceptibility to Chondroitinase ABC from Proteus
Hyaluronic acid	D-GlcNAc	D-GlcUA	. .	+
Chondroitin	D-GalNAc	D-GlcUA	. .	+
Chondroitin-4-SO_4	D-GalNAc	D-GlcUA	O-SO_4	+
Chondroitin-6-SO_4	D-GalNAc	D-GlcUA	O-SO_4	+
Heparan sulfate	D-GlcNAc	D-GlcUA L-IdUA	N-SO_4 O-SO_4	-
Heparin	D-GlcNAc	D-GlcUA L-IdUA	N-SO_4 O-SO_4	-
Dermatan sulfate	D-GalNAc	L-IdUA D-GlcUA	O-SO_4	+
Keratan sulfate I	D-GlcNAc	Gal	O-SO_4	-

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Heparin differs from other acid mucopolysaccharides in many ways. D-glucuronic acid and D-glucosamine are the principal constituents but L-iduronic acid is also present. The sulfate content varies, though in preparations of high biological activity, the ratio approaches 2.5 sulfate residues per disaccharide. Nearly all glucosamine residues are bound in sulfamide linkages (Cifonelli, 1968). Sulfate groups are also linked to carbon-6 and carbon-3 of hexosamine and on carbon-2 of uronic acid.

Heparan sulfate, though it shows many features of heparin, appears to be a family of compounds with variable amounts of total sulfate and variable ratios of N-sulfated glucosamine to N-acetylated glucosamine (Cifonelli and Dorfman, 1960).

Keratan sulfate is characterized by considerable molecular heterogeneity. It is principally composed of repeating disaccharide units of N-acetyl glucosamine and galactose. No uronic acid is present (Meyer et al., 1953). Sulfate content is variable, with ester sulfate present on carbon-6 of both galactose and N-acetylglucosamine. In addition to the principal monosaccharides, mannose, fucose, and sialic acid are also present.

Sulfated acid mucopolysaccharides are covalently bound to protein. The basic structural concept of such proteoglycans was first proposed by Matthews and Lozaityte (1957). Their structure contained a number of polysaccharide chains bound covalently to serine residues of a protein core through a trisaccharide linkage region.

Nearly all structural information on protein-mucopolysaccharide complexes is based on material extracted from cartilage (chondromucoprotein). From this work, it now appears that chains of keratan sulfate

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and chondroitin-4/6-SO_4 can be attached to the same protein core (Tsiganos and Muir, 1969; Rosenblum and Cifonell, 1967). Matthews (1968) has shown that polysaccharide chains are spaced along the protein backbone in doublets, with two chains separated by a relatively small number of amino acids and a larger number of amino acids separating the doublets.

Little is known of the structure of other mucopolysaccharides with protein. A protein-polysacchride complex of dermaton sulfate was isolated containing 50% protein. The complex was bound to collagen and its molecular weight was 100,000 to 200,000. This is considerably less than that of the protein complexes of chondroitin sulfate (Toole and Lowther, 1968).

The carbohydrate chains of mammalian mucopolysaccharides are synthesized from the non-reducing end of the growing chain by the serial addition of glycose residues from sugar nucleotides (Telser et al., 1966). The extensive literature on this subject has been reviewed by Dorfman (1972). The glycosyltransferases appear to be associated with membranes. Microsomal preparations of cultured mastocytoma cells are able to synthesize chondroitin-4-sulfate (Lewis et al., 1972). Horwitz and Dorfman (1968) demonstrated in chick embryo chondrocytes that all enzymes necessary for the synthesis of the linkage region to protein and for chain elongation of chondroitin were present in smooth and rough microsomes. Synthesis of the linkage region was localized in rough microsomes, while enzymes for chain completion were highest in the smooth fraction. Sulfation enzymes have also been localized in the smooth endoplasmic rediculum (Suzuki et al., 1967; Horwitz and Dorfman, 1968).

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Though mucopolysaccharides are primarily synthesized by fibroblasts, there is evidence that they may be present in low levels in other cell types. Kraemer (1971a) has reported a soluble and plasma membrane-bound form of heparan sulfate and heparan sulfate now appears to be a generally occurring cell-surface molecule on many cultured animal cells (Dietrich et al., 1970; Kraemer, 1971b). As previously mentioned, a chondroitin-sulfate-like substance has been isolated from the chromaffin granules of adrenal medulla.

Sulfated Glycoproteins

In the biosynthetic pathway of glycoproteins, most of the sugar residues are added sequentially after the synthesis of the polypeptide and after its release from the ribosome into the cisternae of the endoplasmic reticulum (Molnar et al., 1965; Spiro and Spiro, 1966; Lawford and Schacter, 1966; Li, Y. T. et al., 1968). However, there is still some uncertainty about the site of addition of the sugar residue linked directly to the polypeptide. Some glucosamine is presumably added to nascent polypeptides because such polypeptides can be released by puromycin from isolated liver polysomes (Molnar and Dalisay, 1967).

Seven sugars account for almost all the carbohydrate residues in mammalian glycoproteins. These are: N-acetyl glucosamine, N-acetyl galactosamine, galactose, mannose, fucose, sialic acid and glucose. All sugars occur in the D-configuration except fucose, which is in the L-form. There are no exceptions to the general observation that, when they are present, L-fucose and sialic acid occupy a terminal position at the non-reducing end of the carbohydrate chain. The innermost

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sugar of the carbohydrate chain is either N-acetyl glucosamine, linked to the amide nitrogen of asparagine (Sharon, 1966) or N-acetyl galactosamine linked to the hydroxyl group of serine or threonine (Harbon et al., 1968; Kabat et al., 1965). In collagen, galactose is linked to the hydroxyl group of hydroxylysine (Cunningham and Ford, 1968).

The existence of sulfated heterosaccharides in glycoproteins which were distinct from sulfated mucopolysaccharides was first demonstrated by Kent and Marsden (1963) in a highly purified preparation from sheep colonic mucin. Since then, sulfated glycoproteins have been obtained from many sources.

In the digestive tract, sulfated glycopeptides are present in human saliva (Haves et al., 1967), human gastric juice (Häkkinen et al., 1965a; Martin et al., 1967), dog gastric secretion (DeGraef and Glass, 1968), dog gastric wall (Häkkinen et al., 1965b) and from a mucus carcinoma of the human stomach (Kimoto et al., 1968). In the intestine, sulfated glycopeptides are isolated from sheep colonic mucus (Kent and Marsden, 1963), pig colonic mucosa (Inoue and Yosiazawa, 1966), and rabbit intestinal mucosa (Nemoto and Yosizawa, 1969).

The physical properties of these compounds are summarized in a review by Yosizawa (1972). Unfortunately, all the sulfated glycoproteins mentioned above were isolated as glycopeptides after extensive proteolysis of the starting material. The sulfated glycopeptides obtained have been carefully characterized with respect to their amino acid and carbohydrate composition but no data is available on the subcellular localization or molecular weight of the native glycoproteins. Some sulfated glycoproteins show similarities to keratan

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sulfate preparations, however, sulfated glycoproteins in mammals are epithelial products in contrast to keratan sulfates which are of mesodermal origin. Therefore, sulfated glycoproteins may represent a unique class of compounds widely distributed in epithelial mucins and cellular membranes.

Carbohydrate Analysis by Gas-Liquid Chromatography

The advantages of the method of gas-liquid chromatography for the estimation of carbohydrates in glycoproteins are its specificity and its sensitivity. It is possible to separate, identify and quantitate, in a single procedure, all of the carbohydrates commonly found in mammalian glycoproteins. The problem of making carbohydrates sufficiently volatile for gas-liquid chromatography was solved by Sweeley et al. (1963) who developed the procedure of using trimethylsilyl ethers to replace hydroxyl hydrogens and thus prevent hydrogen binding.

The practical limit of detection of carbohydrates using the method of Bhatti et al. (1970) is 0.2 to 0.4 nanomoles. This procedure uses methanolysis rather than aqueous hydrolysis. In methanolysis, the liberated reducing group is protected as a methyl glycoside, reducing side reactions. Also, the carboxyl group of sialic acid is, at the same time, converted to a methyl ester, making it suitable for derivitization. A further advantage of methanolysis is that each monosaccharide gives rise to at least 2 isomer peaks. Multiple peaks make identification easier and in the case where a sugar peak is obscured by background material, the monosaccharide can still be calculated from the unobscured peaks and the known peak area

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proportions. The use of this procedure for the identification of carbohydrates in biological samples has been reviewed by Clamp, Bhatti, and Chambers (1972).

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MATERIALS AND METHODS

Materials

Reagents for Tissue Incubation Medium

L-amino acids	Sigma Chemical Co., St. Louis, Mo.
phenolsulfopthalein (phenol red)	Sigma Chemical Co.

Electrophoresis Reagents

acrylamide	Canalco. Inc. Rockville, Md.
N, N, N', N'-tetramethyl-ethylenediamine (TEMED)	Bio-Rad Laboratories, Inc. Richmond, Va.
N, N'-methylenebis-acrylamide	Canalco, Inc.
sodium dodecylsulfate, sequanal grade	Pierce Chemical Co. Rockford, Ill.
ammonium persulfate	Canalco, Inc.
Coomassie brilliant blue R	Sigma Chemical Co.
dimethyldichlorosilane	Sigma Chemical Co.
pyronin B	Harleco, Philadelphia, Pa.

Reagents for Gas-Liquid Chromatography

3% silicone rubber SE-30 on chromosorb W.H.P., 80-100 mesh	Hewlett-Packard, Avondale, Pa.

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H_2O,

L-mannitol	Sigma Chemical Co.
hexamethyldisilazane (HMDS) specially purified grade	Pierce Chemical Co. Rockford, Ill.
trimethylchlorosilane (TMCS) specially purified grade	Pierce Chemical Co.

Reagents and Enzymes for Analytical Procedures

soluble starch reagent (for amylase assay)	Nutritional Biochemicals Corp., Cleveland, Ohio
bovine serum albumin	Sigma Chemical Co.
N-acetylneuraminic acid	Sigma Chemical Co.
Chondroitinase-ABC, 5 units/ampoule	Miles Laboratories Kankakee, Ill.
Neuraminidase, Type VI from Clostridium perfringens, 0.04 units/mg protein, using bovine submaxillary mucin	Sigma Chemical Co.
Pronase, Type VI from Streptomyces griseus	Sigma Chemical Co.
Papain, 2x crystallized	Sigma Chemical Co.
Bovine submaxillary mucin	Sigma Chemical Co.

Radiochemicals

L-leucine [4,5-^{3}H(N)], 23 Ci/n mole	Amersham/Searle Arlington Heights, Ill.
L-leucine [1-^{14}C], 20-30 m Ci/m mole	New England Nuclear
D-glucosamine hydrochloride [6-^{3}H(N)], 5-15 Ci/m mole	New England Nuclear
$H_2{}^{35}SO_4$, carrier free, in $^{2}H_2O$, 43 Ci/mg	New England Nuclear

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Miscellaneous

Soybean trypsin inhibitor	Sigma Chemical Co.
Triton X-100	Rohm & Haas Philadelphia, Pa.
Sucrose, ultrapure	Schwarz/Mann Orangeburg, N.Y.

Animals

Sprague-Dawley rats were obtained from Spartan Research Animals, Haslett, Michigan. Dog pancreas was obtained through the courtesy of Drs. M. D. Bailee, C. C. Chou, and J. Scott, of the Department of Physiology, Michigan State University.

Methods

Tissue Incubation Medium

The tissue incubation procedure used was similar to that of Jamieson and Palade (1967). The medium consisted of a Krebs-Ringer buffer (KRB) minus pyruvate, fumarate and glutamate. Sulfate ion was omitted when tissues were to be labeled with $H_2{}^{35}SO_4$. The concentrations of salts in KRB and in calcium-free KRB are listed in Table 2. Calcium-free buffer was prepared by omitting $CaCl_2$ and by replacing part of the bicarbonate with isotonic phosphate buffer.

KRB was supplemented with L-amino acids at concentrations specified for Eagle's Minimal Essential Medium (MEM), (Eagle, 1959) with increased levels of leucine, methionine, and arginine as indicated in Table 3. Soybean trypsin inhibitor was present at a concentration of 0.1 mg/ml to prevent tissue damage by secreted proteolytic enzymes. Choline, folic acid and inositol were omitted in the present study

Table 2.--Salt concentrations of Krebs-Ringer Buffers (Krebs, 1950).

Krebs-Ringer Bicarbonate Buffer

Salt	g/l	mM/l
NaCl	5.530	95.0
KCl	0.353	5.0
$CaCl_2 \cdot 2H_2O$	0.374	2.5
KH_2PO_4	0.160	1.2
$MgSO_4 \cdot 7H_2O$[a]	0.290	1.2
$NaHCO_3$	2.100	25.0

Krebs-Ringer Buffer, Calcium-Free

Salt	5/1	mM/l
NaCl	5.750	98.0
KCl	0.352	5.0
KH_2PO_4	0.162	1.2
$MgSO_4 \cdot 7H_2O$[a]	0.294	1.2
$NaHPO_4 \cdot 2H_2O$	1.970	1.6
$NaH_2PO_4 \cdot 2H_2O$	0.382	1.6
$NaHCO_3$	0.300	3.6

[a]In sulfate-free medium, $MgSO_4$ was excluded and the Mg^{+2} concentration was maintained by addition of 1.2 mM $MgCl_2$ (0.243 g. $MgCl_2 \cdot 6H_2O$/1).

Table 3.--L-amino acid concentrations in modified Eagle's minimal essential medium.

Amino Acid	mg/l	M/l
arginine-HCl	160	758
cysteine	24	198
glutamine	292	1657
histidine	31	200
isoleucine	53	400
leucine	79	600
Lysine	58	331
methionine	60	403
phenylalanine	32	194
threonine	48	412
tryptophane	10	49
tryrosine	36	199
valine	46	393

and the glucose concentration (14mM) was 2.5 times that present in normal Eagle's Minimal Essential Medium. The medium was incubated, before addition of tissue pieces, at 37°C in a Dubnoff Metabolic Bath with agitation at 110 cycles/min.

The medium was sterilized by passage over a Millipore filter (GSWP 09025, pore size 0.22 μm). Since the addition of penicillin and streptomycin (5 μ/ml) had no effect on ^{35}S-sulfate incorporation and incubation times were short (5 hours maximum), anti-bacterial agents were not added to the medium.

Tissue Labeling Procedures

Excised pancreas tissue was trimmed free of fat and was placed in chilled Krebs-Ringer salt solution. In experiments involving measurement of labeling kinetics, the tissue was cut into slices of uniform thickness with a Stadie-Riggs tissue slicing blade. For experiments in which large quantities of pancreas tissue (15 to 20 grams) were labeled for subsequent fractionation, the tissue was cut into pieces approximately 1.5 mm square using Von Graefe Cataract knives (#1) in sterile Petri dishes. The tissue pieces were transferred to an Erlenmeyer flask containing the incubation medium prewarmed to 37°C and gassed with a mixture of 5% CO_2, 95% air to adjust the pH to 7.4. Labeling was initiated by addition of the radioactive compound and the medium was gassed throughout the labeling period to maintain the pH.

Labeled tissue was recovered by filtering the medium and collecting the tissue pieces from Whatman #1 filter paper. To remove

ions which might interfere with membrane fractionation, the tissue was rinsed five times with 100 ml volumes of cold 0.3M sucrose.

Tissue Homogenization

Homogenization and all subsequent procedures were performed at 4°C. Each gram of labeled tissue was resuspended in 10 volumes of 0.3M sucrose (Mann Ultrapure) containing 0.1 mg/ml of soybean trypsin inhibitor (SBTI). The cells were sheared with 4 up and down strokes of a glass-teflon Potter-Elvehjem homogenizer (clearance of 0.007 inch, Kontes Glass Co.) driven at 620 RPM. The homogenate was filtered through 2 layers of cheesecloth to remove debris.

Isolation of Subcellular Fractions

Zymogen granules.--Zymogen granules were isolated using the procedure of Jamieson and Palade (1967a) modified by MacDonald and Ronzio (1972). The filtered homogenate was centrifuged at 500 xg (1600 RPM, HS-4 rotor, I. Sorval Co.) to remove debris. Zymogen granules were obtained by centrifugation at 1730xg (2800 RPM, HS-4 rotor) for 30 minutes.

Mitochondria.--Mitochondria were isolated from the 1600xg supernatant by centrifugation at 8700xg (8500 RPM, SS-34 rotor) for 25 minutes. Contaminating zymogen granules were removed by resuspending the mitochondrial pellet and recentrifuging.

Microsomes.--The post mitochondrial supernatant was centrifuged at 93,00xg (40,000 RPM, Type 40 rotor, Beckman Instruments) for one hour to pellet microsomes. The non-sedimentable material was

designated as the post microsomal supernatant. Whole microsomes were subfractionated into rough and smooth microsomes by sucrose density centrifugation according to the method of Ronzio (1973a). The microsomal subfractions obtained by this procedure have been well characterized (Ronzio, 1973b).

Preparation of Membranes from Subcellular Fractions (Procedure of MacDonald, 1974)

Zymogen granule membranes.--Purified zymogen granules resuspended in 0.17M NaCl containing 0.66mg SBTI/ml were lysed by addition of 0.2M $NaHCO_3$, pH 8.2. Lysis required approximately 30 minutes for completion and could be monitored by a visible decrease in turbidity. Nine-ml of lysed granule solution was layered over a three-ml cushion of 1.0 M sucrose. The gradient was centrifuged for one hour at 192,000xg (40,000 RPM, SW41 rotor, Beckman Instruments). The two membrane fractions obtained have been characterized (MacDonald, 1974). The pellet, which contained high cytochrome c oxidase activity, consisted mainly of mitochondria. Zymogen granule membrane banded at the bicarbonate-1.0 M sucrose interface (Meldolesi et al., 1971a). This membrane band was designated as crude zymogen granule membrane (CZGM). The soluble fraction above the 1.0M sucrose cushion contained the protein contents of the zymogen granule, designated as the zymogen granule lysate.

The crude zymogen granule membrane band was removed carefully with a long-tipped Pasteur pipet to avoid contaminating the zymogen granule lysate. The crude zymogen granule membrane was sonicated in 0.25M NaBr for 10 seconds at maximum setting (Biosonik sonic oscillator,

Bronwill Scientific). This step removed adsorbed proteins. The membrane was recovered after a centrifugation at 192,000xg for one hour. The pellet was designated as zymogen granule membrane and the soluble fraction as NaBr supernatant. A schematic diagram of zymogen granule fractionation is presented in Figure 1.

Mitochondrial and microsomal membranes.--Mitochondrial and microsomal membranes were prepared in a manner identical to zymogen granule membranes. Mitochondrial and microsomal pellets were resuspended in 0.2M $NaHCO_3$, pH 8.2 using a Dounce glass homogenizer, and were lysed by sonication at maximum setting for ten seconds. Membranes were sedimented at 192,000xg for one hour. The membrane pellets were then sonicated in 0.25M NaBr and were sedimented by centrifugation at 192,000xg for one hour.

SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gels were run following the procedure of Fairbanks et al. (1970) as modified by MacDonald and Ronzio (1972). Samples containing 1% SDS and 2% 2-mercaptoethanol were dissolved in protein solvent (0.01M Tris-HCl, pH 8.0, 5mM EDTA) by heating at 100°C for 15 minutes. Pyronin B was added to mark the ion front.

The 9% acrylamide gels were cast in dimethyl-dichlorosilane-coated glass tubes 0.5 or 0.6cm in diameter. During electrophoresis, the gels were maintained at 13°C to minimize band curvature. Gels were pre-run at 5v/cm for 30 minutes. After sample was applied, gels were run at a potential gradient of 10v/cm. The current was not allowed to exceed 6m amperes/gel.

Figure 1. Diagram of zymogen granule subfractionation. Zymogen granules were lysed and subfractions were obtained as described in Methods. The abbreviation ZGM is used for zymogen granule membrane.

ZYMOGEN GRANULE FRACTIONATION

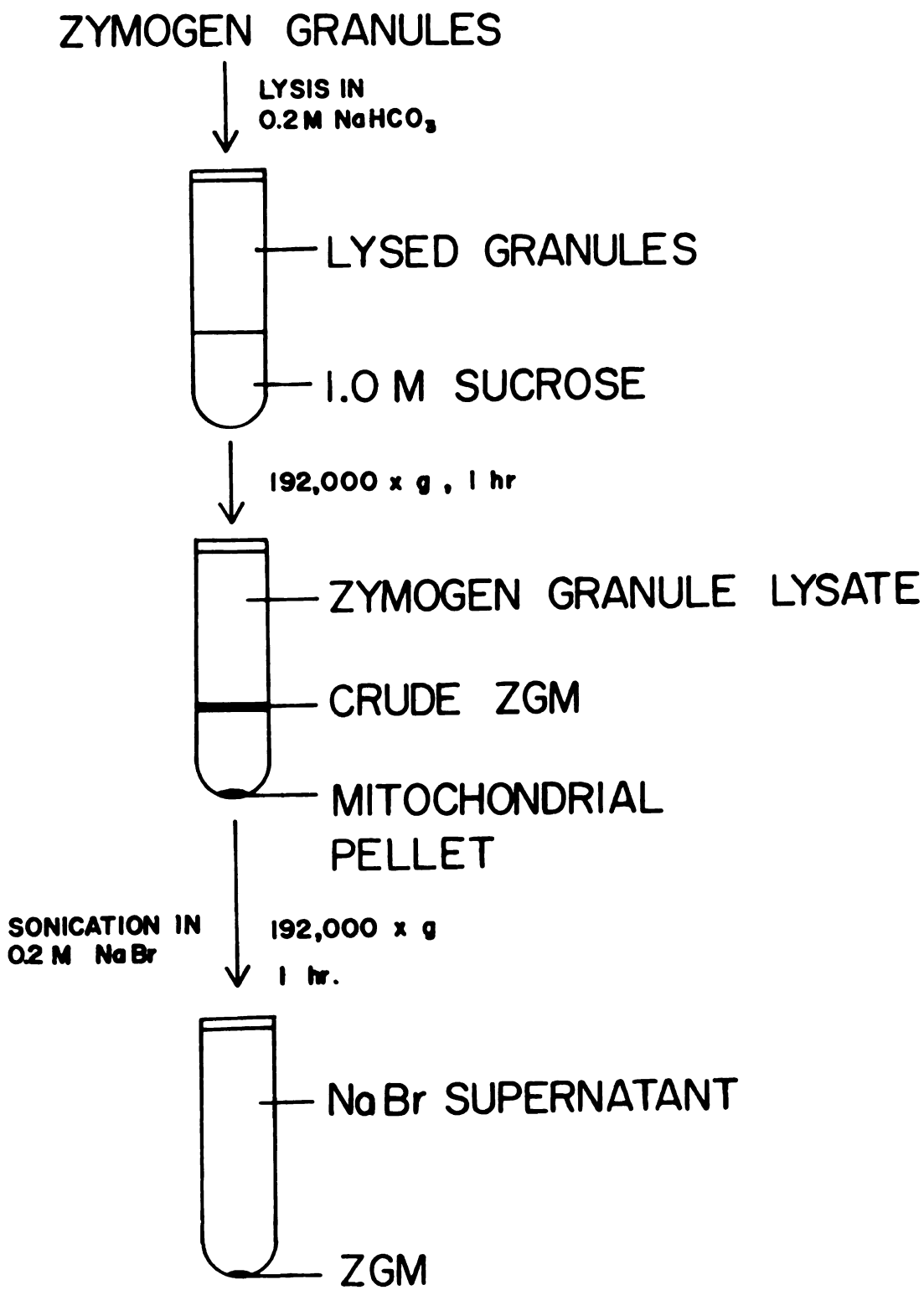


Figure 1

Gels were stained for protein in 0.4% Coomassie Blue R in 10% TCA containing 33% methanol (Johnson et al., 1971). To destain, the gels were incubated in 10% TCA-33% methanol for eight to ten hours. Removal of background stain was completed in 10% TCA at 37°C. Coomassie blue-stained gels were scanned at 550nm using a Gilford Linear Transport.

Fractionation and Determination of Radioactivity in Polyacrylamide Gels

Polyacrylamide gels containing radioactivity were fractionated with a Savant Autogeldivider (Savant Instruments, Inc.) (Maizel, 1966) using a nontransfer system in which gels were extruded directly from electrophoresis tubes. Gels were fractionated within two hours after electrophoresis to minimize diffusion and broadening of bands. A typical 8.7cm gel yielded 82 to 90 fractions. Each fraction is equivalent to approximately 0.1cm of gel. Fractions containing extruded gel were extracted with 0.7 milliliter of 1% SDS-0.1N NaOH at 60°C for a minimum of 12 hours. Ten milliliters of a Triton X-100-toluene based scintillation fluid (Mostafa et al., 1970) was added. Vials were shaken and counted in a Packard liquid scintillation spectrophotometer.

Liquid Scintillation Counting

^{14}C and ^{35}S were routinely counted in a refrigerated Packard Tri-Carb at a gain of 12% with a window setting of 50-1000. The efficiency was 73% for ^{14}C. Tritium was optimally counted at 55% gain with window settings at 50-1000; efficiency was 26%.

In dual labeling experiments, tritium counts were corrected for spillover of ^{14}C or ^{35}S. The counting efficiencies in this case were 36% for ^{14}C, and 18% for tritium. The counting efficiency for ^{35}S was not determined.

Carbohydrate Analysis

Preparation of column for gas-liquid chromatography.--A six-foot U-shaped glass column with an internal diameter of 3mm was cleaned prior to packing by the following procedure: The column was washed with concentrated HCl, rinsed with water and acetone, and dried. It was coated with a mixture of 10% dimethyldichlorosilane (DMDS) in hexane and allowed to dry for 15 minutes. Excess DMDS was removed with hexane followed by dry methanol and a final acetone rinse. The column was then dried thoroughly and was packed with 3% SE-30 on chromosorb W (Hewlett-Packard). It was conditioned overnight at 250°C. Additional conditioning at 200°C over a period of several days was often necessary to reduce "bleeding" of the liquid phase at higher temperatures and to obtain a steady baseline at low signal attenuations.

Gas-liquid chromatography was performed using a Model 402 Hewlett Packard Gas Chromatograph (Hewlett-Packard Co.) equipped with a flame ionization detector and a Honeywell recorder and disc integrator. The gas chromatograph injector temperature was 190°, the detector temperature was 240°, and the column temperature was programmed from 100° to 210° at 1° per minute. Flow rates were as follows: N_2 = 30cc/min (measured at 165°); H_2 = 25cc/min; and O_2 = 30cc/min.

Preparation of samples for gas-liquid chromatography (procedure of Bhatti, Chambers and Clamp, 1972.--Solutions of carbohydrate standards or samples of known protein concentration were placed in 2-ml glass ampoules (Sargent-Welch Co.) and lyopholyzed. They were further dried in a vacuum dessicator over P_2O_5 for a minimum of five hours. Protein samples which had to be delipidated were extracted three times with 1 ml of chloroform:methanol (2:1) and a drop of diethyl ether. The sedimented, nonextractable material was thoroughly dried *in vacuo* over P_2O_5 after addition of a known amount of the internal standard, D-mannitol. 0.9 ml of anhydrous methanolic-HCl, prepared according to Bhatti, Chambers and Clamp (1972), was added to the dried sample. A stream of nitrogen gas was passed into the ampoule for 30 seconds and the ampoule was immediately sealed. Samples were hydrolyzed at 85°C for eight hours.

Hydrolyzed samples were cooled, neutralized with solid silver carbonate and amino sugars were re-N-acetylated by addition of 50µl of acetic anhydride. The ampoules were mixed well, centrifuged, and the supernatant was removed to a fresh test tube. The residue of silver carbonate in the ampoule was extracted with three additional aliquots of anhydrous methanol which were pooled with the first supernatant. The samples were evaporated with a stream of nitrogen and were dried *in vacuo* over P_2O_5.

The samples were derivatized by addition of a mixture of Pyridine:HMDS:TMCS (1:0.25:0.25) according to the procedure of Sweeley et al. (1963).

Determination of molar relative response factor.--Quantitative determination of carbohydrate content was obtained by utilizing the

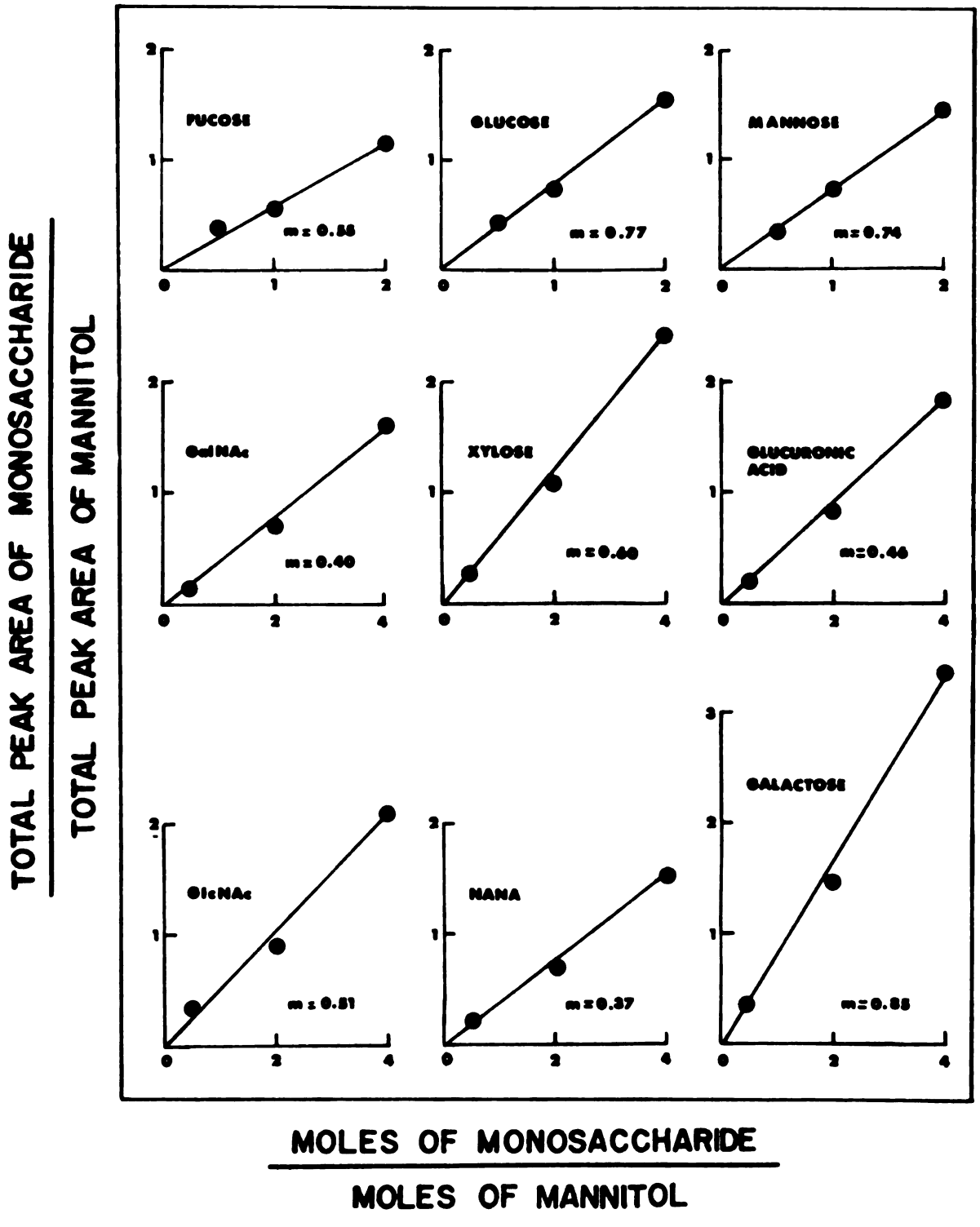
technique of internal standarization. A known amount of the internal standard, in this case, D-mannitol, was added to the unknown sample before hydrolysis. Any losses of material during the procedure affect both the unknown and the standard to the same extent, and the ratio of the total peak area of the unknown compound to the total peak area of mannitol directly measures the amount of the unknown compound in the original sample. However, equal amounts of different carbohydrates do not produce equal peak areas and it is necessary to determine the "molar relative response factor" for each carbohydrate relative to the internal standard. To do this varying known amounts of carbohydrate standards were added to a known, fixed amount of mannitol and the ratio of the total peak area of each monosaccharide relative to the total peak area of mannitol was graphed versus the mole ratio of monosaccharide to mannitol. This gave a straight line whose slope was the "molar relative response factor." The "response factor" for a number of monosaccharides was determined for use in this study. The data is graphed in Figure 2 and the values are tabulated in Table 4.

Quantitation of monosaccharides.--The total peak area of the isomers of a given monosaccharide were summed to give the total peak area of the monosaccharide. The total peak area ratio is then obtained by dividing the total peak area by the peak area of the internal standard. The quantity in nanomoles of an individual monosaccharide in a given sample is calculated using the following equation:

$$\frac{\text{total peak area ratio x amount of internal standard (nM)}}{\text{molar relative response factor}}$$

Figure 2.--Molar relative response factors of selected methyl glycosides. Molar relative response factors were determined as described in the text.

Figure 2


TOTAL PEAK AREA OF MONOSACCHARIDE
TOTAL PEAK AREA OF MANNITOL
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GLUCOSE
m = 0.77
MANNOSE
m = 0.74
GalNAc
m = 0.40
XYLOSE
m = 0.60
GLUCURONIC ACID
m = 0.46
GlcNAc
m = 0.51
NANA
m = 0.37
GALACTOSE
m = 0.85
MOLES OF MONOSACCHARIDE
MOLES OF MANNITOL

Figure 2

Table 4.--Molar relative response factor of selected methyl glycosides relative to mannitol.[a]

Monosaccharide	Response Factor
Fucose	0.55
Xylose	0.60
Mannose	0.74
Galactose	0.85
Glucose	0.77
N-acetylglucosamine	0.51
N-acetylgalactosamine	0.40
NANA	0.37
Glucuronic Acid	0.46

[a]Heating of samples, with mannitol included, was carried out for 12 hours at 82°C in 1.5N methanolic-HCl.

The nM carbohydrate/mg protein is obtained by dividing the above result by the amount of protein present in the original sample.

Enzyme Assays

α-Amylase was assayed by the reductometric method of Bernfeld (1955). Amylase activity was expressed as milligrams of maltose/3 minutes at 37°C/1-ml enzyme solution. Specific activity is expressed as amylase activity/mg protein.

Analytical Procedures

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Sucrose, which interferes with the Lowry determination, was removed by precipitation of the protein sample with an equal volume of 10% TCA. Precipitates were

collected by centrifugation for five minutes (Beckman Microfuge), washed two times with additional 10% TCA, and redissolved in 0.5 N NaOH prior to analysis. Sialic acid was analyzed colorometrically by the procedure of Warren (1959), using N-acetylneuraminic acid as a standard.

Determination of Specific Radioactivity of Subcellular Fractions

Specific radioactivity is defined as counts per minute of a given radioisotope bound to macromolecule per µg of protein. Specific radioactivity was determined in this study using three methods: Biogel P-4 column chromatography; cellulose acetate strip electrophoresis; or by recovery of trichloroacetic acid-phosphotungstic acid (TCA-PTA) precipitable counts on glass fiber filters.

Biogel P-4 Chromatography

Samples were heated in 1% SDS to dissolve membranes and disaggregate lipid-protein complexes. The sample was applied to a column made from a 10-milliliter glass pipette containing 9 milliliters of hydrated Biogel P-4 equilibrated with 1% SDS. Fractions were eleuted from the column with 1% SDS. "Macromolecule" was defined as the material which eleuted in the void volume (i.e., with dextran blue). The exclusion limit of Biogel P-4 is molecular weight of 4,000 or greater.

The elution position of free sulfate was determined by applying a $H_2{}^{35}SO_4$ standard. Fractions were collected from the column and aliquots were counted to determine the percent distribution of ^{35}S counts in macromolecule and retained material. Recovery of applied counts from such columns was routinely 85% to 95%.

Cellulose Acetate Strip Electrophoresis

Radioactive samples were applied to buffer-moistened cellulose acetate strips (Sepraphore III, Gelman Instrument Co.) and run in 1% sodium tetraborate at 100v for 20 minutes. Free sulfate migrated rapidly away from macromolecule bound sulfate, which remained near the origin. The strips were cut into 1/2 centimeter pieces and counted. The percentage of total counts remaining at the origin was equal to the percentage of total counts bound to macromolecule.

There was good agreement between the results obtained by P-4 chromatography and by cellulose acetate strip electrophoresis. However, 15µl was the maximum amount of sample which could be applied to the strip without streaking so this technique could not be used for dilute fractions.

TCA-PTA Precipitation

Aliquots of up to 1 ml of radioactive sample were added to 1 ml of ice cold 10% TCA-5% PTA and were allowed to precipitate at 0°C for 30 minutes. In the case of samples containing little protein, 300 g of carrier BSA was added. The precipitates were collected on glass fiber filters (Whatman GF/A) and were washed six times with 2-milliliter aliquots of 5% TCA-2.5% PTA. Filters were then rinsed with acetone and were counted.

Preparation of Materials for Electron Microscopy

Pelleted membrane fractions were fixed for four hours at 0°C in 0.1M Na cacodylate buffer, pH 7.4; containing 2% glutaraldehyde. Glutaraldehyde was then removed from the pellet by several washes

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with 0.1M cacodylate buffer. The fixed material was dehydrated, stained and sectioned by personnel at the Electron Microscopy Laboratory, Center for Electron Optics, Michigan State University, East Lansing, Michigan. Micrographs were taken using a Philips-300 electron microscope.

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RESULTS

I. Incorporation of Labeled Compounds Into Pancreatic Macromolecules *in vitro*

Pancreatic tissue was cultured for short periods (one to five hours) using the culture conditions described in Methods. To determine the viability of pancreas tissue slices under these culture conditions in our hands, the ability of the tissue to incorporate ^{3}H-leucine and ^{35}S-sulfate was tested.

^{3}H-Leucine Uptake and Incorporation Into Pancreas Slices

Tissue slices from a single rat pancreas were incubated for varied times in 0.3 ml of Eagle's Minimal Essential Medium buffered with Krebs-Ringer salts, containing 0.4 mM leucine and 10 μCi/ml ^{3}H-leucine. The kinetics of ^{3}H-leucine incorporation into TCA-precipitable and soluble material is shown in Figure 3.

The incorporation of label into total protein proceeded at a linear rate for three hours. After three hours the rate of incorporation into TCA-precipitable material slowed, and leveled off between four and five hours (data not shown). The soluble leucine pool equilibrated very quickly and reached a steady state within the first fifteen minutes of incubation.

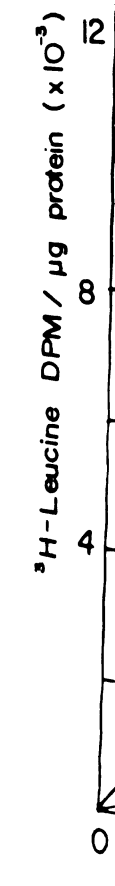

Figure 3.--^{3}H-leucine uptake and incorporation into TCA-precipitable and soluble pancreatic proteins in vitro.

Pancreas tissue slices were incubated for varied times in Eagle's MEM containing Krebs-Ringer salts, 0.4 mM leucine and 10 µCi/ml ^{3}H-leucine. Incorporation was terminated by removal and washing of the slice followed by sonication in 1% SDS. Aliquots of the sonicate were precipitated with 15% TCA. A control experiment indicated that 1% SDS, used to solubulize tissue proteins, did not interfere with the TCA precipitation of radioactive macromolecules. The precipitate and a portion of the TCA-soluble filtrate were counted.

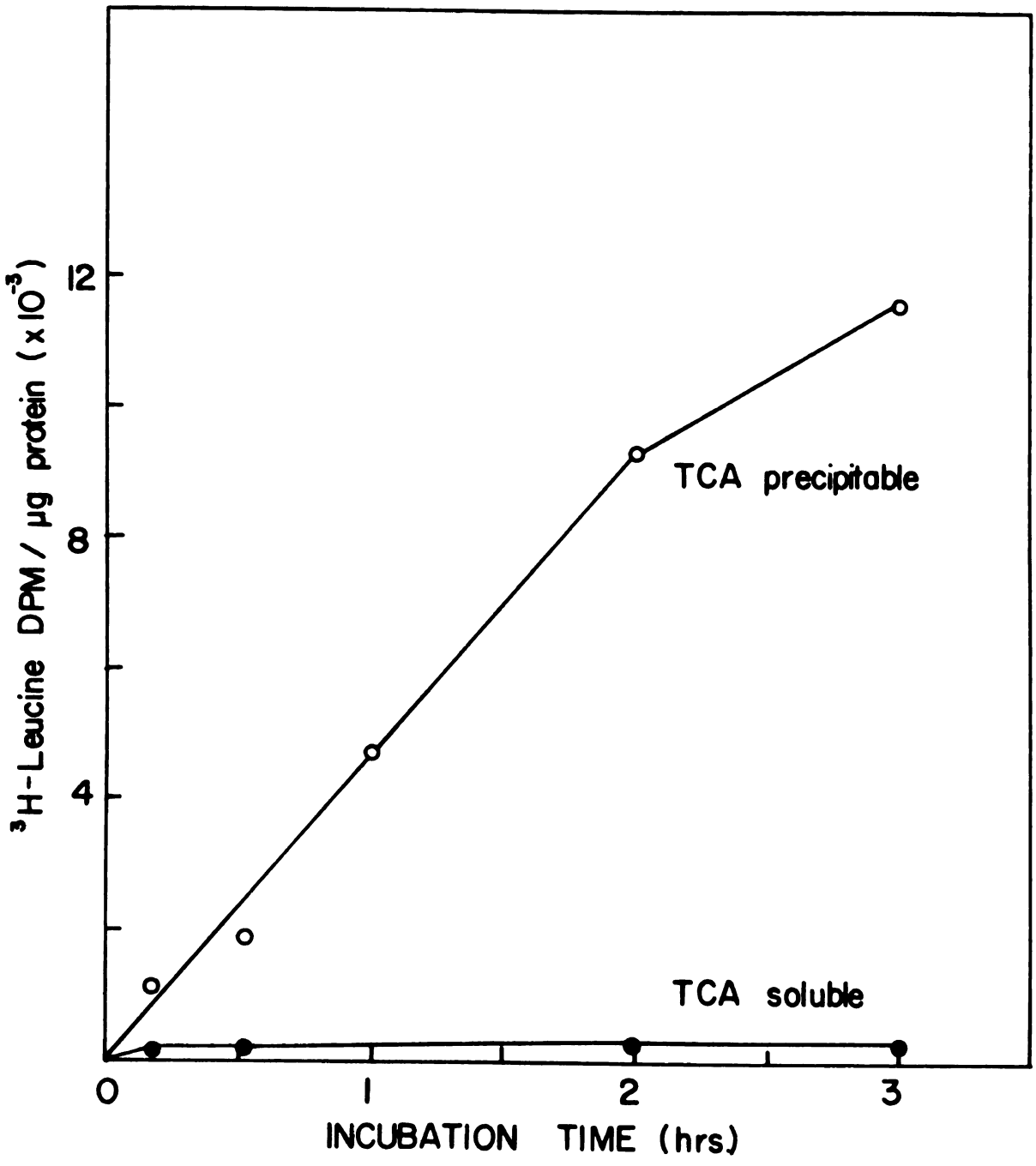
^{3}H-Leucine DPM / µg protein (x 10^{-3})
12
8
4
0
1
2
3
INCUBATION TIME (hrs)
TCA precipitable
TCA soluble

Figure 3

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^{35}S-Sulfate Incorporation Into Pancreatic Macromolecules in vitro

Tissue slices from a single rat pancreas were incubated for various times in 25-ml Erlenmeyer flasks containing 15 ml of sulfate-free Eagle's Minimal Essential Medium, Krebs-Ringer salts and 0.005 mCi/ml $H_2{}^{35}SO_4$. Sulfate-^{35}S incorporation is graphed as a function of time in Figure 4.

^{35}S-sulfate was incorporated into macromolecules by pancreas tissue at a linear rate for a period of five hours. Under culture conditions in defined chemical medium adult pancreas tissue was demonstrated to synthesize proteins, as demontrated by leucine incorporation, and to incorporate sulfate from $H_2{}^{35}SO_4$ into macromolecules at linear rates for periods of four to five hours.

Secretory Activity of Pancreas Tissue Slices in the Presence and Absence of Calcium and Sulfate Ions

To determine the effect of culture conditions on the secretory activity of pancreas tissue slices and to demonstrate whether the removal of sulfate from the medium in $H_2{}^{35}SO_4$ labeling experiments had an effect on secretion, a series of experiments was done in which release of amylase into the medium was quantitated as a function of time. The results are graphed in Figure 5.

The upper curve shows that the presence or absence of 1.2 mM sulfate ion in normal medium did not affect the rate of secretion. However, after 240 minutes of incubation (the usual time when labeled tissue pieces were harvested), approximately 90% of the total amylase had been secreted. This finding confirmed an empirical observation

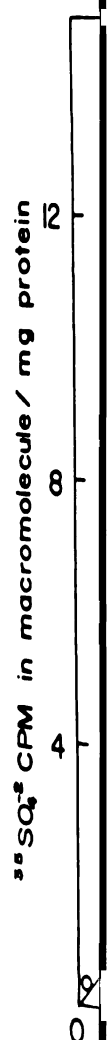


Figure 4.--^{35}S-sulfate incorporation into pancreatic macromolecules *in vitro*.

Tissue slices were incubated in sulfate-free Eagle's MEM buffered with Krebs-Ringer salts and containing 5 μCi/ml $H_2{}^{35}SO_4$. Slices, removed from the incubation at specified times, were rinsed with cold Krebs-Ringer salts, and were sonicated in 1% SDS. Total CPM/μl was determined for a portion of the sonicate and another portion was electrophoresed on cellulose acetate strips, as described in Methods, to determine the percentage of total ^{35}S-sulfate counts bound to macromolecule. The presence of SDS did not affect the migration of radioactive macromolecules on cellulose acetate strips. Counts per minuts of ^{35}S-sulfate bound to macromolecules per milligram of total protein were graphed as a function of time.

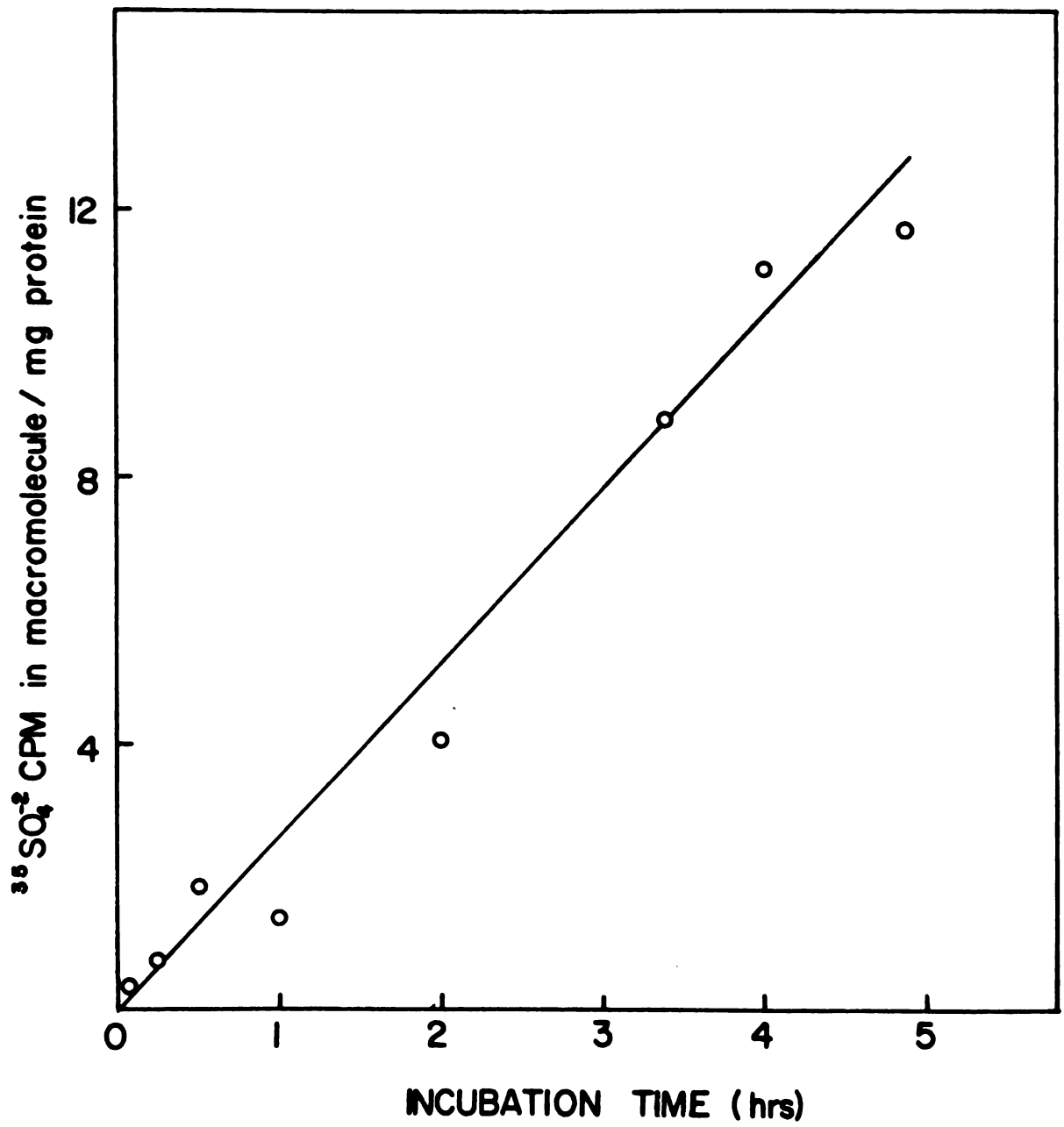
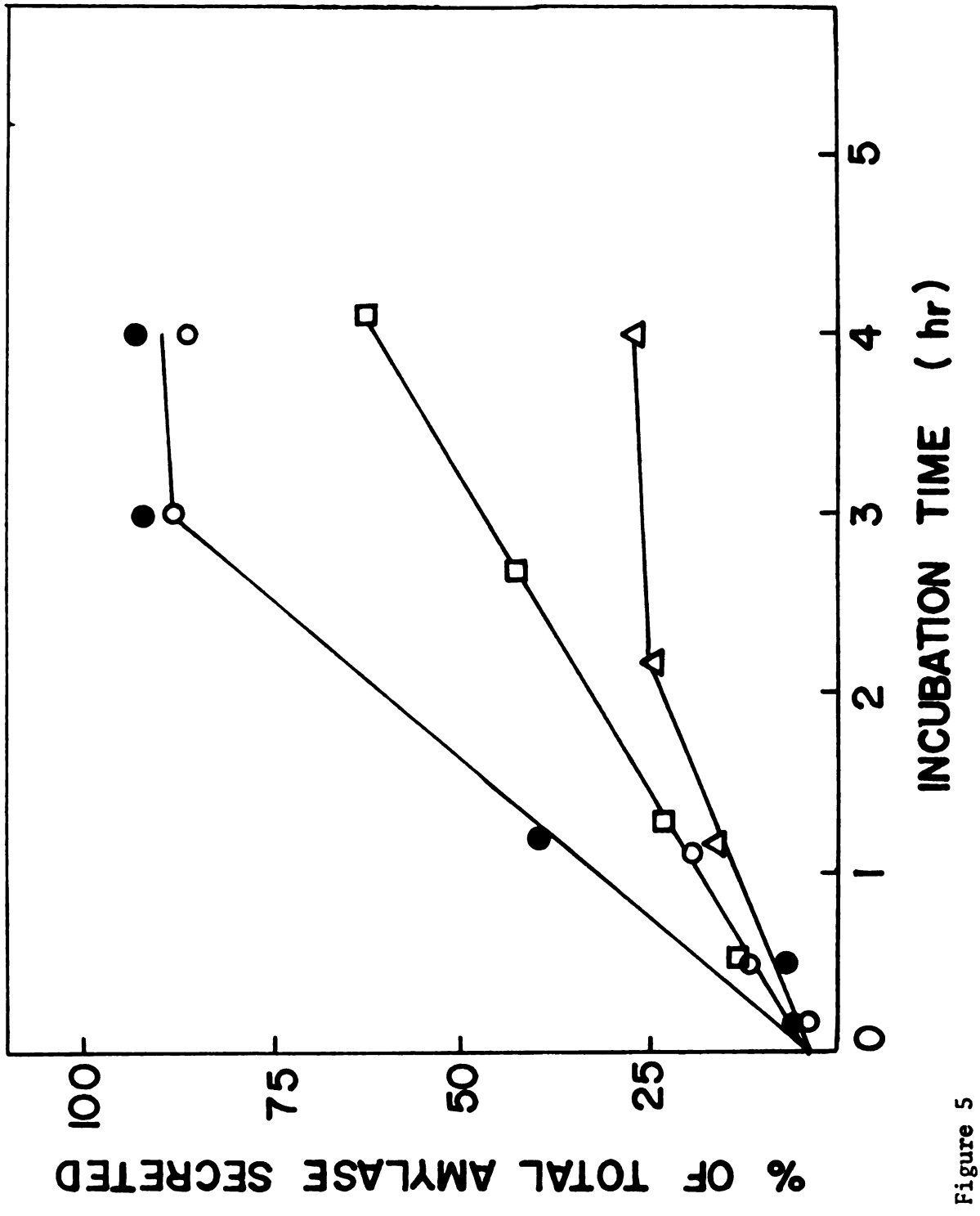

$^{35}SO_4^{-2}$ CPM in macromolecule / mg protein
12
8
4
0
1
2
3
4
5
INCUBATION TIME (hrs)

Figure 4

Figure 5.--α-amylase release from pancreas tissue in vitro.

Tissue slices were incubated in 10-ml of a specified medium per time point. At intervals, slices were removed, rinsed and placed in 2-ml of water. The tissue was homogenized by sonication for 20 seconds at maximum setting (Biosonik oscillator, Bronwill Scientific). The tissue homogenate and the filtered medium were assayed for amylase using the method of Bernfeld (1955). Amylase secreted into the medium is expressed as percentage of total amylase present in tissue plus medium.

The upper curve represents amylase release in medium containing 1.2 mM calcium ion in the present (●) and absence (O) of 1.2 mM sulfate ion. The lower curves show amylase release in calcium-free medium in the presence (Δ) and absence (□) of 1.2 mM sulfate ion.


% OF TOTAL AMYLASE SECRETED
100
75
50
25
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INCUBATION TIME (hr)

Figure 5

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Discussio

that the yield of zymogen granules from labeled tissue was smaller than that from an equivalent amount of fresh pancreas.

The phenomenon of high secretory activity of pancreas slices in Krebs-Ringer bicarbonate buffer has been noted by others. Heisler et al. (1972) reported that the spontaneous release of amylase in untreated tissues incubated in Krebs-Ringer bicarbonate buffer at calcium concentrations greater than 0.1 mM approached the release of the enzyme from stimulated tissues. Therefore, amylase release in calcium-free medium was studied. Soyben trypsin inhibitor was excluded from the medium in these experiments because of the possibility that it contained traces of calcium salts. The results are shown in the two lower curves of Figure 5. Removal of calcium decreased the amount of total amylase released.

With calcium absent, a possible effect of sulfate ion on secretion was indicated. After 240 minutes of incubation in the presence of 1.2 mM sulfate ion, 26% of the total amylase was released. In the absence of sulfate, amylase release rose to 60%. It is not possible to postulate a differential effect of sulfate on amylase secretion since more data points at late times (180-300 minutes) are needed to establish a significant difference between the two curves. Nevertheless, the demonstrated effect of high calcium concentrations on amylase secretion confirmed the earlier work of others and explained the apparent lower yield of zymogen granules from tissues incubated in Krebs-Ringer buffer.

The high calcium concentrations were used in the medium in all subsequent experiments. Reasons for this will be presented in the Discussion.

Isolation o
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Isolation of ^{35}S-Sulfate Labeled Lipid

To determine the nature of the sulfate-labeled macromolecules synthesized *in vitro*, pancreatic lipids were studied. Pancreas tissue was labeled four hours in standard sulfate-free medium containing 0.25 mg SBTI/ml and 0.050 mCi $H_2{}^{35}SO_4$/ml. Lipids were extracted from the tissue and the extract was applied to a silica gel thin-layer plate (Quantum) and chromatographed in chloroform:methanol:water (100:42:6) along with a sample of crude pancreatic lipid extracted from unlabeled tissue; a standard bovine sulfatide preparation (Applied Science Laboratories); and a control consisting of 1 µCi of $H_2{}^{35}SO_4$ in 10 µl of 10 mM carrier $MgSO_4$. Radioactivity on the thin layer plate was detected with a Berthold Radioscanner. The results are shown in Figure 6.

The lipid extract from ^{35}S-labeled pancreas displayed two radioactive peaks. The first, containing 30% of the radioactivity, did not migrate from the origin and consisted of free $^{35}SO_4{}^{-2}$ or labeled protein. The second peak, 70% of the radioactivity, represented sulfatide by virtue of its migration with a commercial bovine sulfatide standard.

The total radioactivity recovered in the lipid extract constituted only 1%-3% of the non-diliazable counts incorporated by pancreas. It can be concluded, therefore, that glycolipid accounts for a very small amount of the ^{35}S-sulfate incorporated into macromolecules during a four hour labeling period *in vitro*.

Figure 6.--Slicia gel thin-layer chromatogram of ^{35}S-sulfate-labeled lipid extract from pancreas tissue.

Tissue was extracted with chloroform:methanol (1:2) and then with chloroform:methanol (2:1). The organic phases were pooled. Water was removed by several additions of anhydrous ethanol followed by rotary evaporation. The dry material was dissolved in chloroform: methanol (2:1) and the solution was briefly centrifuged to sediment denatured protein. The clear supernatant was removed and the pellet was washed three times with additional aliquots of chloroform:methanol (2:1). All supernatants were pooled. The chloroform: methanol extract was evaporated in a tared ampoule. The weight of the residue was 0.103 gm.

This material was dissolved in 10 ml of chloroform:methanol (2:1), was placed in a dialysis bag and was dialyzed against running tap water for six hours to remove free $^{35}SO_4^{-2}$. The dialyzed material was dried with several washes of absolute ethanol in a rotary evaporator. The lipid extract was dissolved in 100 µl of chloroform:methanol.

Samples were applied to an unactivated Quantum silica gel thin-layer plate. The plate was developed for 150 minutes in a tank equilibrated with chloroform:methanol:water (100:42:6). Samples are identified as follows:

Lane 1. 1 mg of bovine sulfatide standard.

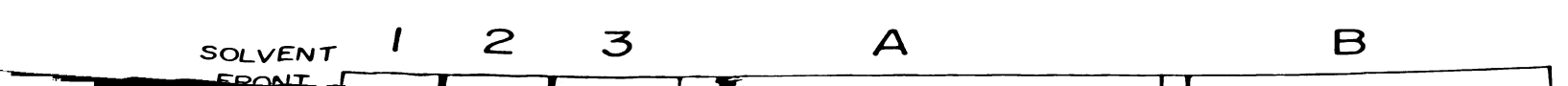
Lane 2. 4 mg of crude pancreatic lipid.

Lane 3. 10 µl of lipid extract from ^{35}S-labeled pancreas tissue.

Radioactivity scans:

Scan A. 25 µl of lipid extract from ^{35}S-labeled pancreas tissue.

Scan B. 1 µCi of $H_2{}^{35}SO_4$ in 10 µl of 10 mM carrier $MgSO_4$.



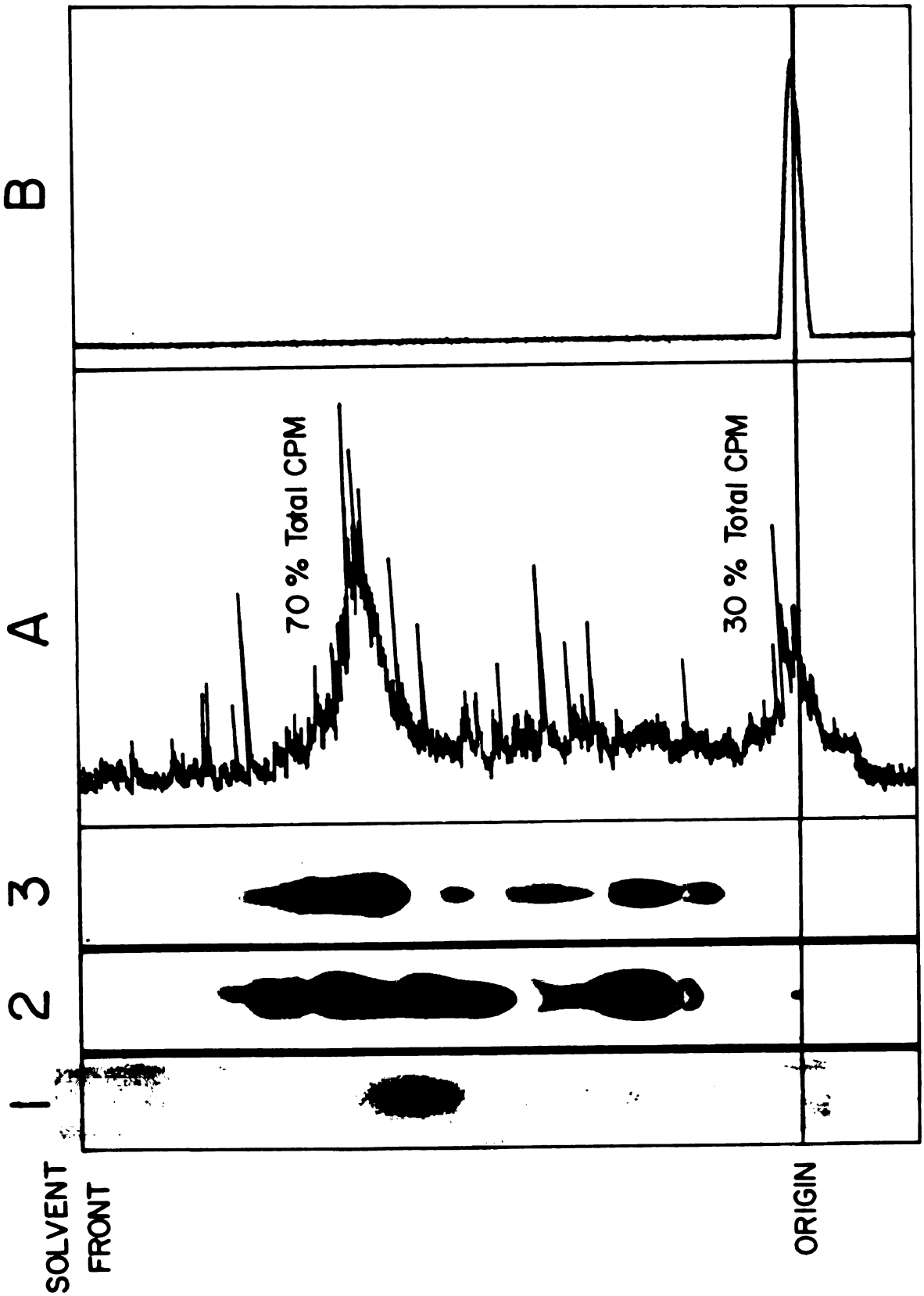


Figure 6

Susceptibility of ^{35}S-labeled Macromolecules to Proteolytic Digestion

To identify the sulfated macromolecules present in total pancreas, labeled tissue was subjected to Pronase digestion. Sulfated mucopolysaccharides and glycolipids yield non-dializable digestion products. Sephadex G-150 chromatography was then used to determine the molecular weight of the digested material.

Labeled pancreas tissue was digested, after lipid extraction, for 72 hours at 55°C with Pronase from Streptomyces griseus. The digested material was dialyzed and applied to a column of Sephadex G-150. The results are presented in Figure 7a. The void volume of the column was determined with dextran blue. The exclusion limit of Sephadex G-150 is 400,000 for globular proteins. From the broadness and heterogeneity of the ^{35}S elution profile, it was suspected that the Pronase digestion was incomplete. Pronase was checked for its ability to digest casein using the method of Narahashi (1970) and was found to be active. A sample of the original Pronase digest was redigested as before, using two additions of Pronase over a period of 48 hours at 55°C. Toluene was present to prevent bacterial growth. The redigested material was rechromatographed on the same Sephadex G-150 column. The ^{35}S elution profile of the redigested sample is shown in Figure 7b. Even after redigestion, the ^{35}S-elution profile appeared to represent a complex mixture of heterogeneous macromolecules which were resistant to further proetolytic cleavage.

Figure 7.--Sephadex G-150 chromatography of ^{35}S-sulfate-labeled pancreatic macromolecules after Pronase digestion.

a.--$^{35}SO_4$-labeled pancreas tissue pieces were lipid extracted. The dried residue was suspended in 0.1 M borate-borax buffer, pH 7.4, containing 5 mM $CaCl_2$ and digested at 55°C with two additions of 3 mg of Pronase over a period of 72 hours. The digested, dialyzed sample containing 2.6×10^4 CPM in 0.5 ml of 0.1 M NaCl was applied to a Sephadex G-150 column (1.5 x 23.8 cm) equilibrated with 0.1 M NaCl. Fractions were eluted with 0.1 M NaCl and counted.

b.--Another aliquot of the digested material chromatographed in Figure 8a was redigested for an additional 48 hours at 55°C, with two additions, each of 7 mg of Pronase and was rechromatographed as described in the text. Results are expressed in terms of ^{35}S CPM per fraction.

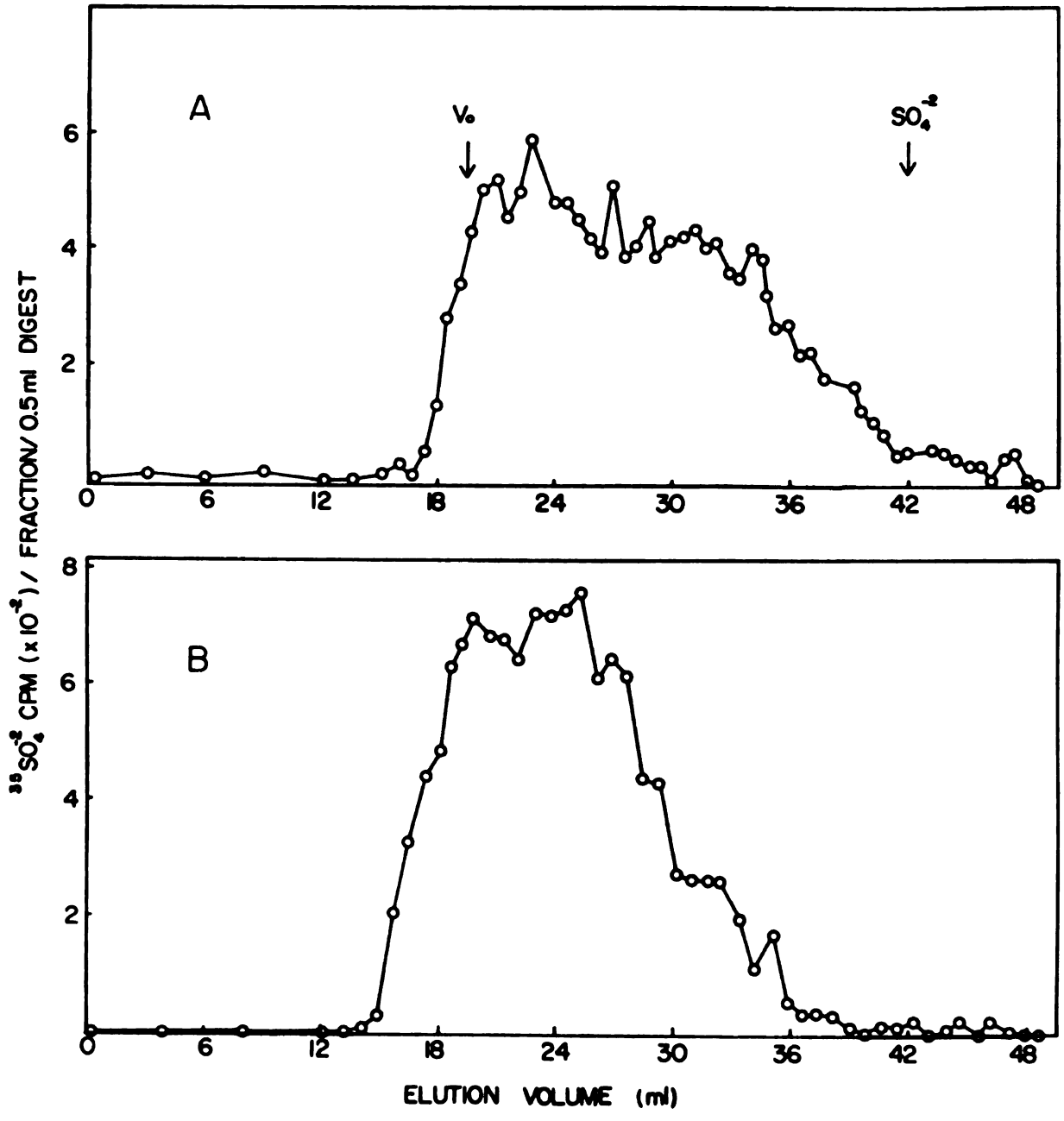

A
V_o
SO_4^{-2}
B
$^{35}SO_4^{-2}$ CPM (x10^{-2}) / FRACTION / 0.5ml DIGEST
ELUTION VOLUME (ml)
0
6
12
18
24
30
36
42
48
2
4
6
8

Figure 7

Stability of $^{35}SO_4$-Labeled Macromolecules to Acid and Base Hydrolysis

To determine the general type of sulfate linkage present in ^{35}S-labeled macromolecules, their stability to acid and base was studied. Tissue pieces were sonicated in 1% SDS to extract ^{35}S-labeled macromolecules. Free $^{35}SO_4^{-2}$ was removed by passing the sonicate over a Biogel P-4 column equilibrated with 1% SDS. The material eluting in the void volume was collected and was then subjected to acid or base treatment. The material was rechromatographed on P-4 in 1% SDS to determine the percentage of $^{35}SO_4^{-2}$ hydrolyzed. The results are shown in Table 5.

The stability of sulfate to mild acid and base hydrolysis indicated the presence of a sulfate ester.

Table 5.--Stability of ^{35}S-labeled macromolecules to acid and base hydrolysis.

Samples of SDS-solubulized pancreatic macromolecules were analyzed as described in the text.

Treatment	Bound ^{35}S-Sulfate Remaining (%)
None	93
0.04 N HCl, 85°C, one hour	90
1.0 N HCl, 102°C, one hour	2
0.05 N KOH, 4°C, 19 hours	95

II. Isolation and Characterization of ^{35}S-Sulfate Labeled Macromolecules in Subcellular Fractions from Pancreas

Protein Distribution and Specific Radioactivity of Subcellular Fractions

Rat pancreas tissue was labeled with $^{35}SO_4^{-2}$ and fractionated as described in Methods. The specific ratioactivity of $^{35}SO_4^{-2}$ in the tissue incubation medium was routinely 17μCi/ml. The $^{35}SO_4^{-2}$ concentration of the medium, calculated from the specific radioactivity of stock $H_2{}^{35}SO_4$ was 3.8μM. Assuming the counting efficiency of ^{35}S at maximum gain is similar to that of ^{14}C (73%), approximately 0.3 to 0.5% of the 10mCi of $H_2{}^{35}SO_4$ used in a typical experiment was recovered in the filtered tissue homogenate. Of these counts, 10% was incorporated into TCA-PTA-precipitable material. This yielded about 5×10^6 CPM of macromolecule-bound ^{35}S, which was distributed among the subcellular fractions. Table 6 shows the percentage of protein recovered in each fraction relative to the crude homogenate, and the specific radioactivity, defined as counts per minute of macromolecule-bound ^{35}S-sulfate per μgm protein.

Cellular debris was pelleted from the crude homogenate during a short, low-speed spin (500xg for ten minutes). This particulate fraction consisted of unlysed cells, nuclei, connective tissue, plasma membranes, and trapped material and contained 25% of both the total protein and bound ^{35}S-sulfate.

The purified zymogen granule fraction contained 5% of the total protein and approximately 14% of the bound ^{35}S-sulfate. When zymogen granules were lysed in 0.2M bicarbonate more than 80% of the protein

Table 6.--Subcellular distribution of ^{35}S-sulfate-labeled macromolecules.

Pancreas tissue was labeled and subcellular fractions were isolated as described in Methods. Bound sulfate recovery was calculated from the experimentally determined protein recovery and specific radioactivity. Numbers in parentheses in the Bound Sulfate Recovery column indicate the range of values obtained in 2 to 4 separate experiments. Figures given with no standard deviation (SD) were based on a single experiment.

Fraction	Specific Radioactivity ±SD	Protein Recovery (% of Homogenate) ±SD	Bound ^{35}S Recovery (% of Homogenate)	
Homogenate	3 ± 2	100	100	
Nuclei, Debris	3 ± 2	25 ± 1	25	(24-26)
Zymogen Granules	6 ± 1	5 ± 1	14	(8-20)
Zymogen Granule Lysate	3 ± 1	5 ± 1	7	(5-8)
Zymogan Granule Membranes	63 ± 15	< 0.1	3	(2-5)
Mitochondria	3 ± 1	10 ± 1	14	(9-18)
Mitochondrial Membranes	5 ± 1	3 ± 2	5	(4-6)
Microsomes	5 ± 1	14 ± 4	30	(21-40)
Microsomal Membranes	5 ± 1	4 ± 1	9	(6-12)
Rough Microsomes	13	5.4	15	
Rough Microsomal Membranes	5	2.7	4.5	
Smooth Microsomes	13	0.8	3.4	
Smooth Microsomal Membranes	23	0.2	1.5	
Post Microsomal Supernatant	3 ± 2	25 ± 2	25	(24-27)

and 30% to 50% of the bound ^{35}S-sulfate was found in the soluble lysate. The final washed zymogen granule membrane pellet, designated as ZGM, contained less than 0.1% of the total protein of the crude homogenate and approximately 3% of the total bound sulfate. The specific radioactivity of the ZGM, as shown in Table 6 was 63 $\pm$ 15 CPM/μg protein, the highest value of any fraction isolated. It was twelve-fold higher than the specific radioactivity of either mitochondrial or microsomal membranes.

Table 7 shows in greater detail the protein and bound ^{35}S-sulfate distribution obtained when zymogen granules were subfractionated. Table 7a outlines the protein distribution and indicates that 70% or more of the zymogen granule protein was non-particulate after lysis of the intact granules. This soluble fraction contained the zymogens, the digestive enzymes which are destined for secretion. The particulate fraction, designated as crude zymogen granule membrane (crude ZGM) was obtained as a band over a 1.0M sucrose cushion after centrifugation as outlined in Figure 1.

When the crude zymogen granule membrane fraction was collected, sonicated in 0.25 M NaBr, and pelleted, 94% of the crude ZGM protein was recovered in the NaBr supernatant and only 5% remained sedimentable. The washed ZGM pellet represented 0.3% of the total zymogen granule protein. This confirmed the findings of MacDonald (1974) that the NaBr sonication effectively removed adsorbed secretory proteins, such as amylase, which were present as contaminants on crude zymogen granule membranes. The sonication was judged to be a fairly gentle treatment since it did not inactivate amylase. However, it was a

Table 7.--Recovery of protein and bound ^{35}S-sulfate after zymogen granule fractionation.

Labeled pancreas tissue was fractionated as described in Methods. The fractionation scheme is also diagrammed in Figure 1.

a.--Recovery of protein.

Fraction	% of Homogenate	% of Zymogan Granules	% of Crude ZGM
Homogenate	(100)	. .	. .
Zymogen granules (ZG)	7.3	(100)	. .
ZG Lysate	5.1	69.4	. .
Crude ZG membranes	0.4	11.0	(100)
NaBr supernatant	0.4	5.5	94.3
ZG membranes	0.1	0.3	5.6

b.--Recovery of bound sulfate.

Fraction	% of Zymogen Granules	% of Crude ZGM
Zymogen granules	(100)	. .
ZG Lysate	27	. .
Crude ZG membranes	29	100
1.0 M Sucrose	14	. .
Mitochondrial Pellet	28	. .
NaBr supernatant	16	56
ZG membranes	13	44

rigorous wash considering the high concentration of salt used (0.25M), the sonication step, and the large dilution faction (1:20).

Table 7b indicates the bound ^{35}S-sulfate distribution after zymogen granule subfractionation. Approximately 30% of the bound ^{35}S-sulfate of the intact granule was present in the lysate and 30% was recovered in the crude ZGM band. Fourteen percent of the bound ^{35}S was found in the 1.0M sucrose cushion and 28% was in the mitochondrial pellet.

Following extraction of crude ZGM with 0.25M NaBr, over 50% of the bound sulfate was found in the NaBr supernatant; 44% remained with the zymogen granule membrane. In a later section, evidence will be presented that the NaBr supernatant contains a sulfated component with the same electrophoretic mobility on SDS-polyacrylamide gels as the sulfated species in zymogen granule membrane. Presumably, this solubulized material was dislodged from the membrane by sonication in NaBr.

Mitochondria isolated from labeled tissue contained 14% of the bound sulfate and 10% of the total protein. Washed mitochondrial membranes contained one-third of the protein and one-third of the bound sulfate of whole mitochondria.

The microsomal fraction isolated from labeled tissue contained 14% of the total protein of the crude homogenate and 30% of the bound ^{35}S-sulfate. When microsomal membranes were prepared, 30% of the bound sulfate remained with the particulate fraction.

Labeled microsomes were subfractionated into rough and smooth membranes. As shown in Table 6, the specific radioactivity of the smooth microsomal membranes was four times that of rough microsomal

membranes and was three-fold lower than that of zymogen granule membranes. The specific radioactivity of mitochondrial and rough microsomal membranes was twelve-fold lower than that of zymogen granule membranes.

The post microsomal supernatant, which represents cytoplasmic proteins plus 5 to 20% of the zymogen granule contents contained 25% of the total protein of the crude homogenate and, though it had a low specific radioactivity (3 CPM/μg protein) it accounted for one quarter of the total bound sulfate.

Investigation of Non-Specific Binding of ^{35}S-Labeled Macromolecules to Membrane Fractions

The presence of sulfate-labeled macromolecules in all membrane fractions suggested that it was important to rule out non-specific binding of ^{35}S-labeled macromolecules to membranes. The post microsomal supernatant was particularly suspect as a source of contamination since it contains fully 25% of the macromolecule-bound sulfate.

An experiment was designed in which fresh, unlabeled mitochondria and microsomes were incubated with ^{35}S-labeled post microsomal supernatant. Zymogen granules were mixed with both labeled zymogen granule lysate and post microsomal supernatant. The unlabeled organelles were incubated for 20 minutes at 37°C and for an additional 30 minutes at 0°C. Soybean trypsin inhibitor was present at a concentration of 0.1mg/ml to prevent proteolysis. It is important to point out that the ratio of protein in unlabeled fractions to protein in post microsomal supernatant and zymogen granule lysate was identical to that of a typical fractionation experiment so that putative "binding

sites" on the organelle membranes would be exposed to an equivalent concentration of radioactive soluble proteins at specific radioactivities comparable to those encountered in tissue homogenates.

The results are presented in Table 8. Mitochondrial membranes bound only 0.2% of the total counts added. The specific radioactivity of this membrane fraction was 0.6 CPM/μg protein, eight-fold lower than that isolated from labeled tissue. Microsomal membranes bound 6% of the total counts and had a specific radioactivity of 0.7 CPM/μg protein, seven-fold lower than that isolated from labeled tissue. Zymogen granule membrane bound 1.4% of the total counts in the incubation and had a specific radioactivity of 21 CPM/μg protein. This is a significant degree of non-specific binding since this value is approximately 30% of that obtained in zymogen granule membranes from labeled tissue. However, zymogen granules in this control experiment were not treated in an analogous fashion to those isolated from labeled tissue. In the actual tissue fractionation experiment, the intact zymogen granules were isolated from the homogenate by differential centrifugation and were washed with a large volume of 0.3M sucrose to remove trapped or adsorbed soluble proteins from the zymogen granule pellet. The washed granules were resuspended in isotonic NaCl and then they were lysed by addition of bicarbonate. In the control experiment, the unlabeled zymogen granules were lysed in the presence of labeled post microsomal supernatant and zymogen granule lysate, exposing both sides of the zymogen granule membrane to the ^{35}S-labeled soluble proteins and possibly allowing an artifically high degree of non-specific binding to be measured.

Table 8.--Non-specific binding of ^{35}S-sulfate labeled proteins to membrane fractions.

Fraction	^{35}S-Labeled Soluble Protein (Total CPM Added)	Total CPM Bound	% CPM Bound	Specific Radioactivity
Mitochondrial Membranes	13,050	22	0.2	0.6
Microsomal Membranes	13,050	780	6	0.7
Zymogen Granule Membranes	185,700	2,650	1.4	21.0

Unlabeled mitochondria, microsomes, and zymogen granules were incubated with ^{35}S-sulfate-labeled post microsomal supernatant. Membrane fractions were prepared from the organelles and their specific radioactivity was determined as described in the text.

It was concluded that, although a small percentage of sulfated proteins from the post microsomal supernatant or zymogen granule lysate was bound, at least 70% of the sulfate-labeled macromolecules isolated in membrane fractions were normal components of these fractions.

Polypeptide Profiles of Particulate and Soluble Fractions on SDS-Polyacrylamide Gels

^{35}S-labeled subcellular fractions were prepared and analyzed on 9% polyacrylamide gels in 1% SDS to monitor the purity of the fractions and to check for proteolytic degradation. Gels were stained with Coomassie Brilliant Blue R and scanned at 550nm, as described in Methods. The polypeptide profiles of subcellular fractions from tissues cultured *in vitro* for four hours were similar to those obtained by MacDonald (1974) using fresh tissue, though small variations in the relative abundance of some components were noted.

It was sometimes necessary in the present study to increase the diameter of the gel to 6mm and to apply 300-800 μg of protein. The gel system was able to accommodate this large protein load without disturbing sample stacking or distorting the gel top, and unbowed protein bands were obtained, though there was a slight decrease in the resolving power of these gels.

The polypeptide profiles are presented in Figure 8. The zymogen granule lysate (Figure 8b) contained nine major components. The major component at 3.1cm has been identified as amylase (Sanders and Rutter, 1972). It has an apparent molecular weight of 52,000 as determined by MacDonald who compared it to proteins of known weight on parallel gels.

In contrast, the zymogen granule membranes (Figure 8a) contained a major, unique component which migrated at 1.7cm and had a reported apparent molecular weight of 75,000 (MacDonald and Ronzio, 1972). The molecular weight of the component running at 1cm was estimated as 130,000 (MacDonald, 1974).

Rough microsomal membrane and postmicrosomal supernatant (Figures 8c and d) displayed complex profiles. The point to be made regarding these fractions is that they did not possess major polypeptide components in the range from 200,000 to 75,000 (0 to 1.7cm).

Electrophoretic Analysis of ^{35}S-Sulfate-Labeled Subcellular Fractions on SDS-9% Acrylamide Gels

To determine if sulfated macromolecules showed coincidence with polypeptide components, sulfate-labeled subcellular fractions were run

Figure 8.--Electrophoretic analysis of polypeptides of subcellular fractions on SDS-9% acrylamide gels.

Electrophoresis was performed on gels 6mm in diameter under standard conditions (9% acrylamide, 1% SDS, pH 7.4) as described in Methods. The following amounts of protein were applied: A, zymogen granule membranes, 32µg; B, zymogen granule lysate, 160µg; C, rough microsomal membranes, 380µg; D, post microsomal supernatant, 330µg. Gels were stained with Coomassie Blue R and scanned at 550 nm. The arrow at the right hand side of each scan marks the distance of migration of the tracking dye, pyronin B.

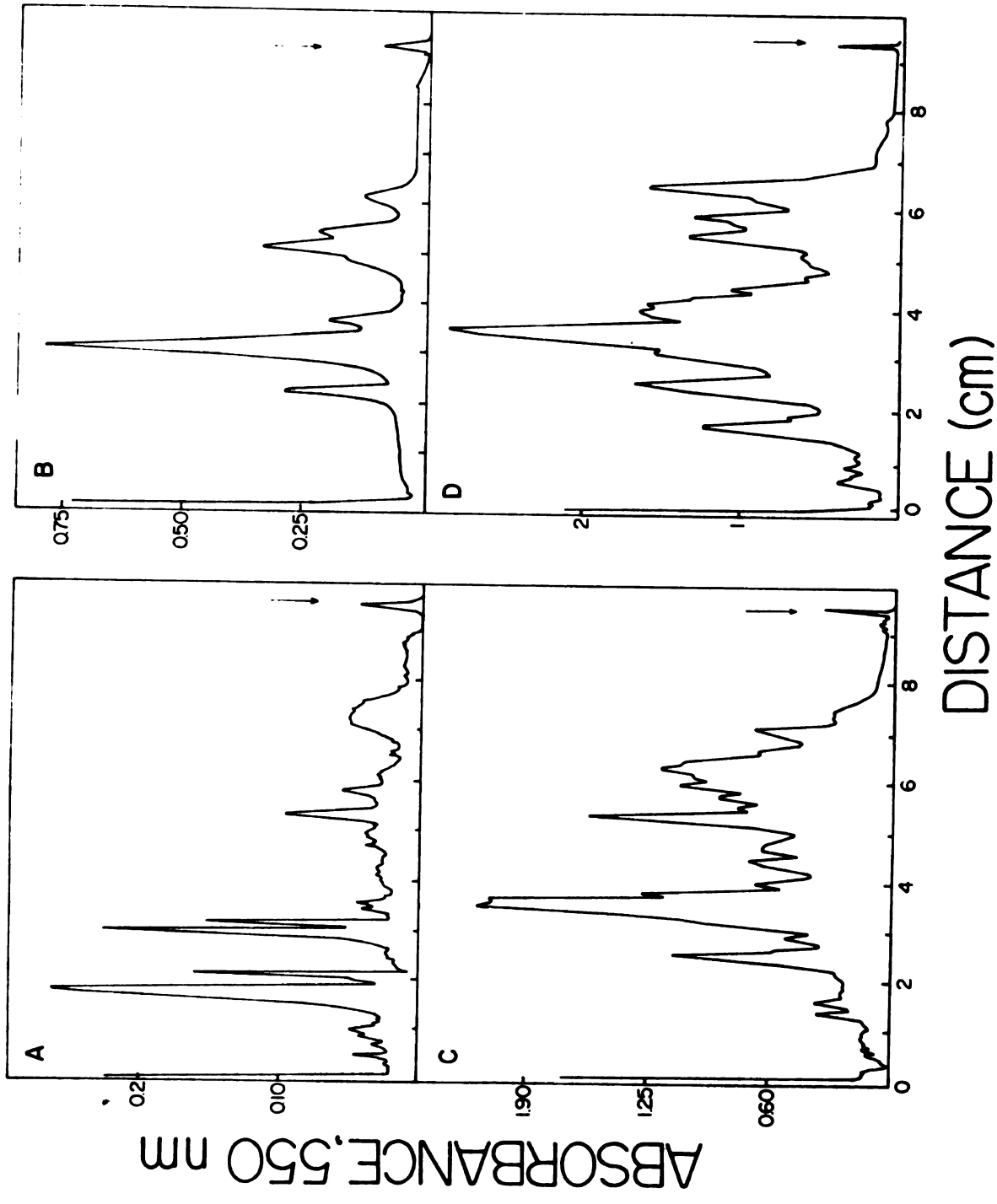

A
B
C
D
0.2
0.10
0.75
0.50
0.25
1.90
1.25
0.60
2
1
0
2
4
6
8
ABSORBANCE,550 nm
DISTANCE (cm)

Figure 8

run on 9% acrylamide gels in 1% SDS. The gels were fractionated and counted as described in Methods. The results are shown in Figure 9.

All subcellular fractions displayed a high molecular weight component which was broadly distributed, suggesting a heterogeneous family of macromolecules. The major component of zymogen granule membranes (Figure 9A) migrated at 0.8cm, indicating an average apparent molecular weight of 140,000. A smaller peak at 1.4cm could be assigned an apparent molecular weight of 84,000. The major component of rough microsomal membranes (Figure 9B) migrated at 1.2cm (apparent molecular weight 87,000) and the sulfated species in mitochondrial membrances (9C) had an apparent molecular weight of 170,000.

Zymogen granule lysate (Figure 9D) had a major peak at an apparent molecular weight 140,000 with a minor peak at 4.2cm of 32,000. The post microsomal supernatant (Figure 9E) displayed several distinct peaks. Apparent molecular weights of 150,000, 87,000, 60,000, and 32,000 could be assigned the components at 0.4cm, 1.2cm, 2.1cm, and 4.2cm respectively.

Figure 10 shows that the ^{35}S-sulfate profile of the NaBr supernatant on the same gel system displayed a major component with an apparent molecular weight of 140,000 which may represent a macromolecule dislodged from the zymogen granule membrane. Coincidence of sulfated macromolecules with Coomassie blue-staining polypeptide components was not noted, except in the zymogen granule membrane, where a sulfated component displayed the same relative mobility as a polypeptide with an apparent molecular weight of 130,000.

Figure 9.--Electrophoretic analysis of ^{35}S-sulfate-labeled subcellular fractions on SDS-9% acrylamide gels.

Electrophoresis was performed on 6mm gels under standard conditions. Gels were fractionated and counted as described in Methods. The following amounts of protein and radioactivity were applied: A, zymogen granule membranes, 15µg, 1600 CPM; B, rough microsomal membranes, 286µg, 1200 CPM, C, mitochondrial membranes, 320µg, 2160 CPM; D, zymogen granule lysate, 412µg, 1800 CPM; E, post microsomal supernatant, 530µg, 5500 CPM. The arrow at the right hand side of each profile marks position of the tracking dye, pyronin B.

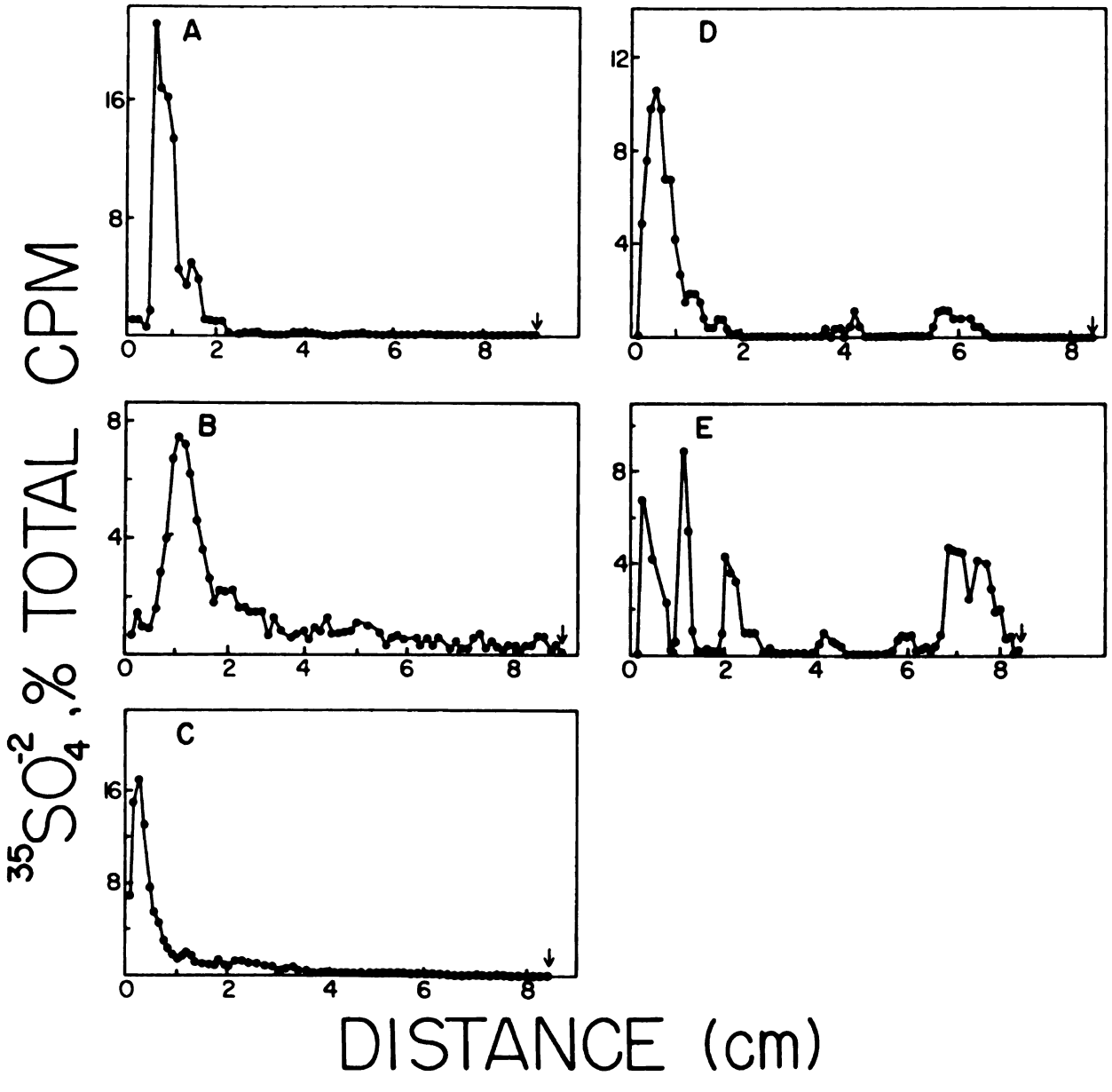


Figure 9

Figure 10.--Electrophoretic analysis of ^{35}S-sulfate-labeled NaBr supernatant on SDS-9% acrylamide gels.

Conditions and procedures were as described in Figure 9. Sixty µg of protein and 500 CPM were applied to the gel. The position of the tracking at 8.6 cm is not shown on the figure.

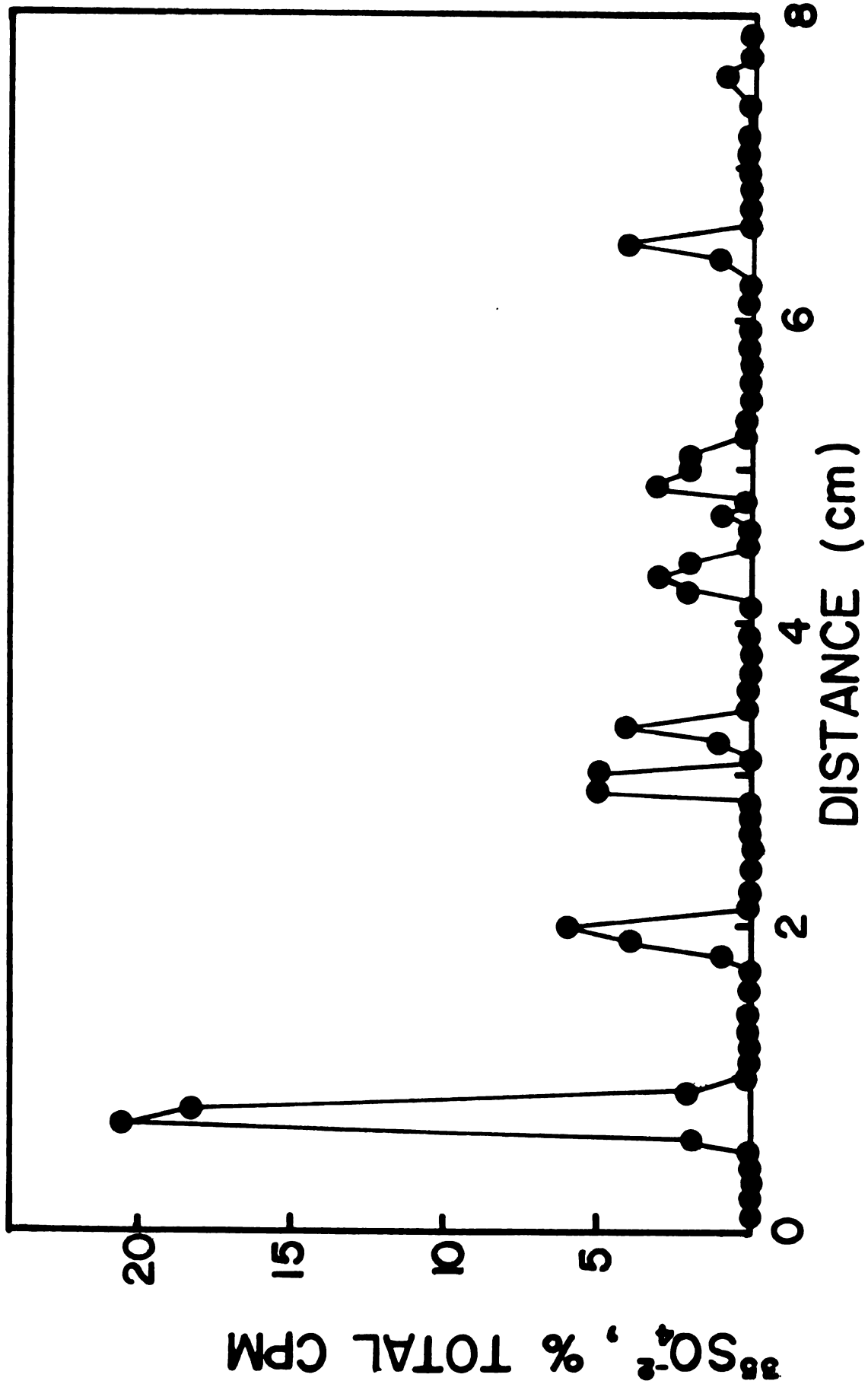


Figure 10

Electrophoretic Analysis of ^{35}S-Sulfate-Labeled Subcellular Fractions on SDS-5% Acrylamide Gels

It was impossible to determine if the high molecular weight sulfated components of the subcellular fractions were distinct from one another because of the small differences in migration of these macromolecules on 9% acrylamide gels. The fractions were run on a 5% polyacrylamide gel system to resolve this problem. The 5% gel was calibrated with proteins of known molecular weight. The results are graphed in Figure 11.

Results obtained with sulfate-labeled fractions are presented in Figure 12. On 5% gels, the sulfated macromolecules displayed a striking heterogeneity. Microsomal membranes (12A) contained sulfated components, ranging in molecular weight from 200,000 to 30,000 with a peak at 80,000. Zymogen granule membranes (Figure 12B) were very similar. However, the principal component of mitochondrial membranes (Figure 12C) appeared to be unique with an apparent molecular weight of 130,000. This confirmed the results obtained on 9% gels where the sulfated species of mitochondrial membrane migrated more slowly than that of zymogen granule membrane or microsomal membrane.

The soluble fractions analyzed, post microsomal supernatant (Figure 12D) and zymogen granule lysate (Figure 12E) were also extremely heterogeneous. Zymogen granule lysate showed a greater proportion of rapidly migrating components on 5% gels than it did on 9% gels, possibly suggesting that the material was partially degraded.

Figure 11.--Calibration of SDS-5% acrylamide gels.

Proteins of known molecular weights were analyzed on 5% acrylamide gels under standard conditions. Mobilities are expressed relative to the tracking dye (arrow). Molecular weights of standard proteins were obtained from surveys by Klotz and Darnall (1960), Weber and Osborn (1969), Smith (1968), and DeCrombruggbe et al. (1966). The table below identifies the standard proteins in the figure.

Protein	Source	Molecular Weight
a. thyroglobulin	pig	165,000
b. β-galactosidase	Escherichia coli	130,000
c. catalase	beef liver	60,000
d. alkaline phosphatase	bacteria	40,000
e. alcohol dehydrogenase	yeast	37,000
f. β-lactoglobulin	human	18,000
g. lysozyme	egg white	14,300

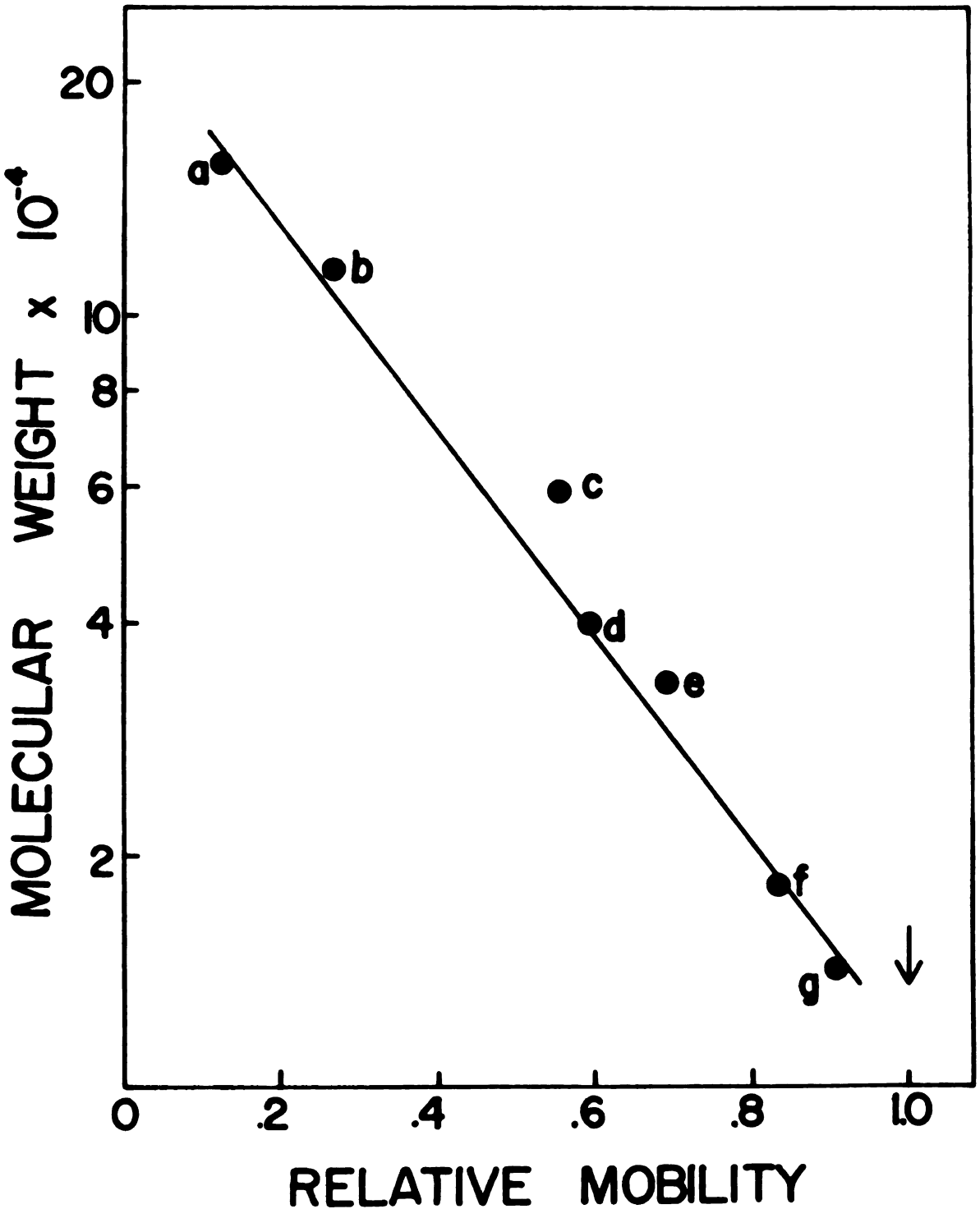

MOLECULAR WEIGHT x 10^{-4}
20
10
8
6
4
2
a
b
c
d
e
f
g
0
.2
.4
.6
.8
1.0
RELATIVE MOBILITY

Figure 11

Figure 12.--Electrophoretic analysis of ^{35}S-sulfate-labeled subcellular fractions on SDS-5% acrylamide gels.

Electrophoresis was performed on 6mm gels under standard conditions as described in Methods. Five percent gels were too soft for fractionation by extrustion. They were sliced into 2mm discs and results were plotted as bar graphs. The following amounts of protein and radioactivity were applied: A, microsomal membranes, 270µg, 1500 CPM; B, zymogen granule membranes, 67µg, 10,300 CPM; C, mitochondrial membranes, 400µg, 2700 CPM; D, post microsomal supernatant, 424µg, 4300 CPM; E, zymogen granule lysate, 413µg, 1800 CPM. The arrow at the right hand side of each profile marks the position of the tracking dye, pyronin B.

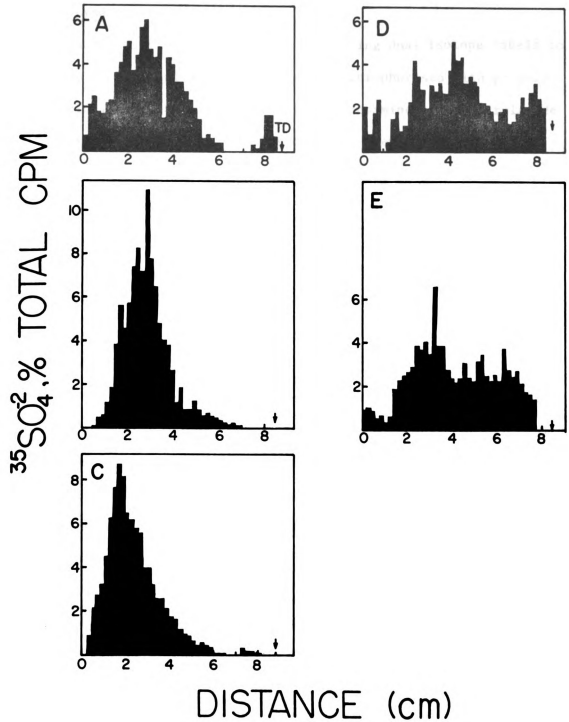


Figure 12

Further Characterization of the Sulfated Components of Zymogen Granule Membrane and Zymogen Granule Lysate

A series of experiments was done using dual isotope labels to determine if sulfated macromolecules coelectrophoresed with protein (^{3}H-leucine) and with carbohydrate (^{3}H-glucosamine) on 9% acrylamide gels. Figure 13 shows the results obtained when ^{35}S-sulfate- and ^{3}H-leucine-labeled fractions were mixed and electrophoresed. In the zymogen granule membranes (Figure 13A) the sulfate profile coincided with one polypeptide component with an apparent molecular weight of 130,000. In the zymogen granule lysate (Figure 13B), no ^{3}H-leucine migrated with the major sulfated component. However, this did not prove conclusively that sulfate in the zymogen granule lysate was not associated with protein.

Figure 14A shows co-migration of leucine and glucosamine in two high molecular weight components of zymogen granule membrane with apparent molecular weights of 130,000 and 75,000. In the lysate (Figure 14B) glucosamine counts coincided with the secretory proteins. However, evidence presented later will show that these glycoproteins are probably a very minor species.

In Figure 15A, sulfate- and glucosamine-labeled zymogen granule membranes showed coincidence in a peak in the molecular weight region of 130,000. The lysate (Figure 15B) also showed coincidence of sulfate and glucosamine in the high molecular weight region.

These data suggested that the sulfated components of zymogen granule membrane were associated with both protein and carbohydrate.

Figure 13.--Electrophoretic analysis of zymogen granule membranes and zymogen granule lysate labeled with ^{3}H-leucine and ^{35}S-sulfate.

Labeled membrane and soluble fractions were prepared and electrophoresed as described in Methods. The following amounts of protein and radioactivity were applied to the gels.

(A) Zymogen granule membranes: ^{3}H-leucine (O), 37µg, 3800 CPM; ^{35}S-sulfate (●), 15µg, 1600 CPM.

(B) Zymogen granule lysate: ^{3}H-leucine (O), 50µg, 9500 CPM; ^{35}S-sulfate (●), 412µg, 1750 CPM.

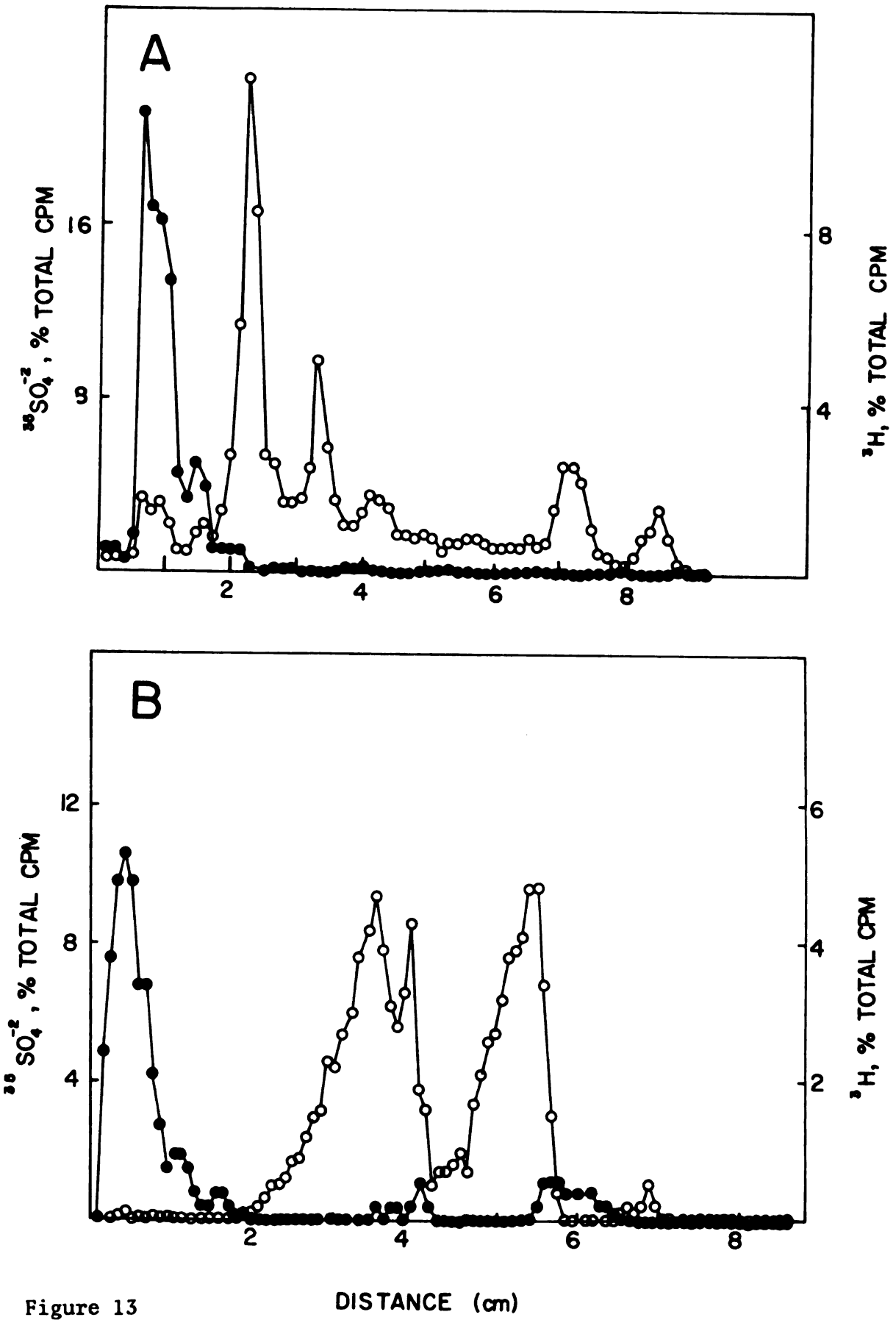


Figure 13

Figure 14.--Electrophoretic analysis of zymogen granule membranes and zymogen granule lysate labeled with ^{3}H-glucosamine and ^{14}C-leucine.

Labeled membrane and soluble fractions were prepared and electrophoresed as described in Methods. The following amounts of protein and radioactivity were applied to the gels:

(A) Zymogen granule membranes: ^{14}C-leucine (●), 45µg, 3800 CPM; ^{3}H-glucosamine (O), 10µg, 4200 CPM.

(B) Zymogen granule lysate: ^{14}C-leucine (●), 90µg, 2000 CPM; ^{3}H-glucosamine (O), 7µg, 1600 CPM.

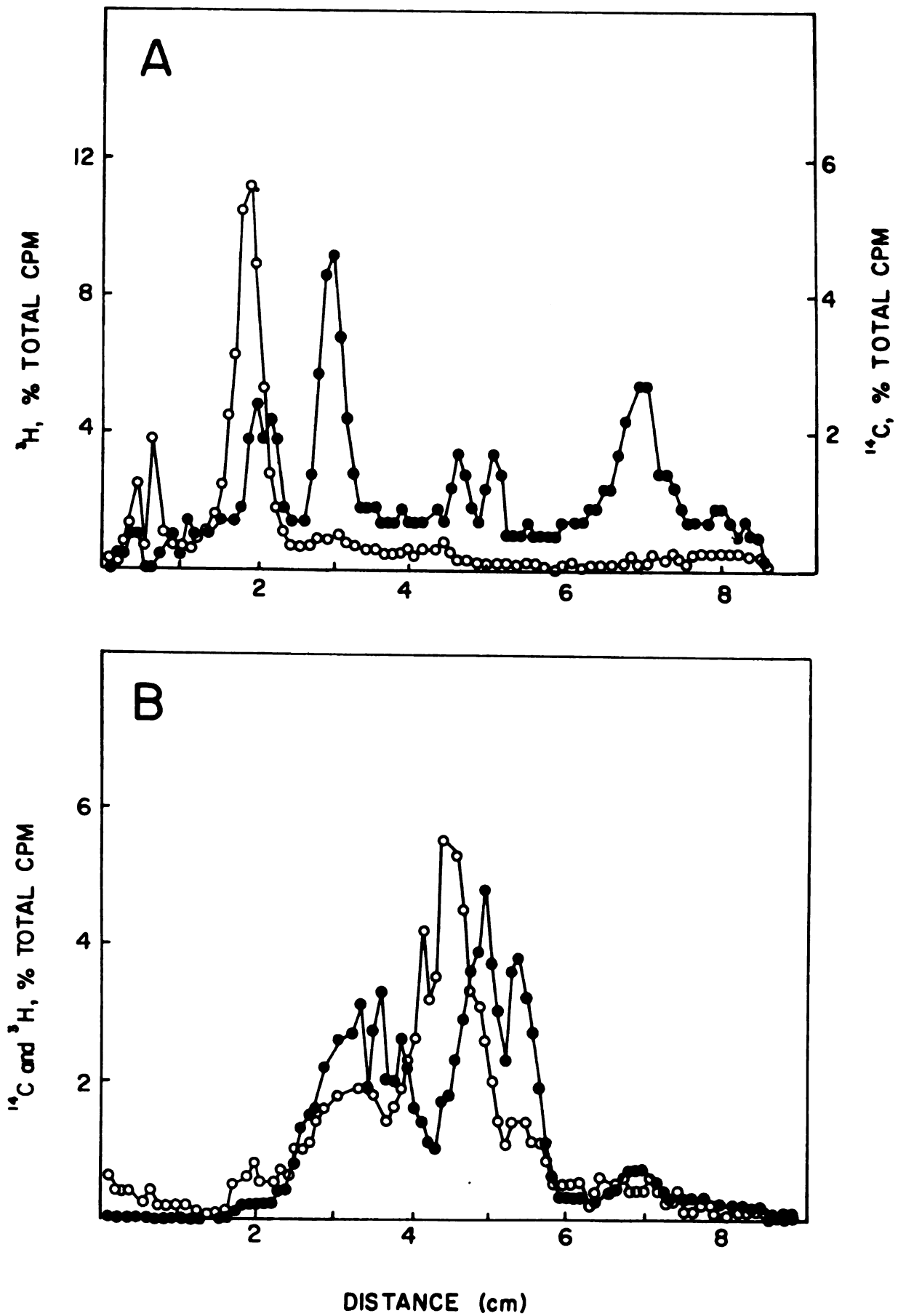

A
B
^{3}H, % TOTAL CPM
^{14}C, % TOTAL CPM
^{14}C and ^{3}H, % TOTAL CPM
DISTANCE (cm)
12
8
4
6
4
2
2
4
6
8

Figure 14

Figure 15.--Electrophoretic analysis of zymogen granule membranes and zymogen granule lysate labeled with ^{3}H-glucosamine and ^{35}S-sulfate.

Labeled membrane and soluble fractions were prepared and electrophoresed as described in Methods. The following amounts of protein and radioactivity were applied to the gels:

(A) Zymogen granule membranes: ^{3}H-glucosamine (O), 92µg, 3900 CPM; ^{35}S-sulfate (●), 121µg, 4200 CPM.

(B) Zymogen granule lysate: ^{3}H-glucosamine (O), 50µg, 9500 CPM; ^{35}S-sulfate (●), 412µg, 1700 CPM.

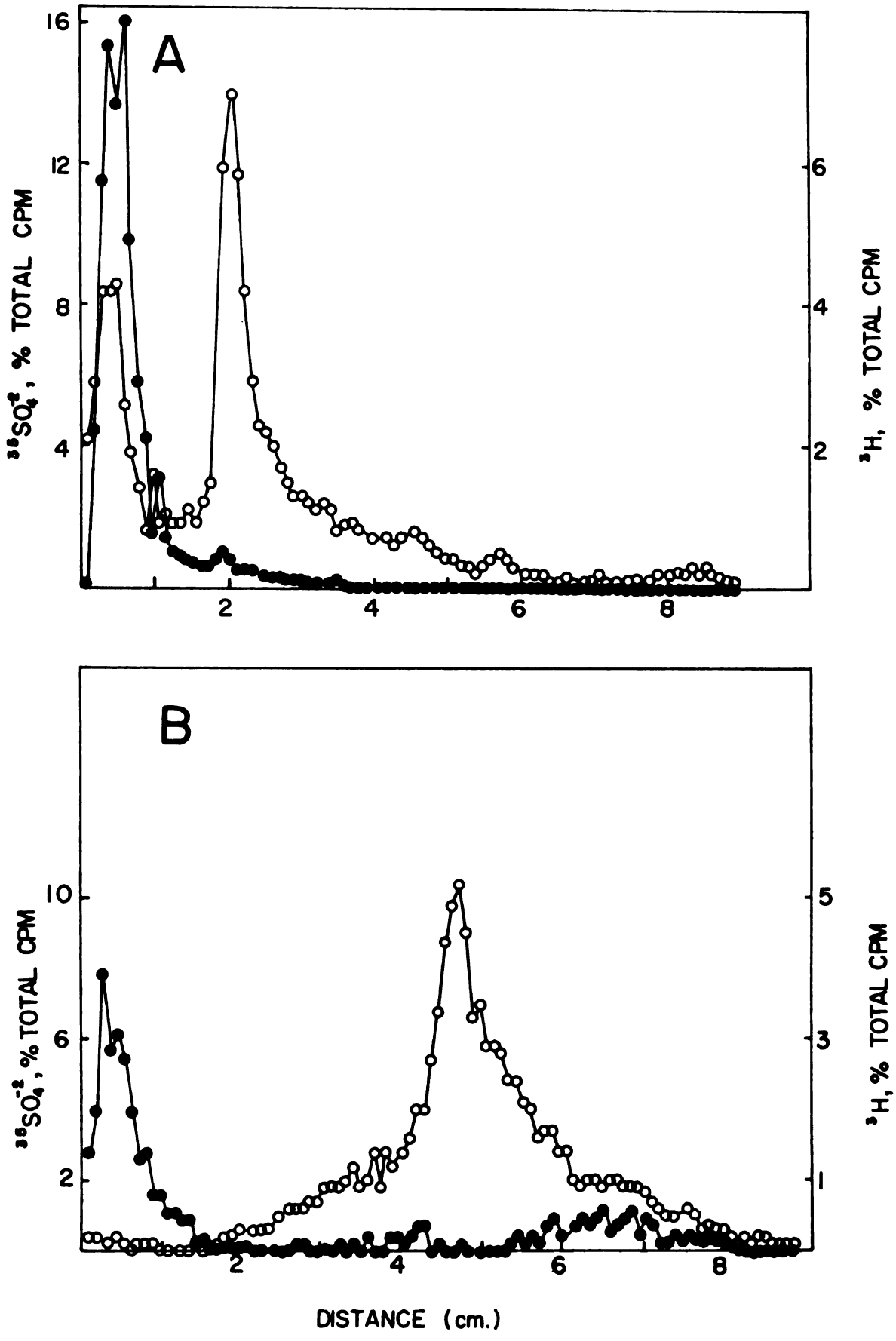


Figure 15

The sulfated macromolecules of zymogen granule lysate also co-migrated with carbohydrate but their association with protein was not yet demonstrated.

Protease Digestion of Sulfate-Labeled Fractions

The previous experiment suggested that sulfate was associated with protein and carbohydrate. This finding would be expected if the sulfated components were glycoproteins or mucopolysaccharides. To distinguish between these two classes of macromolecule, sulfate labeled fractions were extensively digested with papain. The digest was electrophoresed and the gels were fractionated and counted. The results are shown in Figure 16.

In every case the sulfated macromolecules were degraded to heterogeneous components which migrated in the molecular weight range between 60,000 and 15,000. This suggested that the sulfated molecules were glycoproteins since chondroitin sulfate-like molecules were reported to migrate rapidly on gels even in the absence of SDS (Baumgartner et al., 1974).

A gel of ^{35}S-sulfate-labeled, digested zymogen granule membrane was stained for protein with Coomassie Blue R and scanned, while an identical gel was fractionated and counted. A plot of absorbance at 550nm plotted over the sulfate profile in Figure 17 showed that only traces of protein, as measured by Coomassie Blue staining, remained in the area of the sulfate label.

The proteolytic digestion products of zymogen granule membranes were characterized using a dual labeling technique, as shown in Figure 18. To determine if sulfate was covalently linked to protein a

Figure 16.--Electrophoretic analysis of ^{35}S-sulfate-labeled fractions after papain digestion.

Fractions were digested in 1mM Tris-acetate buffer, pH 8.0, containing 2mM EDTA, 5mM cystein, and 0.1mg papain for 48 hours at 37°C. After 24 hours incubation, an additional aliquot of papain and cystein was added. Digests were made 1% in SDS and 2% in β-mercaptoethanol and electrophoresis was carried out under standard conditions on 9% acrylamide gels. Gels were fractionated and counted as described in Methods. The following amounts of protein and radioactivity were present in the sample before digestion: A, zymogen granule membranes, 25µg, 1000 CPM; B, rough microsomal membranes, 381µg, 1500 CPM; C, mitochondrial membranes, 692µg, 1200 CPM; D, zymogen granule lysate, 576µg, 1800 CPM; E, post microsomal supernatant, 444µg, 3100 CPM. The arrow at the right hand side of each profile indicates the position of the tracking dye, pyronin B.

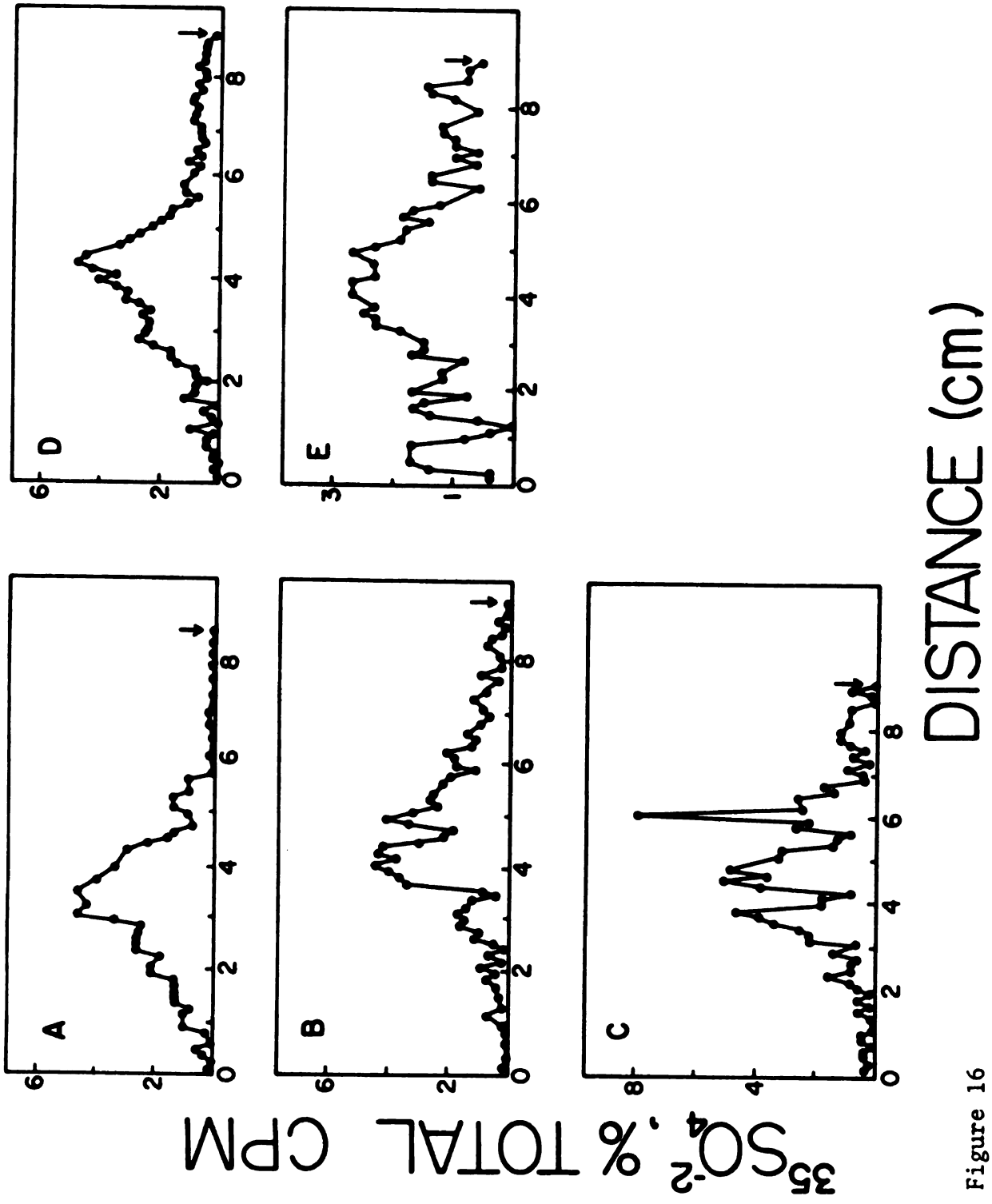


Figure 16

Figure 17.--Polypeptide profile of papain digested, ^{35}S-sulfate-labeled zymogen granule membranes.

Duplicate samples of ^{35}S-labeled zymogen granule membranes, each containing 41µg protein and 2900 CPM, were digested with papain as described in the legend to Figure 16. Samples were electrophoresed on 9% acrylamide gels under standard conditions. One gel was fractionated and counted. The other was stained with Coomassie Blue R and scanned at 500nm, as described in Methods.

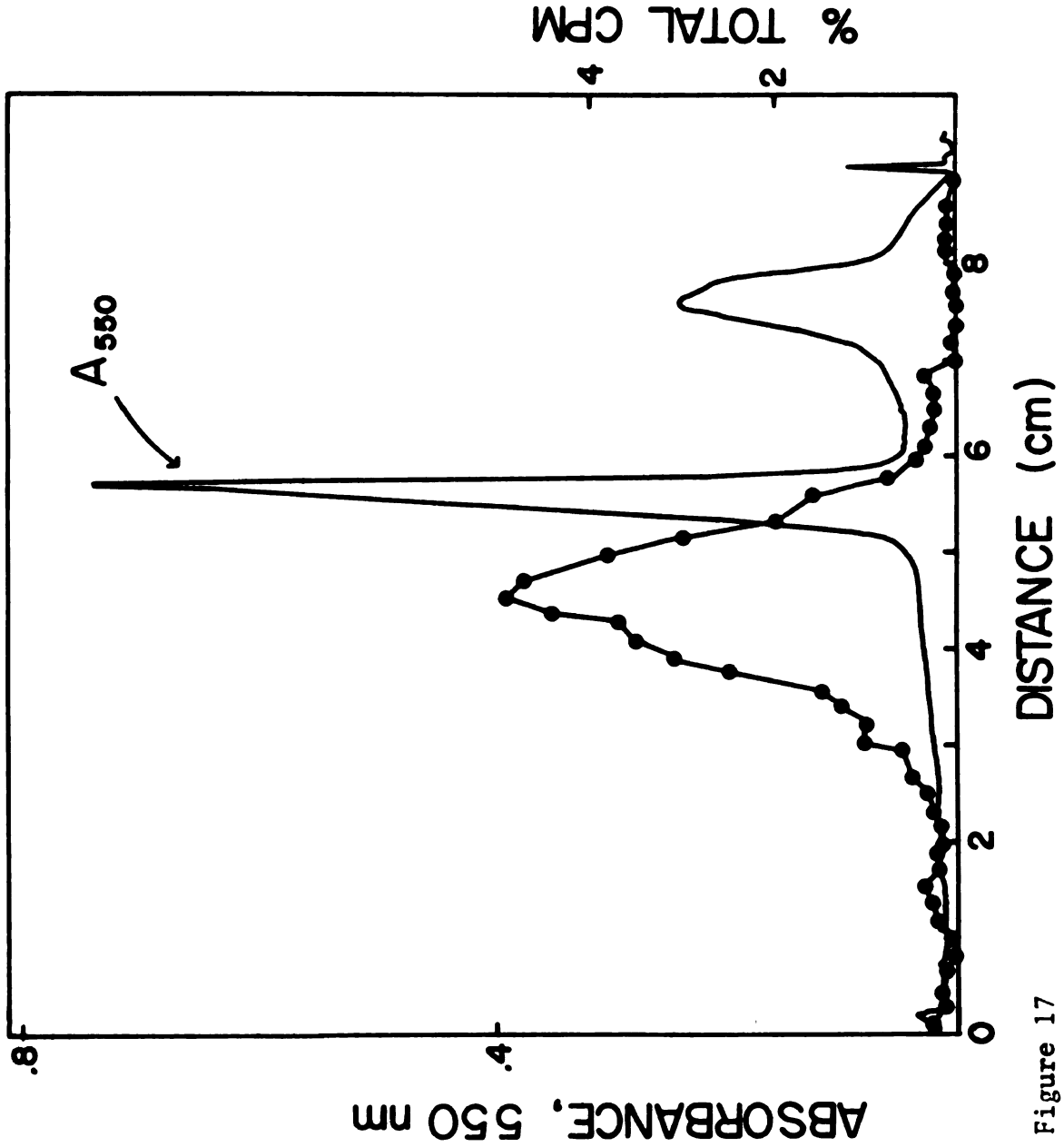


Figure 17

Figure 18.--Electrophoretic analysis of protease-digested zymogen granule membranes labeled with ^{35}S-sulfate, ^{3}H-leucine and ^{3}H-glucosamine.

Labeled membranes were prepared, electrophoresed under standard conditions on SDS-9% acrylamide gels and were fractionated as described in Methods. Papain digestion was carried out as stated in the legend to Figure 16. Pronase digestion was done in 1mM Tris-acetate buffer containing 5mM $CaCl_2$ and 0.2mg pronase for 48 hours at 37°C. The following amounts of protein and radioactivity were applied to the gels:

Left hand panel: ^{3}H-leucine (black line) and ^{35}S-sulfate-labeled (shaded area) zymogen granule membranes before treatment (A) and after treatment (B): ^{35}S-sulfate, 35µg, 2000 CPM; ^{3}H-leucine, 5µg, 2000 CPM.

Right hand panel: ^{3}H-glucosamine (black line) and ^{35}S-sulfate-labeled (shaded area) zymogen granule membranes before treatment (A) and after treatment (B): ^{35}S-sulfate, 30µg, 1000 CPM; ^{3}H-glucosamine, 60µg, 2500 CPM.

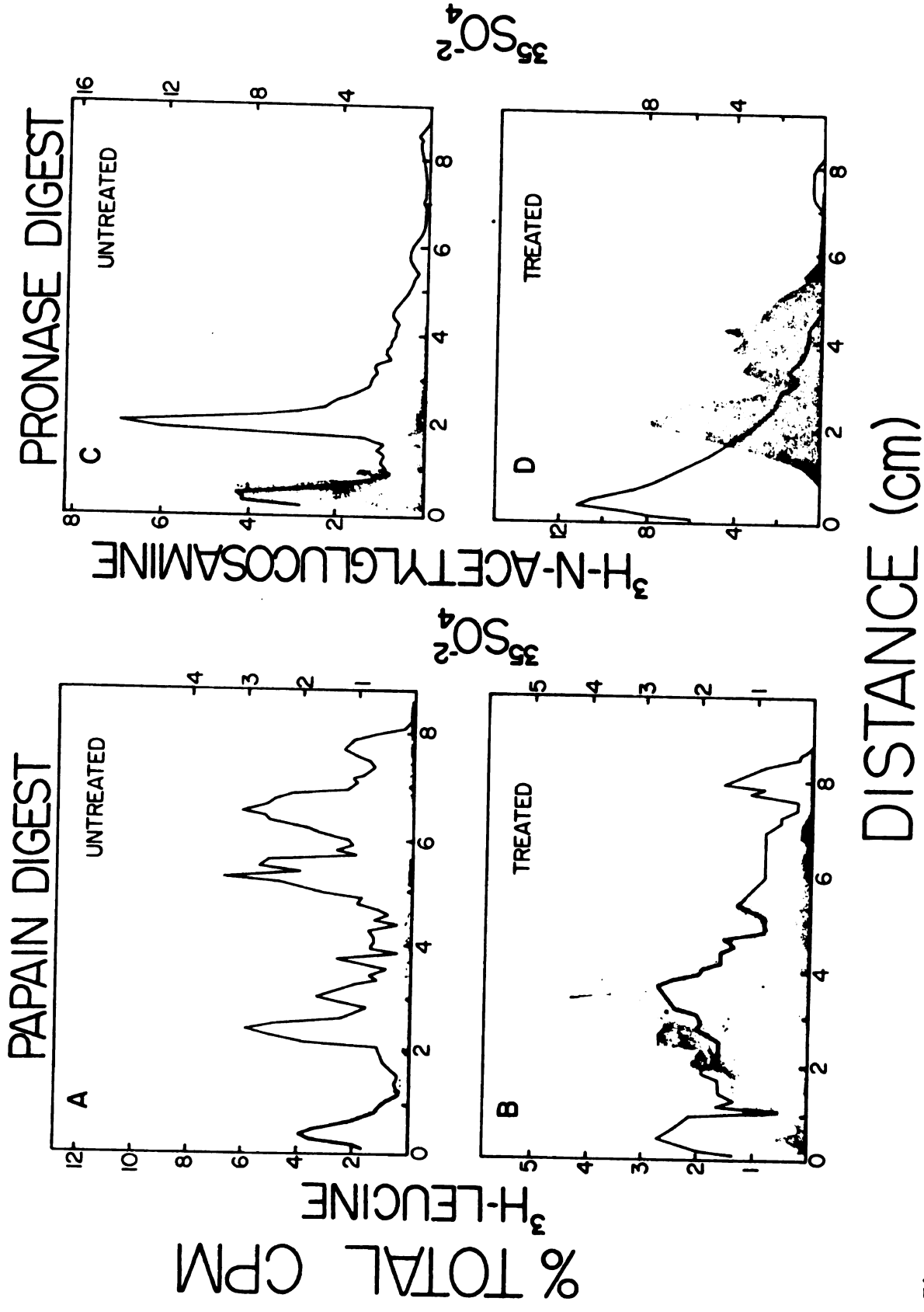

PAPAIN DIGEST
PRONASE DIGEST
A
UNTREATED
B
TREATED
C
UNTREATED
D
TREATED
% TOTAL CPM
^{3}H-LEUCINE
$^{35}SO_4^{-2}$
^{3}H-N-ACETYLGLUCOSAMINE
$^{35}SO_4^{-2}$
DISTANCE (cm)

Figure 18

mixture of ^{3}H-leucine and ^{35}S-sulfate-labeled zymogen granule membranes was digested with papain, as shown in the panel on the left. After digestion, 100% of the sulfate counts were recovered, although they were shifted to a lower molecular weight. In contrast, 50% of the leucine counts migrated off the gel, indicating extensive degradation.

In a similar experiment with ^{3}H-glucosamine and sulfate-labeled zymogen granule membranes, shown in the right hand panel of Figure 18, pronase similarly shifted the molecular weight of the sulfated species. The lower molecular weight glucosamine peak was effectively degraded but a large part of the high molecular weight glucosamine component was resistant indicating that some, but not all of the glucosamine label in the high molecular weight peak was associated with sulfate. These experiments strongly indicate that sulfate is bound to carbohydrate and is also associated with, but not covalently bound to, protein.

Acid Stability of ^{35}S-Sulfate-Labeled Fractions

The acid stability of the sulfate linkage in five subcellular fractions was determined. The O-sulfonate linkage of sulfate esters is more stable to mild acid (Meyer and Schwarz, 1950) than the sulfamide linkage, which is found in only one class of compounds: heparan sulfates. A membrane-bound form of heparan sulfate had been reported (Kraemer, 1971) so the present experiment was done to determine if heparan sulfate was present in pancreas.

The results are presented in Table 9. All fractions except the post microsomal supernatant displayed an acid stability typical of

Table 9.--Acid stability of ^{35}S-labeled fractions.

^{35}S-labeled fractions were incubated in 0.04 M HCl for 60 minutes at 100°C. The percentage of sulfate hydrolyzed was measured as a loss in TCA-PTA precipitable counts compared to an unheated sample.

Fraction	% of Total CPM Liberated in 0.04 M HCl, 100°C, 60 Minutes
Zymogen Granule Lysate	12%
Zymogen Granule Membranes	14%
Mitochondrial Membranes	17%
Rough Microsomes	17%
Post Microsomal Supernatant	37%

O-sulfonate linkages. The hydrolysis measured in the supernatant may reflect a difference in the nature of the hydroxyl group esterified or may indicate the presence of heparan sulfate.

Chondroitinase-ABC Treatment of ^{35}S-Labeled Fractions

Sulfate labeled fractions were treated with Chondroitinase-ABC according to the procedure of Suzuki et al. (1968) to reveal the presence of susceptible mucopolysaccharides: hyaluronic acid, chondroitin A, B, and C, and dermatan sulfate. The activity of the enzyme was verified by its ability to digest authentic chondroitin sulfate.

The results of Chondroitinase-ABC digestion of labeled subcellular fractions are shown in Table 10. No Chondroitinase-sensitive counts were found in any fraction except the incubation medium. The few percentage of sensitive counts seen in other fractions is within

Table 10.--Chondroitinase-ABC treatment of sulfate-labeled fractions.

Sulfate-labeled fractions were lyopholyzed to dryness and were digested with Chondroitinase-ABC from Proteus vulgaris according to the procedure of Suzuki et al. (1968). the reaction mixture contained 10µl of enriched Tris buffer (3 gram of Tris (hydroxymethyl) amino-methane 2.4 gram of sodium acetate, 1.46 gram of NaCl and 50 mg of crystalline bovine serum albumin in 100 ml of 0.13 N HCl, pH 8.0) and 0.1 unit of Chondroitinase-ABC in a total volume of 50µl. Blank mixtures contained no enzyme. Tubes were incubated at 37°C for 90 minutes. The mixture were spotted on Whatman 1 paper strips 5 x 22 inches. The strips were subjected to descending chromatography using 1-butanol: acetic acid:1N ammonia (2:3:1, v/v) and were then cut into 1 cm pieces and counted. The products of Chondroitinase digestion, 4,5-unsaturated disaccharides, migrated from the origin but protein did not.

The percentage of total counts remaining at the origin in the control sample minus the percentage of total counts remaining at the origin in the digested sample gave the percentage of Chondroitinase-sensitive counts in the fraction.

Fraction	Chondroitinase ABC-Sensitive Counts
Zymogen Granule Lysate	7% ± 5
Zymogen Granule Membranes	1% ± 0.5
Mitochondrial Membranes	5% ± 5
Microsomal Membranes	15% ± 5
Post Microsomal Supernatant	7% ± 5
Incubation Medium	37% ± 5

experimental error due to the low number of counts available for study. A small amount of non-dialyzable, sulfate-labeled material was recovered in the medium and 40% of these sulfate counts were digested by Chondroitinase. The origin of this enzyme-sensitive material is uncertain. It is not a zymogen granule secretion product, however, because it is not present in the zymogen granule lysate.

Electrophoretic Analysis of ^{35}S-Sulfate- and ^{3}H-Glucosamine-Labeled Macromolecules Isolated from Incubation Medium

A high molecular weight sulfated macromolecule was present in the zymogen granule lysate. During the course of tissue labeling, zymogens were secreted and, therefore, it was reasonable to assume that a high molecular weight sulfated macromolecule would also be present in the incubation medium. Attempts to demonstrate its presence were unsuccessful. Nine percent SDS-acrylamide gels of the macromolecules in the medium showed a ^{35}S-sulfate profile that appeared degraded, while, at the same time, the polypeptide profile of the medium, obtained by Coomassie Blue staining, looked identical to zymogen granule contents and showed only very minor amounts of the low molecular weight material indicative of minimal proteolysis. These results are illustrated in Figure 19.

Figure 19A shows the polypeptide profile of zymogen granule lysate and Figure 19B shows the position of ^{35}S-sulfate-labeled (closed circles) and ^{3}H-glucosamine-labeled components (open circles) of the zymogen granule lysate. Figure 19C shows the polypeptide profile of the ^{35}S-labeled incubation medium. In the high molecular weight regions of the gel, the polypeptide profile of the medium was

Figure 19.--Electrophoretic analysis of ^{35}S-sulfate- and ^{3}H-glucosamine-labeled macromolecules isolated from incubation medium.

Labeled fractions were electrophoresed and stained or counted as described in Methods. The following amounts of protein and radioactivity were applied: A, zymogen granule lysate, 40µg; B, zymogen granule lysate labeled with ^{35}S-sulfate (●), 412µg, 1700 CPM, and ^{3}H-glucosamine (O), 50µg, 9500 CPM; C, ^{35}S-labeled incubation medium, 55µg; D, ^{35}S-labeled incubation medium, 55µg, 700 CPM, E, ^{3}H-glucosamine labeled incubation medium, 80µg; F, ^{3}H-glucosamine labeled incubation medium, 110µg, 3700 CPM. The position of the tracking dye, pyronin B, is indicated by the right hand spike on the polypeptide scans, and by the last data point on the radioactivity profiles.

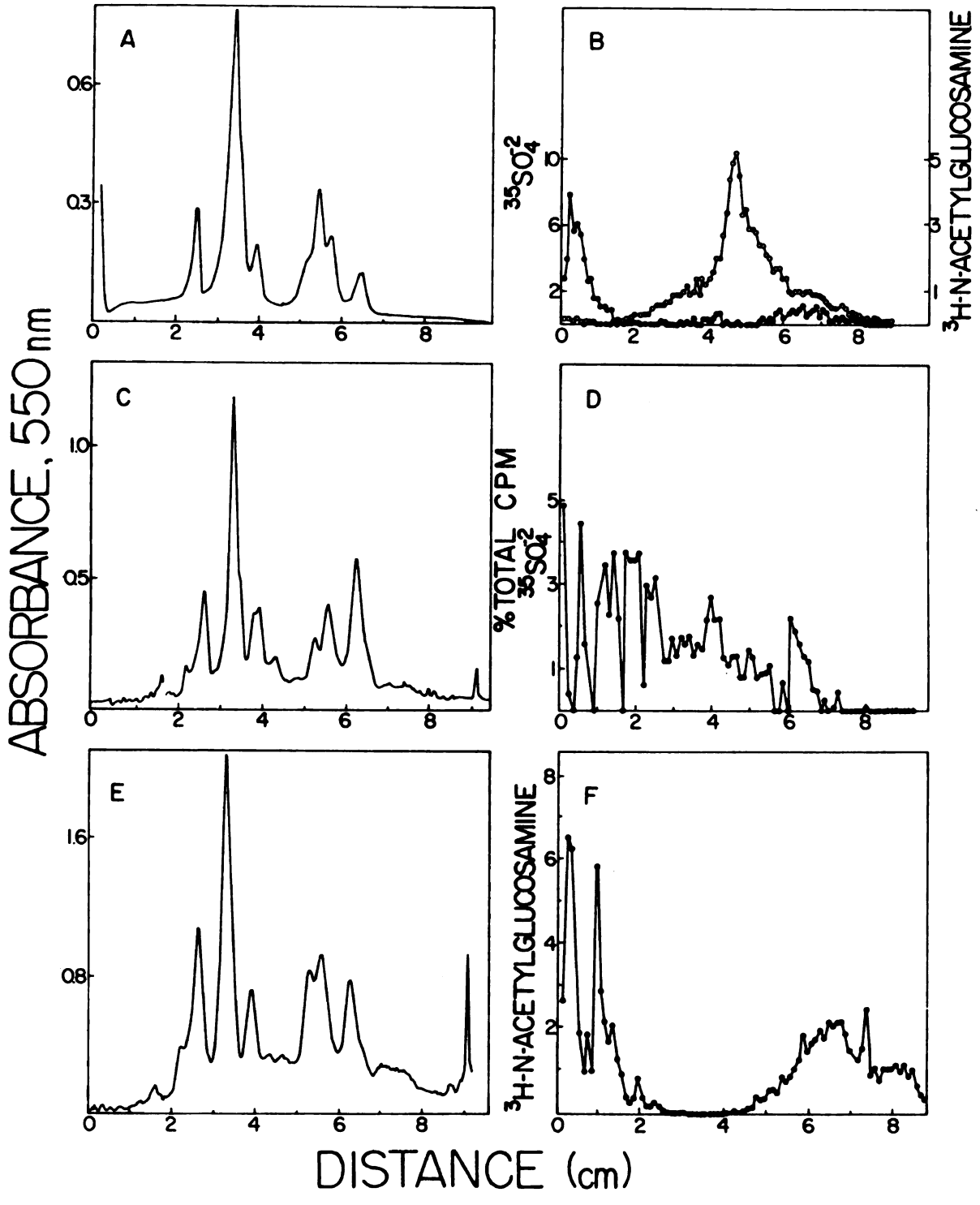

A
B
C
D
E
F
ABSORBANCE, 550 nm
$^{35}SO_4^{-2}$
^{3}H-N-ACETYLGLUCOSAMINE
% TOTAL CPM $^{35}SO_4^{-2}$
DISTANCE (cm)

Figure 19

identical to that of zymogen granule lysate but small differences in the low molecular weight polypeptides were seen. Figure 19D shows the position of ^{35}S-labeled macromolecules in the medium. A comparison of the bound ^{35}S profile of the zymogen granule lysate (Figure 19B, filled circles) and of the incubation medium (Figure 19D) showed that the migration position of the major sulfate containing peak had shifted. The irregularity of the incubation medium ^{35}S profile was due to the dispersion of counts on the gel and reflected degradation of the sample.

To determine if a "sulfatase" in the medium was responsible for degrading the lysate macromolecule, fresh medium from unlabeled tissue was incubated with ^{35}S-labeled zymogen granule lysate for varying times at 37°C. There was no decrease in the specific radioactivity of the lysate, indicating that the sulfate-macromolecule bond was not the site of cleavage.

Figure 19E shows the polypeptide profile of incubation medium from a ^{3}H-glucosamine labeling experiment and Figure 19F shows the ^{3}H-glucosamine profile of the medium. Two interesting observations were noted from a comparison of ^{3}H-glucosamine-labeled macromolecules in the lysate (Figure 19B) and in the incubation medium (Figure 19F). First, there was a high molecular weight glucosamine peak in the medium which was absent from the lysate. This peak may be the secretion product of another cell type or it may have come from damaged cells. Also, the second ^{3}H-glucosamine peak in the medium, which was assumed to be derived from the glucosamine peak of the lysate, was shifted to a lower molecular weight, indicating degradation following secretion.

These data suggest that certain components of the zymogen granule lysate which would logically be expected to appear in the

incubation medium following secretion appeared to be degraded. This finding could be accounted for by the presence of proteases which would have to be selective for only certain polypeptides, since the polypeptide profile of the medium appeared generally to be identical to that of the lysate. Alternatively, glycosidases in the medium may have been acting in a general way on both ^{35}S-sulfate- and ^{3}H-glucosamine-labeled glycopolypeptides.

III. Carbohydrate Analysis of Zymogen Granules from Rat and Dog Pancreas

Carbohydrate Analysis of Rat Zymogen Granules and Granule Subfractions

Quantitative analysis of rat zymogen granule subfractions was carried out using gas-liquid chromatography to determine the carbohydrate composition and the extent of glycosylation of zymogen granule proteins. Glucuronic acid was carefully determined since its presence would indicate that zymogen granules contained mucopolysaccharides.

Carbohydrate analysis of lipid-extracted samples was done as described in Methods. Mannitol was used as the internal standard. The result of a separation of standard sugars commonly found in glycoproteins and mucopolysaccharides is presented in Figure 20.

The carbohydrate composition of rat zymogen granule lysate is presented in Table 11. The lysate contained 0.23% carbohydrate by weight and no detectable glucuronic acid or N-acetylgalactosamine. The practical limit of quantitation of sugars using this gas-liquid chromatographic technique is 0.2-0.4 nM and the limit of detection is slighly lower. A typical separation of carbohydrates in zymogen granule lysate is presented in Figure 21. The high amount of glucose

Figure 20.--Gas-liquid chromatographic separation of standard carbohydrates.

25 nM of each sugar was chromatographed as the O-trimethylsilyl ether of the re-N-acetylated methyl glycoside. Details of the procedure are outlined in Methods. Peaks were identified as follows: fucose 1,2,3; xylose, 4,5; glucuronic acid 6,13; mannose 7,9; galactose 8,10,11; glucose 12,14; mannitol 15,17; N-acetyl-galactosamine 19; N-acetylglucosamine 16,18,20; N-acetyl neuraminic acid 21.

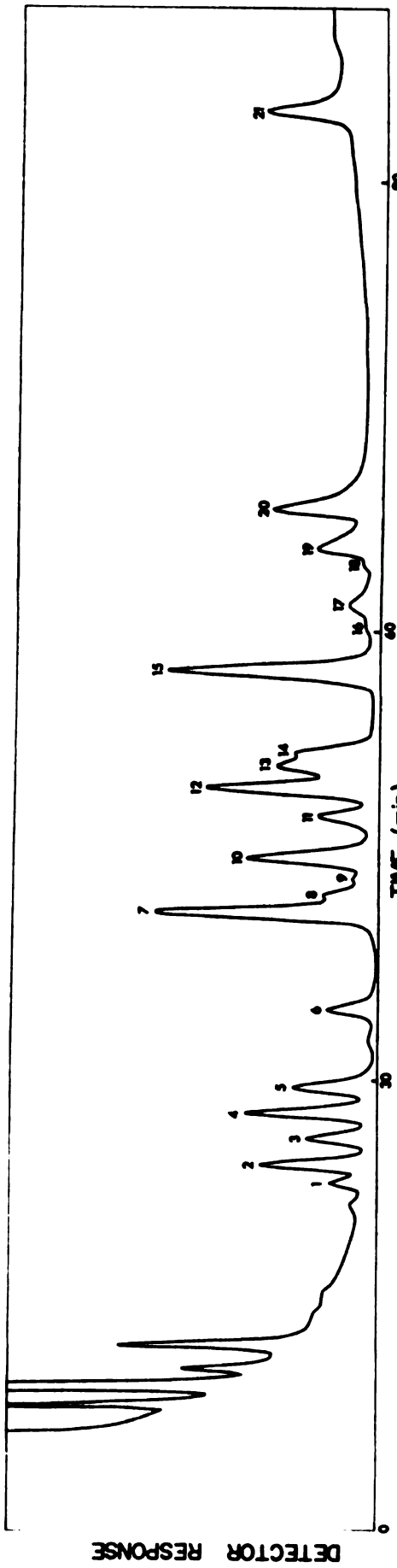

DETECTOR RESPONSE
TIME (min)
0
30
60
90
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21

Figure 20

Table 11.--Carbohydrate composition of rat zymogen granule lysate.

Quantiative gas-liquid chromatography was carried out as described in Methods. Values reported are averages of four separate determinations on a single lysate preparation from 10 adult male rats. SD = standard deviation.

Sugar	nmoles Carbohydrate per Milligram Protein ±SD	Grams Carbohydrate Per 100 Grams Protein	Error
Fucose	2.7 ± 0.5	0.05	19%
Mannose	5.7 ± 0.2	0.10	4%
Galactose	2.6 ± 0.2	0.05	8%
N-Acetylglucosamine	4.0 ± 0.3	0.08	8%
Sialic Acid (as NANA)	1.2 ± 0.2	0.04	16%

Figure 21.--Gas-liquid chromatographic analysis of carbohydrates from rat zymogen granule lysate.

A sample of lipid-extracted zymogen granule lysate containing 387μg of protein and 2 nM of mannitol was hydrolyzed and re-N-acetylated. Carbohydrates were derivatized as O-trimethylsilyl ethers. The sample was chromatographed as described in Methods. Peaks were identified as follows: fucose 1,2,3, mannose 4,4'; galactose 5,6,7; glucose 8,9; mannitol 10,11; N-acetyglucosamine 12; N-acetylneuraminic acid 13. Unidentified contaminants interfered with the direct quantitation of peaks 5 and 7 of galactose and peak 11 of mannitol. The values for these peaks were calculated from experimentally determined isomer peak area ratios and the area of the unaffected galactose and mannitol peaks.

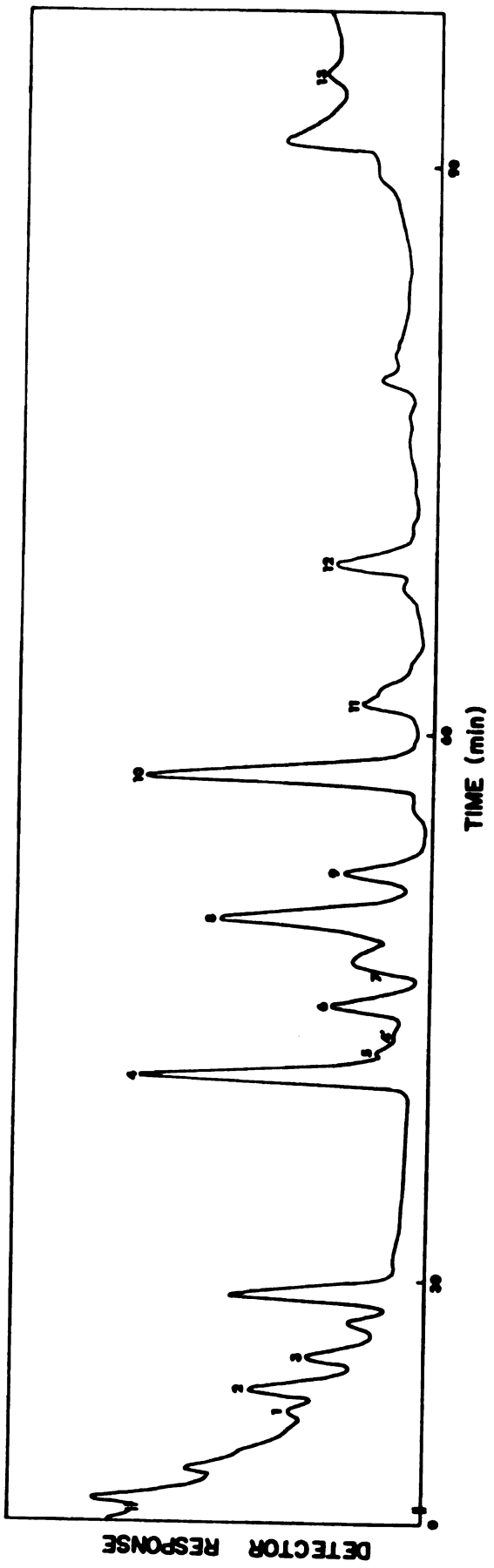


Figure 21

present in the lysate is not reported in Table 11 because it is probably a contaminant from sucrose used in the granule isolation.

In contrast to the lysate, zymogen granule membranes were heavily glycosylated. The carbohydrate composition is presented in Table 12. The membrane protein contained 22.1% carbohydrate by weight. Again, no glucuronic acid or N-acetylgalactosamine were present. Approximately 165 nM of glucose/mg protein was present in the zymogen granule membrane, an amount too high to be due to sucrose contamination in the washed membrane sample. Some portion of the glucose is probably an actual membrane glycoprotein constituent. A typical separation of carbohydrates in zymogen granule membranes is shown in Figure 22.

The carbohydrate composition of intact zymogen granules is presented in Table 13. Zymogen granule proteins were 1.32% carbohydrate by weight. They contained the same types of sugars present in the isolated membrane and lysate.

The NaBr extract of zymogen granule membranes was analyzed because it was suspected that loosely associated glycoproteins as well as adsorbed secretory proteins were being extracted from the zymogen granule membranes. The carbohydrate composition is shown in Table 14. The protein recovered in the NaBr extract contained 2.4% carbohydrate by weight.

Table 15 shows the carbohydrate distribution in subfractions of rat zymogen granules. 16% of the total carbohydrate and 70% of the total protein of intact zymogen granules were present in the lysate. Zymogen granule membrane, though heavily glycosylated, contained only 5% of the total carbohydrate, while 51% of the total carbohydrate and 28% of the total protein were found in the NaBr extract. Proteins in

Table 12.--Carbohydrate composition of rat zymogen granule membranes.

Quantiative gas-liquid chromatography was carried out as described in Methods. Values reported are averages of four separate membrane preparations, each of 30 adult rat pancreata. SD = standard deviation.

Sugar	nmoles Carbohydrate per Milligram Protein ±SD	Grams Carbohydrate per 100 Grams Protein	Error
Fucose	234.4 ± 9.3	4.2	4%
Mannose	236.1 ± 30	4.2	13%
Galactose	311.7 ± 30	5.6	10%
N-Acetylglucosamine	258.2 ± 24	5.4	9%
Sialic Acid	87.7 ± 11	2.7	12%

Figure 22.--Gas-liquid chromatographic analysis of carbohydrates from rat zymogen granule membranes.

A sample of lipid-extracted zymogen granule membranes containing 25μg of protein and 5 nM of mannitol was hydrolyzed and re-N-acetylated. Carbohydrates were derivatized as O-trimethylsilyl ethers. The sample was chromatographed as described in Methods. Peaks were identified as follows: fucose 1,2,3; mannose 4; galactose 5,6,7; glucose 8,9; mannitol 10,11; N-acetylglucosamine 12; N-acetylneuraminic acid 13.

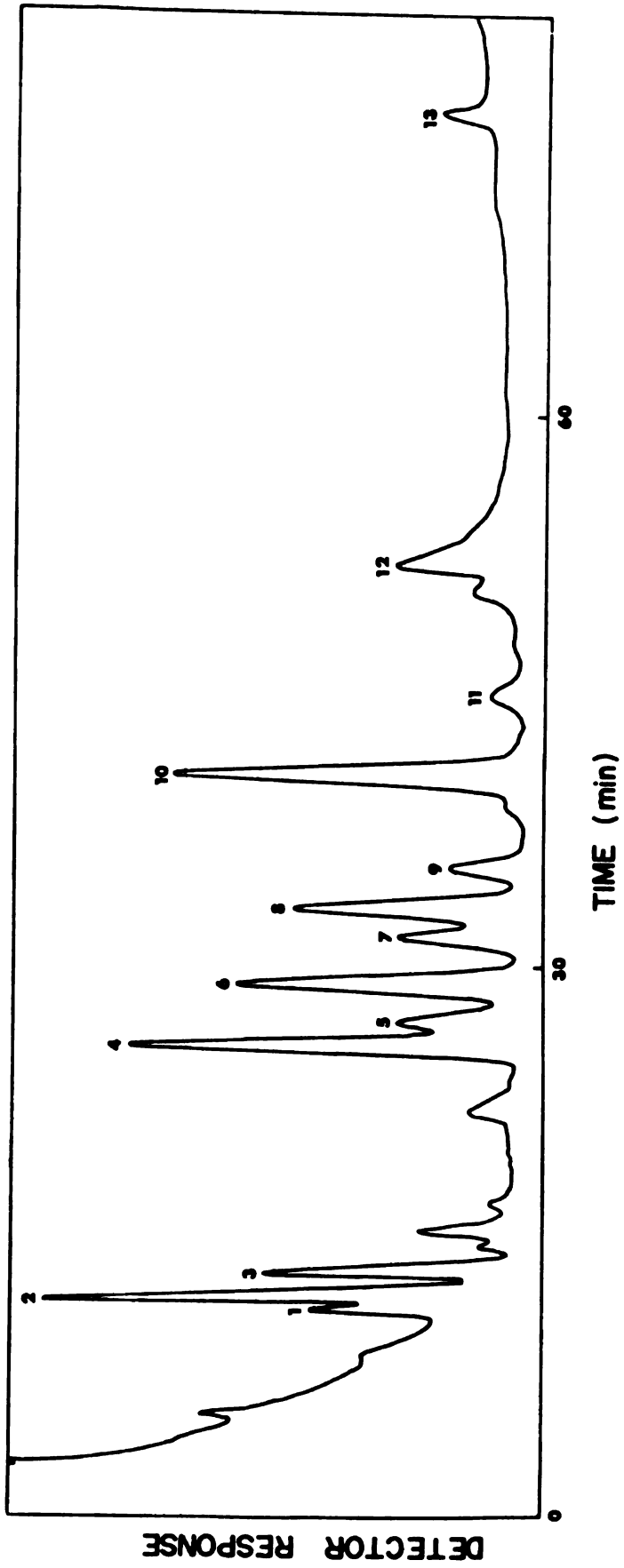


Figure 22

Table 13.--Carbohydrate composition of rat zymogen granules.

Quantitative gas-liquid chromatography was carried out as described in Methods. Values reported are averages of 3 separate granule preparations, each from 5-10 adult rats. SD = standard deviation.

Sugar	nmoles Carbohydrate per Milligram Protein ±SD	Grams Carbohydrate per 100 Grams Protein	Error
Fucose	9.9 ± 1.1	0.06	11%
Mannose	13.3 ± 2.0	0.24	15%
Galactose	12.8 ± 1.7	0.23	13%
Glucuronic Acid	<0.2	. .	. .
N-Acetyglucosamine	24.2 ± 0.6	0.51	2%
N-Acetylgalactosamine	<0.2	. .	. .
Sialic Acid (as NANA)	5.8 ± 1.1	0.18	19%

Table 14.--Carbohydrate composition of NaBr extract of zymogen granule membranes.

Quantitative gas-liquid chromatography was carried out as described in Methods. Values reported are averages from 4 separate determinations on a single preparation from 30 adult rats. SD = standard deviation.

Sugar	nmoles Carbohydrate per Milligram Protein ±SD	Grams Carbohydrate per 100 Grams Protein	Error
Fucose	21.3 ± 1.2	0.35	6%
Mannose	24.5 ± 3.0	0.44	12%
Galactose	33.6 ± 1.8	0.61	6%
N-Acetylglucosamine	34.6 ± 2.1	0.73	6%
Sialic Acid (as NANA)	8.5 ± 0.7	0.26	8%

Table 15.--Carbohydrate distribution in subfractions of rat zymogen granules.

Calculations are based on data presented in Tables 6A, 10, 11, 12, and 13.

Fraction	Total Protein (% of Whole Zymogen Granules)	Carbohydrate Content (Grams Carbohydrate per 100 gm Protein)	Total Carbohydrate (% of Whole Zymogen Granules)	Relative Carbohydrate Content $\frac{\%\ \text{Total Carbohydrate}}{\%\ \text{Total Protein}}$
Zymogen Granules	(100)	1.32	(100)	1
Zymogen Granule Lysate	70	0.32	12	0.2
Zymogen Granule Membrane	0.3	22.1	5	16
NaBr Supernatant	28	2.4	51	2

the NaBr supernatant had electrophoretic mobilities identical to components of the zymogen granule contents, as shown by Coomassie Blue staining on SDS-acrylamide gels. MacDonald (1974) reported that the polypeptides which were solubilized from the membrane by NaBr and which could not be correlated with any secretory proteins represented only minor species.

Calculations indicated that if only adsorbed secretory proteins (which were not heavily glycosylated) were being extracted from the zymogen granule membrane, then the NaBr supernatant would contain only 5% of the total zymogen granule carbohydrate, instead of 51%. The data strongly suggested that membrane-associated proteins present in small quantities but rich in carbohydrate were being removed by the NaBr wash.

Table 16 shows a comparison of the molar ratios of carbohydrate in zymogen granule subfractions relative to fucose. Since fractions contain a heterogeneous mixture of glycoproteins, molar ratios can only indicate gross differences between fractions. It appeared from Table 16 that the zymogen granule membrane and the NaBr extract were quite similar, suggesting that a unique population of glycopolypeptides was not being selectively removed from the membrane in this wash step.

Carbohydrate Analysis of Dog Zymogen Granule Membranes

To verify the results of Ronzio and MacDonald (1972) that zymogen granule membranes from other species of animals also contained glycoproteins, carbohydrate analysis was carried out on dog zymogen granules. Table 17 reports the values obtained from two dogs. Zymogen granule membrane proteins from the first dog (Table 17A) were 16.5%

Table 16.--Molar ratios of carbohydrates in zymogen granule subfractions.

Ratios are calculated relative to fucose = 1 from data presented in Tables 10, 11, 12, and 13.

Sugar	Zymogen Granules	Zymogen Granule Lysate	Zymogen Granule Membranes	NaBr Extract of Zymogen Granule Membranes
Fucose	1	1	1	1
Mannose	1.3	2	1	1
Galactose	1.3	1	1.3	1.6
N-Acetyglucosamine	2.4	1.5	1	1.6
Sialic Acid	0.6	0.4	0.4	0.4

Table 17.--Carbohydrate composition of dog zymogen granule membranes.

Carbohydrate analysis was performed on zymogen granule membranes isolated from two adult male dogs. Quantitative gas-liquid chromatography was carried out as described in Methods. Values reported are averages of three separate determinations. SD = standard deviation.

(A) Sugar	nmoles Carbohydrate per Milligram Protein ± SD	Grams Carbohydrate per 100 Grams Protein	Error
Fucose	279.5 ± 21	4.6	8%
Mannose	239.7 ± 19	4.6	8%
Galactose	232.8 ± 34	4.2	15%
N-Acetylglucosamine	81.2 ± 4	1.7	5%
Sialic Acid (as NANA)	45.7 ± 10	1.4	22%

(B) Sugar	nmoles Carbohydrate per Milligram Protein	Grams Carbohydrate per 100 Grams Protein	Error
Fucose	210.6 ± 8	3.4	4%
Mannose	152.6 ± 3	2.7	2%
Galactose	166.3 ± 12	3.0	7%
N-Acetylgalactosamine	18.5 ± 1.5	0.4	8%
N-Acetylglucosamine	199.0 ± 17	4.2	9%
Sialic Acid (as NANA)	32.0 ± 7	1.0	21%

carbohydrate by weight. Granule membrane proteins from the second dog (Table 17B) were 14.7% carbohydrate. Also, a small amount of N-acetylgalactosamine was detected in the latter sample.

Both dog and rat zymogen granule membranes were heavily glycosylated and they also displayed the same high fucose: sialic acid ratio. An interesting findings was that membranes from individual dogs showed variations in carbohydrate composition. This was not seen in rats since an inbred strain was used and the values obtained were averaged among approximately 30 individual rats per membrane preparation. The individual variation seen in dog membranes suggested that the glycoproteins on zymogen granule membranes might be related to blood group substances.

IV. Assymetrical Distribution of Sialic Acid on Zymogen Granule Membranes

The optimal conditions for stability of zymogen granules were sought since, to study surface components selectively, the integrity of the zymogen granule membrane is essential. The fragility of zymogen granules in solutions containing ions, especially divalent cations, was reported by Burwen and Rothman (1972). They found that granules were stable in distilled water, 0.3 M sucrose, or 0.3 M urea. These findings were confirmed in some initial experiments. Table 18 shows the stability of zymogen granules in various solutions. Buffered glutaraldehyde did not fix the granules before lysis or leakage of contents occurred. Glutaraldehyde fixation in 0.3 M sucrose was also unsuccessful since fixed granules, washed 3 successive times, lost 10-12% of their total protein with each wash. 0.3 M sucrose was

Table 18.--Stability of zymogen granules in ionic and non-ionic media.

Zymogen granules were incubated for times and at temperatures indicated. Granules were sedimented and protein in the pellet and supernatant was measured.

Medium	Temperature	Time	% of Total Protein Released
Glutaraldehyde-fixed granules in 0.05 M Tris-HCl, pH 8.0	37°	30 min	46%
Unfixed granules, 0.05 M Tris-HCl, pH 5.5	37°C	10 min	70%
0.3 M sucrose	20°C	10 min	8%
0.3 M sucrose	27°C	40 min	15%
Distilled water	27°C	40 min	32%

selected as the medium of choice since only 15% of the total granule protein was recovered in the supernatant after 40 minutes at 27°C.

The distribution of sialic acid on the inner and outer surface of zymogen granule membranes was studied. Intact zymogen granules and lysed zymogen granule membranes were incubated with Neuraminidase from Clostridium perfringens. It was assumed that intact granules would have only the outer surface, and lysed zymogen granules both inner and outer surfaces, accessible to enzyme.

After treatment, the intact zymogen granules and the zymogen granule membranes were sedimented and carbohydrate analysis was performed on the pellet. Neuraminidase-treated fractions were compared with control fractions incubated with boiled enzyme or with water and the difference represented the amount of sialic acid enzymatically released.

The commercial Neuraminidase preparation contained 0.002 U of protease activity/mg protein, based on a casein digestion assay. Samples of Neuraminidase-treated and untreated zymogen granule membranes were electrophoresed on SDS-acrylamide gels to check for proteolytic degradation. Figure 23 shows the results of electrophoretic analysis. There was no apparent proteolytic damage to membrane polypeptides after a 40-minute exposure to the enzyme.

Electron microscopy of the zymogen granule membrane pellet shown in Figure 24 revealed the presence of membrane sheets and vesicles.

The results of Neuraminidase treatment of zymogen granules and granule membrane are presented in Table 19. No sialic acid was removed from the surface of the intact granules. In contrast, 50%

Figure 23.--Electrophoretic analysis of Neuraminidase-treated and untreated zymogen granule membranes on SDS-acrylamide gels.

Membranes were electrophoresed under standard conditions on SDS-9% acrylamide gels. Gels were stained with Coomassie Blue R and scanned at 550 nm as described in Methods. The arrow at the right of each scan marks the position of the tracking dye, pyronin B.

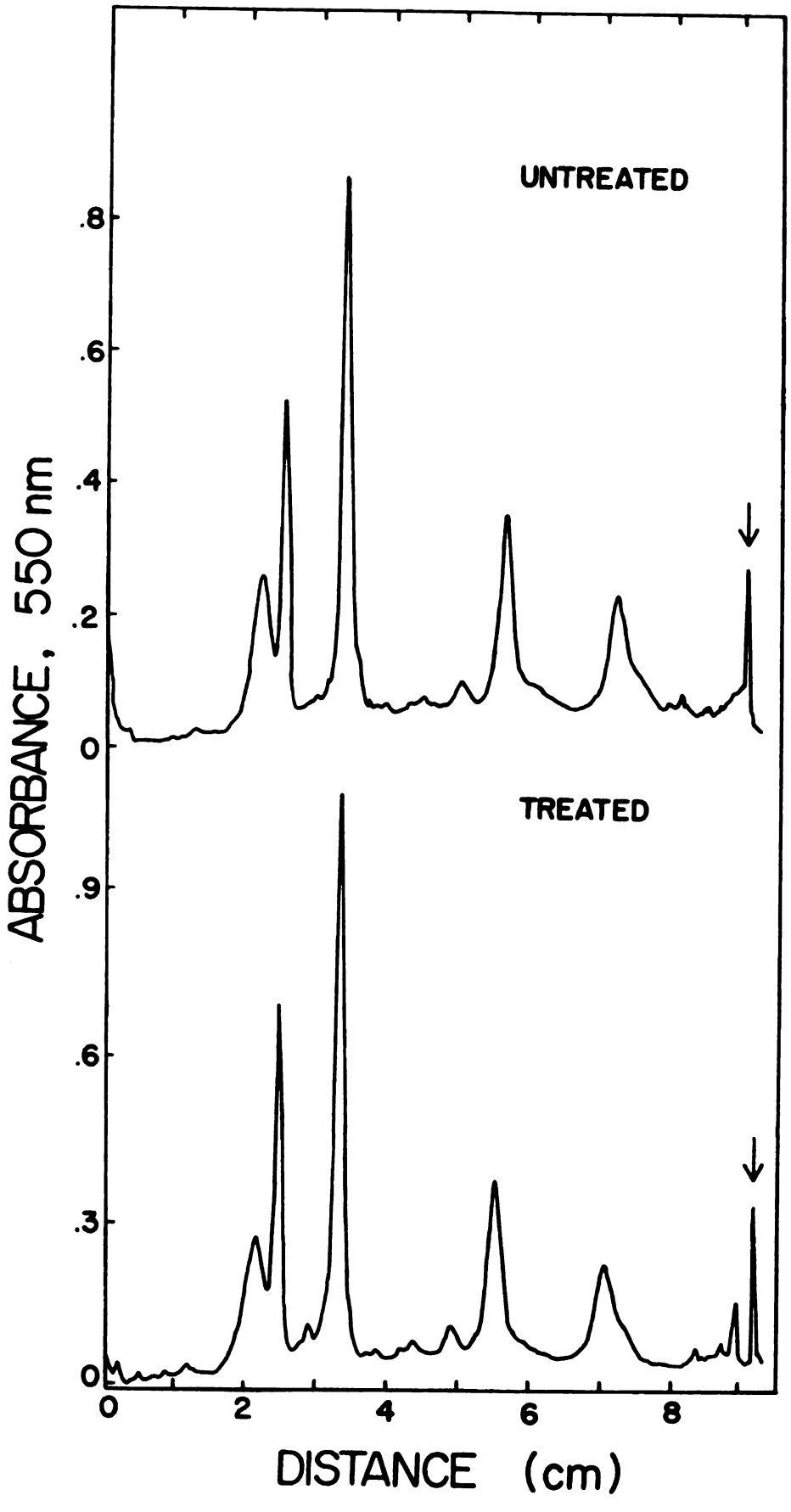

UNTREATED
TREATED
ABSORBANCE, 550 nm
.8
.6
.4
.2
0
.9
.6
.3
0
0
2
4
6
8
DISTANCE (cm)

Figure 24.--Electron microscopy of zymogen granule membranes.

Membrane preparation, fixation, staining and microscopy were performed as described in Methods. Magnification in all figures is 21,000X.

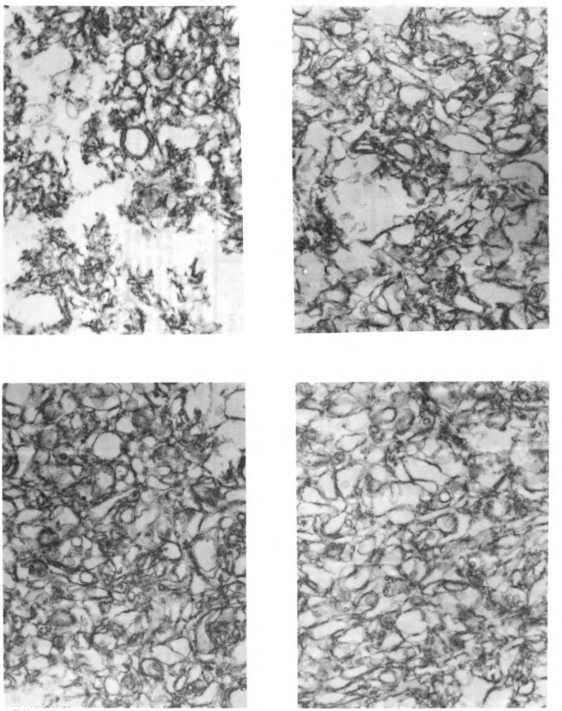


Figure 24

Table 19.--Assymetric distribution of sialic acid on zymogen granule membranes.

Samples containing 5 mg of intact zymogen granules or 0.37 mg of lysed zymogen granule membrane were incubated in 0.3 M sucrose containing 0.001% BHT and 0.064 units of Neuraminidase. Enzyme activity was assayed using bovine submaxillary mucin as substrate. Enough enzyme was present to liberate approximately 30 nM of sialic acid in 40 minutes at 27°C. 5 mg of zymogen granules were calculated to contain a maximum of 28 nM of sialic acid and 0.37 mg of granule membranes a maximum of 33 nM, based on previous determinations of the carbohyrate composition of these fractions. SD = standard deviation. Numbers in parentheses indicate the percentage of error in the carbohydrate composition.

Carbohydrate Composition (nmoles/mg protein) ± SD

Zymogen Granules	Fucose	Mannose	Galactose	Glc NAc	NANA
+ Neuraminidase	9.1 ± 0.3 (3%)	14.1 ± 0.6 (4%)	10.6 ± 1.8 (17%)	11.3 ± 1.3 (12%)	3.3 ± 0.7 (21%)
+ Boiled Enzyme	8.8 ± 1.9 (22%)	13.0 ± 1.3 (10%)	19.0 ± 1.1 (6%)	16.0 ± 0.2 (1%)	3.3 ± 0.9 (30%)
+ H_2O	7.8 ± 0.2 (3%)	12 ± 1.4 (12%)	10.7 ± 1.4 (13%)	7.8 ± 0.1 (1%)	2.9 ± 0.9 (31%)

Zymogen Granule Membranes	Fucose	Mannose	Galactose	Glc NAc	NANA
+ Neuraminidase	143 ± 11 (7%)	130 ± 12 (9%)	175 ± 5 (3%)	152 ± 20 (13%)	13 ± 2.6 (20%)
+ Boiled Enzyme	146 ± 3 (2%)	147 ± 0.5 (1%)	179 ± 2 (1%)	175 ± 5 (3%)	25 ± 3 (13%)
+ H_2O	149 ± 3 (2%)	144 ± 6 (4%)	196 ± 21 (11%)	142 ± 2 (1%)	31 ± 3 (10%)

to 60% was removed from the lysed granule membrane. There are two possible interpretations from these results. The first is that bicarbonate lysis exposed "buried" sialic acid residues on the granule surface; the second that sialic acid was localized on the interior surface of the zymogen granule and was, therefore, inaccessible to enzyme before granule lysis.

DISCUSSION

^{35}S-Sulfate Distribution and Specific Radioactivity of Subcellular Fractions

The results of tissue fractionation indicate that sulfate-labeled macromolecules are present in the membranes of all subcellular organelles studied, and that their presence is not due to binding of labeled molecules from the soluble fraction. It should be noted that while exocrine cells represent 82% to 90% of the total glandular volume of pancreas tissue (Bolender, 1974), the contribution of the other 10% (islet cells, duct cells, and fibroblasts) to the sulfate distribution pattern may be significant, but was not estimated in this study.

Berg and Young (1971) reported that, though they observed scattered silver grains due to $^{35}SO_4^{-2}$ over nuclei, rough endoplasmic reticulum and mitochondria, the grains, when counted and related to the area of the components, demonstrated that labeling of these fractions was negligible. They concluded that the metabolism of sulfate in acinar cells was restricted to the Golgi complex, the condensing vacuoles, and zymogen granules. However, it is likely in an experiment like Berk and Young's, where tissue was essentially exposed to a sulfate pulse, that sulfate would not have been detectable above background in all fractions.

The results of the biochemical study presented here are more complex than those reported in the electron micrographic study. Isolated nuclei were not studied. Rough endoplasmic reticulum, as isolated and characterized by Ronzio (1973a) has the same specific radioactivity as smooth endoplasmic reticulum. The mitochondrial fraction, which contains 14% of the total bound sulfate, may also contain some zymogen granules as contaminants, but the electrophoretic mobility of the sulfate-labeled macromolecules from mitochondrial membrane is distinct from that of zymogen granule membrane on polyacrylamide gel electrophoresis, demonstrating that mitochondria contain unique sulfated components. Heterogeneous sulfated components are also present in the cytoplasmic fraction though their location there in high amounts may be the result of organelle shearing during homogenization.

The origin and significance of the sulfate-labeled material in the zymogen granule contents is controversial. Berg and Young (1971) speculated that it was an acid mucopolysaccharide whose presence might somehow aid in the packaging or release of digestive enzymes. The same hypothesis was put forward by Tartakoff et al. (1974). The latter have isolated secretion products from guinea pig pancreas lobules and find a component which labels with inorganic sulfate. Its chemical nature has not been determined. After isoelectric focusing, this sulfated material ran with the most acidic protein peaks and was isoelectric at a pH near 3.4. A sulfated molecule isolated from guinea pig zymogen granules had the same mobility. The bulk of the secretory proteins, on the other hand, were basic. The charge complimentarity of the sulfated material and the secretory proteins suggested

to these workers a role for the sulfated macromolecules in the concentration of zymogen granule contents.

In purified chromaffin granules (Margolis and Margolis, 1973) and in secretions from adrenal glands (Margolis et al., 1973), a chondroitin-sulfate-like substance was present which was postulated to have a role in the storage and release of biogenic amines. The present study has proven that in rat the sulfated material of the zymogen granule contents is not a mucopolysaccharide.

Sonication of zymogen granule membranes in 0.25 M NaBr releases membrane-associated glycoproteins and bound sulfate. It is possible that the shock of alkaline lysis in 0.2 M sodium bicarbonate also releases membrane-bound sulfate-labeled material and that the sulfated macromolecule seen in the lysate is not a normal constituent of the zymogen granule contents. Indirect evidence supporting this hypothesis is the similarity in electrophoretic mobility of bound sulfate from zymogen granule membranes and zymogen granule lysate on SDS-polyacrylamide gel electrophoresis. Also, when cells are allowed to secrete their zymogen granule contents, a sulfated macromolecule with the same electrophoreic mobility as that of the lysate cannot be recovered in the medium. If a more gentle method of lysis using low salt were found to significantly reduce the amount of sulfated material present in the lysate, the artifactual nature of this soluble constituent would be demonstrated.

The medium used in the present study for all <u>in vitro</u> labeling experiments contains 1.2 mM calcium ion. The secretory activity of pancreas tissue in such medium is extremely high. After 4 hours, 90% of the total amylase is present in the medium. Therefore, the

unusually high specific radioactivity of zymogen granule membranes compared to other intracellular membranes may be caused by the depletion of stored granules due to secretion and the selection of a pool of newly-synthesized and more highly labeled zymogen granules. The reason for such hypersecretion in medium containing high calcium has not been explained but the adventitious phenomenon has proved extremely useful. Yield of granule membrane is so low that a high specific radioactivity is a necessity in this fraction.

Analysis of Sulfate-Labeled Macromolecules on SDS-Polyacrylamide Gels

Renolds and Tanford (1970) demonstrated, using several soluble proteins, that at low ionic strength and pH 7, 1.4 gm of SDS is bound per milligram of protein. The constant charge density resulting from this uniform binding stoicheometry is the basis for the regular relationship between molecular weight and electrophoretic mobility of proteins on SDS gels. Membrane glycoproteins, however, display anomolous behavior. The interaction between SDS and protein is principally hydrophobic (Rosenberg et al., 1969) and membrane polypeptides, which usually contain extensive hydrophobic regions, bind more SDS (Spatz and Strittmatter, 1973) and therefore have a greater electrophoretic mobility than less hydrophobic proteins. Also, the carbohydrate regions of glycoproteins do not bind SDS resulting in an underestimation of their molecular weight (Bretscher, 1971; Russ and Polakora, 1973).

The problem is complicated by the negative charges contributed by sialic acid and sulfate esters associated with sulfated glycoproteins which tend to increase the electrophoretic mobility of these

compounds. Consequently, the molecular weight estimates of sulfated soluble and particulate glycoproteins are subject to considerable uncertainty. In this study, apparent molecular weights have been assigned to sulfated macromolecules on gels but only for purposes of comparison of one sulfated species with another.

The comparison of electrophoretic mobilities of sulfated macromolecules on 9% and 5% gels is difficult to interpret, especially in the soluble fractions, because of the extreme heterogeneity of the sulfated products on 5% gels. With glycoproteins, more accurate molecular weights are obtained on gels of higher acrylamide concentrations since here, the charge of the protein-SDS complex becomes less important than the seiving effect of the gel. The extent of glycosylation and sulfation of the labeled macromolecules may be variable and this may be responsible for their greater heterogeneity on 5% gels.

Carbohydrate Analysis of Biological Samples

The analysis of carbohydrates using the gas-liquid chromatographic technique of Clamp, Bhatti, and Chambers (1972) is an extremely sensitive and useful tool. Accurate quantitation of a complete spectrum of carbohydrates can be obtained from as little as 15 to 20µg of zymogen granule membrane and 300 to 500µg of zymogen granule lysate, the percentage by weight of carbohydrate in these fractions being approximately 20% and 0.3% respectively.

In zymogen granule membranes, variation in the total amount of carbohydrate per mg protein occurs from preparation to preparation. Only the highest values obtained in four separate preparations have been reported. Differences such as the age and sex of the rats used

or slight changes in the preparation procedure may affect the total amount of carbohydrate present in the final zymogen granule pellet. However, the molar ratios of the carbohydrates are quite constant, indicating that the types of glycoproteins present on the membrane are not different.

Characterization of Sulfated Macromolecules in Subcellular Fractions

Zymogen Granule Membranes

The zymogen granule membrane contains a sulfated component of high apparent molecular weight which co-migrates with glucosamine and leucine on SDS-9% acrylamide gels. Proteolysis of this fraction shifts the sulfated macromolecule to a lower molecular weight. No sulfate appears to be esterified directly to protein since no sulfate counts migrate off of the gel after extensive proteolytic degradation. Sulfate and glucosamine counts are coincident on gels both before and after proteolysis, indicating that sulfate is associated with carbohydrate.

The sulfated macromolecule in zymogen granule membrane is stable to mild acid hydrolysis which excludes the presence of sulfamino linkages and, therefore, heparin and heparan sulfate. No Chondroitinase ABC-sensitive counts are present, excluding chondroitin-4-sulfate, chondroitin-6-sulfate, hyaluronic acid, and dermatan sulfate. The results are confirmed by carbohydrate analysis in which no glucuronic acid was detected. The membranes contain fucose, galactose, mannose, N-acetylglucosamine, and sialic acid. Glucose is also present in zymogen granule membrane but may, in part, represent contamination by

sucrose. Sulfatides, which would migrate behind the tracking dye on SDS-acrylamide gels (MacDonald, 1974), are not detected. No experiments have been done which would differentiate between keratan sulfate-like molecules and sulfated glycoproteins. Carbohydrate analysis does not demonstrate a high galactose: N-acetylglucosamine ratio which would be typical of keratan sulfate but this ratio may be obscured by the presence of other glycoproteins in the membrane. Pancreas is a epithelial tissue and keratan sulfate is usually of mesodermal origin. Determination of the molecular weight of the sulfated glycopeptides by column chromatography after extensive proteolysis would definitely distinguish between keratan sulfate and a sulfated glycopeptide.

Zymogen Granule Lysate

A sulfated macromolecule with a high apparent molecular weight is present in the zymogen granule lysate. On SDS gels the sulfated material shows coincidence with N-acetylglucosamine both before and after proteolysis, suggesting that sulfate is associated with carbohydrate. The sulfated component is stable to mild acid hydrolysis and is not sensitive to Chondroitinase-ABC. Carbohydrate analysis does not detect the presence of glucuronic acid in the soluble contents of zymogen granules. The sulfated material present in the lysate may reflect a distinct intracellular form of soluble sulfated glycoprotein or may be an artifact extracted from the zymogen granule membrane.

Mitochondrial and Microsomal Membranes

These fractions contain a component with a high apparent molecular weight which demonstrates a stability to weak acid and to

proteolysis which is similar to that of zymogen granule membranes. The sulfated material in mitochondrial membranes and 90% of that in microsomal membranes is insensitive to Chondroitinase-ABC, indicating that mucopolysaccharides are not present.

Post Microsomal Supernatant

The post microsomal supernatant displays several distinct sulfated species on polyacrylamide gels. The acid stability of this fraction indicates that it may contain some heparin or heparan sulfate. Preliminary carbohydrate analysis of this fraction indicates the presence of 5 to 10 nanomoles of glucuronic acid per milligram of protein. The post microsomal supernatant therefore appears to contain both a sulfated mucopolysaccharide and another type of sulfated macromolecule.

Incubation Medium

The incubation medium contains a sulfate-labeled, non-diliazable macromolecule which appears on SDS gels to be polydisperse, possibly due to degradation. There is no data on the acid stability of this fraction. 40% of the sulfate counts are Chondroitinase-sensitive. The origin of the Chondroitinase-sensitive material is unknown. It is not from the zymogen granule contents, since this fraction is not digested by Chondroitinase.

Physiological Significance

The present study has demonstrated that macromolecules with the properties of sulfated glycoproteins or possibly of keratan sulfate are present, though in small amounts, on many intracellular membranes and

in soluble fractions from rat pancreas. Also present, and complicating the picture in some fractions such as the post microsomal supernatant and the incubation medium, are small amounts of sulfated mucopolysaccharides. The latter molecules may be normal constituents of acinar cells or may be contributed by other pancreatic cell types.

Sulfated glycoproteins are isolated from submaxillary glands, from saliva, gastric juice, gastric mucosa, the intestinal tract (Yosizawa, 1972), and now from pancreas. The organs listed above produce large amounts of digestive enzymes and glycosidases.

Martin et al. (1968), demonstrated in human gastric juice that sulfated glycoproteins inhibited isoagglutination of red blood cells of the ABO (H) and Lewis systems, suggesting a resemblance between blood group substances and sulfated glycoproteins. Both were similar in their lack of mannose, high fucose and low sialic acid content. Moreover, the gastric sulfated glycoproteins inhibited peptic protease.

It is logical that cells which synthesize digestive enzymes might contain inhibitory agents for those enzymes on their cell membranes, especially on the surface of the granule in which they are packaged. If sulfated glycoproteins are localized on the inside of the secretion granule, then they are positioned on the exterior of the cell after granule fusion with the plasma membrane, where they can defend the cell should any digestive enzymes become activated in the lumen. If sulfated glycoproteins are such inhibitory agents, this would explain their presence on many intracellular membranes and in the cytoplasmic fraction.

An alternative role for sulfated glycoproteins can be postulated. The granules of the adrenal medulla (Mathews et al., 1972) and the

neurohypophysis (Poisner and Douglas, 1968) possess a net negative charge on their exterior surface. A significant fraction of this charge has been attributed to external sialic acid residues (Mathews et al., 1972). Assuming that zymogen granules possess a similar anionic surface, it is unlikely that it is contributed by sialic acid, since the position of this sugar on the interior of the zymogen granule was demonstrated. Sulfate on external membrane glycoproteins could account for this negative charge. Such sulfated glycoproteins on secretion granule surfaces could play a role in membrane fusion or as intracellular recognition sites. Knowledge of the interior or exterior localization of the sulfated carbohydrate on the surface of the zymogen granule membrane would give support to one of the above hypotheses. The physiological role of sulfated glycoproteins will, hopefully, become more clearly understood as information about the minor constituents of cellular membranes increases.

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