UCSF
UC San Francisco Electronic Theses and Dissertations

Title
Protein transport in the pancreas

Permalink
https://escholarship.org/uc/item/0k6304w8

Author
Liebow, Charles,

Publication Date
1973

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

PROTEIN TRANSPORT IN THE PANCREAS

by

Dr. Charles Liebow
A.B., University College, New York University, June 1966
D.M.D., Harvard School of Dental Medicine, June 1970

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHYSIOLOGY

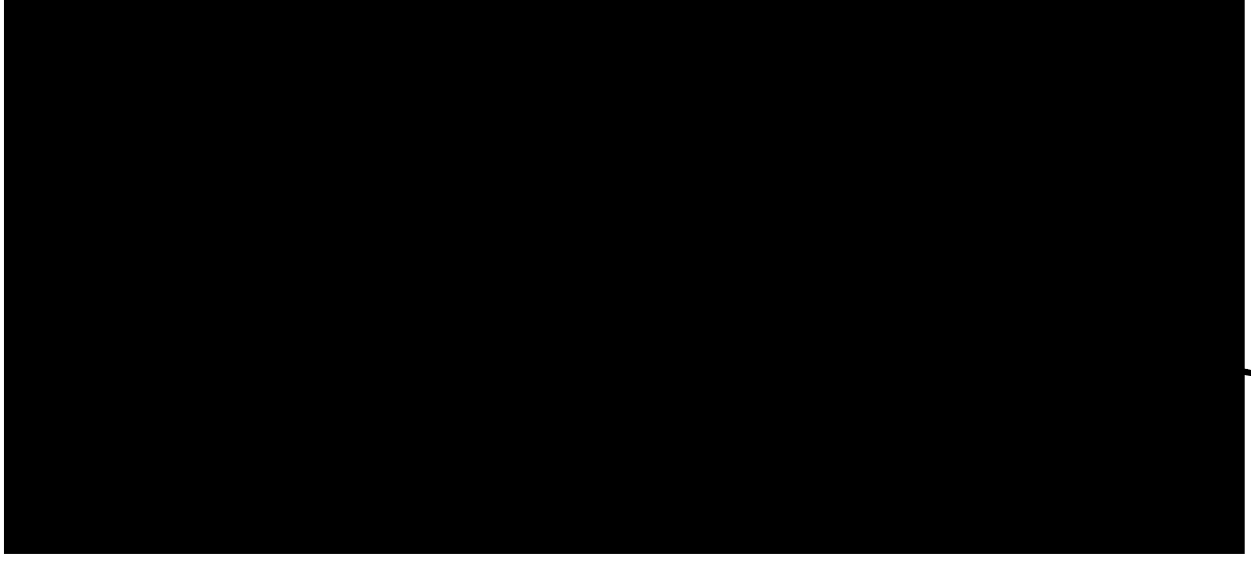
in the

GRADUATE DIVISION

(San Francisco)

of the

UNIVERSITY OF CALIFORNIA



© 1973

CHARLES LIEBOW

ALL RIGHTS RESERVED

DEDICATION

This work must be dedicated to three people:

Raymond Liebow, my father, who instilled in me the willingness and the desire to be skeptical of accepted ideas when facts warranted such skepticism;

Stephen S. Rothman, my scientific father, who not only gave me the tools and the abilities to undertake this work, but more importantly, the inspiration;

Roslyn L. Liebow, my wife, who in spite of personal sacrifices encouraged me to aim only for my highest goals and never to compromise.

ACKNOWLEDGEMENTS

Recognition of credit for other people's contributions to this work must start with Stephen S. Rothman. Not only were the basic ideas for this work born with him, but they grew and matured, as I myself did, under his tutorage. Recognition and thanks for this effort could never be adequately expressed.

Others were also important in the development of these ideas. The collegues I worked with, Susan J. Burwen, Joel Adelson, Michelle Rossier, and Lois Isenman, not only contributed to the background for this thesis, but were ready and helpful testing grounds for the ideas here expressed. My thesis committee members, Walter Stoeckenius and Richard Durbin must be thanked for helping to direct, improved and refine this work. Lastly, I must express my appreciation for the help of Carmen Guillen and Annette Loewe in transforming these ideas into words and pictures.

ABSTRACT

In the introduction, evidence was presented from the literature suggesting that a cytoplasmic route for enzyme secretion might exist. Such a route would require that some membranes are permeable to some secretory enzymes. The aim of this work was to investigate whether such a route actually exists and, if it does, to determine some of its characteristics.

The first approach involved investigation of zymogen granules membrane permeability to secretory enzymes in vitro. Secretory proteins inside granules were in equilibrium with proteins in the bathing media. This was seen by: (1) increased enzyme release from granules in response to increasing suspending volumes; (2) reversibility of this release by reconcentrating suspensions; (3) association of exogenous enzyme with isolated granules; and (4) steady state release of enzyme functionally determined by both time and flow. This equilibrium appears to be mediated by a membrane transport system within the granule membrane, as opposed to nonspecific permeability due to membrane being damaged in the isolation procedure. This was shows by: (1) release specificity not dependent on physical parameters (larger amylase molecules are released more rapidly than smaller chymotrypsinogen molecules); (2) a pronounced temperature sensitivity for release (not consistent with simple diffusion); (3) a slow approach to equilibrium; (4) removal of the delay in approaching equilibirum with removal of the membrane barrier; and (5) in situ granules, known by their in situ state to be intact, equilibrating with cytoplasmic enzyme.

This last demonstration of membrane transport of enzyme, that is, the in situ demonstration, begins the second approach to the investigation of a

cytoplasmic route for secretion. This involves investigation of the uptake of enzyme into cells and into various cellular compartments. This investigation demonstrated enzyme uptake into cells with the distribution of exogenous tritiated chymotrypsinogen paralleling distribution of endogenous enzyme after 60 minute incubation. The apparent equilibration of exogenous and endogenous enzyme in the cytoplasmic and granule pools suggests that entry in response to 1 mg/ml chymotrypsinogen solution is significant in magnitude and comparable to maximally stimulated secretion. Entry appears to occur most rapidly into the cytoplasmic pool, less rapidly into the zymogen granules and very slowly into the microsomes. The serosal border of the acinar cell appears to be at least in the same order of magnitude as permeable as the mucosal border.

The serosal permeability, along with certain observations in the literature, suggested the possibility of enteropancreatic circulation of pancreatic secretory proteins, the final object of investigation in this work. Tritiated α-chymotrypsinogen crossed from the bathing medium to ductal content of the in vitro pancreas at a rate approximately 20 times that of iodinated albumin. This transpancreatic flux of enzyme was increased by addition to the bath of AMeSNI, a mixture of peptides extracted from the hog duodenum containing at least one peptide known to stimulate chymotrypsinogen secretion. Gut sacs also transport chymotrypsinogen with maximal transport apparently occurring in the ileum. This segment of gut displayed a 4:1 $J_{M \cdot S}/J_{S \cdot M}$ flux ratio. Exogenous tritiated α-chymotrypsinogen (Bovine) when injected into rabbit gut appeared in part as intact tritiated α-chymotrypsinogen (Bovine) in pancreatic secretion within 1/2 hour.

The evidence presented suggests that cell and granule membranes are permeable to enzyme and that the cytoplasmic enzyme pool functions with a role in secretion. The extent to which this pathway is utilized remains to be determined. Also, the capability for enteropancreatic circulation of enzyme was demonstrated, although its magnitude, scope, physiological role and relation to enzyme activation remain to be investigated.

CONTENTS

CONTENTS - page 2

Index of Tables and Figures

Figures

Tables

INTRODUCTION

I. Background

In order to fully appreciate current opinion on mechanisms of pancreatic secretion, a certain knowledge of the genesis of concepts of pancreatic function is helpful. This knowledge will aid in understanding why certain models were suggested and from where they derive their strengths.

In 1856 Claud Bernard obstructed the pancreatic duct and thereby prevented pancreatic juice from entering the small intestine. Accompanying the obstruction Bernard noted an impairment of digestion. Through this observation, Bernard postulated that pancreatic secretion is a necessary element for proper digestion of foods. Kühne (1867) noted that pancreatic juices developed proteolytic activity on standing. These observations form the basis from which Heidenhain formulated his theories about pancreatic function and the secretory cycle which provide the ground work for current understanding of pancreatic function.

Heidenhain (1875) in the latter part of the last century, related the zymogen granule to the storage and secretion of pancreatic enzymes. Through microscopic observations, he noted the unique appearance of small round particles, densely packed in the apical portion of the pancreatic acinar cell. These granules though large and numerous in the fasted animal appeared to be fewer in number and smaller in size during feeding. Not only were the size and number of these granules reduced, but their distribution was changed from almost exclusively in the apical portion of the cell, to more randomly distributed

throughout the cell content. From this rearrangement of granule order during the feeding cycle, Heidenhain postulated that these granules must be directly related to the digestive process. He confirmed this hypothesis by removing the pancreas of animals during various phases of the feeding cycle and correlating the proteolytic activity of pancreatic homogenates with the number of granules found in the pancreas at that specific phase of the pancreatic cycle. Furthermore, Heidenhain attempted to deplete the gland of both granules and enzymes in order to show in absolute terms the relationship of enzymes and granules. His initial attempts to deplete the animal by stimulation were unsuccessful until he created a pancreatic fistula and removed pancreatic secretion from the gut completely. Upon doing this, he was able to greatly deplete the gland of both proteolytic activity and zymogen granules, and, therefore, made the relationship that proteolytic enzymes were contained in zymogen granules. Heidenhain postulated, therefore, that zymogen granules served to store proteolytic enzyme, and that a cycle existed of storage and, at feeding time, depletion.

Heidenhain's demonstration of the granule as a storage vesicle opened two broad questions. The first was, how could these storage vesicles release their content into the environment, and the second was, what was the nature of these storage vesicles? The study of the mechanism of discharge of vesicles into the environment was approached using live specimens and utilizing the difference between refractivity of granules and the surrounding medium. Kühne and Lea (1882) and Mathews (1899) studied the movements of granules and their change in

appearance during the secretory cycle. Mathews specifically studied the morphological changes in the acinar cells of this living preparation produced by pilocarpine. This work suggested that pilocarpine stimulation increased migration of intact granules toward the luminal borders and release of material at this luminal border. Covell (1928), described the release of intact granules into the lumen, the process of extrusion. The process of extrusion was the first suggestion of the process known today as exocytosis. This movement of granules with a flow of protein through the cell and final release via an exocytic mechanism provides the basic framework on which later models for pancreatic protein secretory mechanism were founded.

Heidenhain noted that these granules were very basophilic and stained deeply. Later workers noted the greater refractivity of the granules but still did not really know the nature of these vesicles. Noll in 1905 through in vitro studies demonstrated that the granules were membrane bound vesicles containing a proteinaceous material in a colloidal form. Bowen (1929) postulated that granules developed from empty membranous vesicles which took up enzyme from the cytoplasm where it was synthesized. The concept of enzymes being stored within granules and secretion being determined by release of granule stores suggested that secretion could be controlled only via the number of granules secreted and control of specific enzyme secretions could not occur except possibly by changes in synthetic rates.

The controversy over whether control of enzyme secretion could be modulated to enhance secretion of one enzyme over another was, and

still is, a heated debate. Pavlov in 1898 suggested that the pancreas was capable of adapting its secretion of enzyme to the predominant food in the diet. The idea that an organism could adaptively increase function in response to increased demand fell out of vogue in the early part of the 20th century with the advent of Darwin's ideas. Babkin (1950), a student of Pavlov, was an early advocate for the parallel transport concept. Parallel transport means that the proportions of different enzymes of the pancreatic secretion remain the same regardless of stimulation. Stimulation, it was assumed by Babkin, would increase the number of granules discharged but could not discriminate between one enzyme and another. The work of Grossman et al (1942) supported Pavlov's early suggestion of control by demonstrating changes in the pancreatic content (enzyme ratios) in response to changes in diet. These changes, however, required long periods of a dietetic regimem (over three weeks) and were thought to occur due to changes in the synthetic rate for different enzymes. The work of Marchis-Mouren (1963) showed that such adaptive changes in fact do result from changes in the rates of synthesis for the various enzymes involved. They made this demonstration by using labeled amino acids and measuring the biosynthetic rates. This view that all enzymes are transported in the cell uniformly and that no discrimination between enzymes can occur after the synthetic process, has had a great deal of effect on the view of how the pancreatic cell works. Some newer evidence, however, has suggested that this may not be the complete picture of control of protein secretion. This new evidence will be presented a little later in our discussion.

If protein secretion occured via an exocytic mechanism as suggested

above, then enzymes must be contained within the zymogen granules in similar proportions to secretion. Evidence presented by Keller, Cohen, and Neurath (1958) and later supported by Greene, Hirs and Palade (1963) demonstrated such a similarity between zymogen granule content and pancreatic secretion. All digestive proteins found in the secretion were found to be in the granule. Furthermore, similar proportions of enzymes were found both in secretion and in granules. This evidence was used to establish a direct linkage between granules and secretion. This linkage was reinforced by the observation of Greene, et al. that at least 90% of the protein in the zymogen granule fraction could be specifically identified as digestive enzyme product. The existence of intracisternal granules in the guinea pig similarly linked the content of the endoplasmic reticulum cisternae to secretion. This evidence showing electron dense aggregates existing both in the cisternae of the endoplasmic reticulum and in the zymogen granule suggested the possibility of a continuity between these spaces.

Another concept in the genesis of models for enzyme secretory function involves the physical concept of intracellular membranous compartments. Evidence had been presented suggesting that all membranes in the cell are derived from the same source and are connected, directly or indirectly (intermittently). Sjöstrand and Hanzon (1954) presented electron microscopic evidence that zymogen granule membrane is derived from Golgi membrane. Both Palade (1959) and Fawcett (1962) independently advanced models postulating that membrane flows from the rough surfaced endoplasmic reticulum, to the smooth surfaced endoplasmic reticulum, to the Golgi apparatus, to the zymogen granule, to the cell surface and somehow, finally back to rough endoplasmic reticulum. This cycle of membrane flow

was developed from some independent electron microscopic evidence and some morphological considerations linked to developing models for pancreatic function. Newer evidence presented by Meldolesi, Jamieson and Palade (1971a, 1971b, 1971c) and Meldolesi and Cova (1971, 1972) establishes reason to doubt this flow of membrane from compartment to compartment. This concept of flow however, was important in the genesis of the membrane packaging model for enzymatic secretion.

If enzyme localization in the cell could be demonstrated and quantified, then the sequence of entry of newly formed enzyme into pools could be investigated. Intracellular distribution and localization of enzyme was approached in two distinct ways. Methods were developed initially by Hokin (1955) using the process of homogenization and differential centrifugation to separate different cell organelles. This allowed isolation and characterization of different cellular compartments and the investigation of enzymatic distribution. The second and at least equally important tool developed for the study of intracellular function was of course the electron microscope. This along with the technique of autoradiography allowed or at least aided in the direct localization of certain cellular elements in situ.

Hokin, using a process of differential centrifugation to fractionate cells, confirmed Heidenhain's hypothesis that digestive enzymes were concentrated in the granules. He found that the relative specific activity of amylase and lipase was 2 to 3 times greater in his reasonably pure fraction of zymogen granules than in the homogenate as a whole. This concentration of enzyme in the zymogen granule fraction supported the work of

earlier investigators suggesting that the zymogen granule was a storage site for digestive enzymes. The distribution of secretory (digestive enzymes) and non-secretory (e.g. - cytochrome oxidase) enzymes was investigated by Van Lancker and Holtzer (1959) also using the methods of differential centrifugation. They confirmed that the secretory granules contain mostly secretory enzymes, and that the mitochondria and other fractions contain lower specific activity of the secretory enzymes but higher concentrations of enzymes that are considered to be retained intracellularly.

Several groups found varying distributions of different enzymes in different cell fractions depending on the animal used, the enzyme studied and the state of the animal. Variations emphasize the importance of standardizing conditions by fasting animals. Some enzymes were found to be relatively more highly associated with this fraction. In the guinea pig pancreas, approximately 65% of the chymotrypsinogen was found associated with the crude zymogen granule fraction (Siekevitz and Palade, 1958a, 1958b, 1960) but only 30-40% of the RNAase (Siekevitz and Palade), trypsinogen (Siekevitz and Palade and Hansson, 1959) and amylase (Hansson) and as little as 3% of the lipase (Hansson). Species variation was considerable with Hokin (1955) finding less than 15% of the amylase in the zymogen granules in the pigeon pancreas.

Once enzymes, at least proteolytic enzymes, were shown to be predominantly distributed in membranous compartments, and zymogen granules acted as storage depots, then the sequence of pool filling could be investigated. The depletion-recovery cycle that Heidenhain described was investigated by a number of people in order to explore this question. Initial

investigation into the depletion phase were conducted by Laird and Barton (1957, 1958) using the rat pancreas and monitoring amylase secretion. They demonstrated that the zymogen granule pool of stored amylase was the first pool to apparently be diminished by excitation (pilocarpine injection). They showed that other pools (especially the post-microsomal supernatant which contains cell sap) diminished somewhat in size, but this change was seen to occur with a slower time course than was the substantial drop in amylase content in the zymogen granule fraction. This suggested that the zymogen granule fraction was the immediate precursor for secretion.

The recovery phase of this cycle was first studied by Hirsch (1932) using the light microscope to observe the reappearance of vacuoles in different sections of the cell after massive pilocarpine stimulation. He observed the presence of these vacuoles first in the Golgi bodies and later their gradual transformation into the zymogen granules. Daly and Mirsky (1952) depleted pancreata again using pilocarpine injection, and then analyzed the tissue for reappearance of protease, amylase and lipase. They noted that these enzymes all reappeared in roughly parallel fashion. Others have verified the observation that recovery occurs over an extended period of time ranging from six to thirteen hours.

The actual recovery path was best elucidated by following the incorporation of labeled amino acids into newly synthesized proteins. Since the synthesis of exportable proteins is considerably greater than the synthesis of intracellular proteins (Siekevitz and Palade, 1960, and Hansson 1959), it has been estimated that most of the examined incorporation of

labeled amino acids, at least in the early phase, is due to incorporation into secretory proteins. Following the appearance of the secretory proteins by cellular fractionation or by autoradiography provided the basis for the determination of the sequence of pool filling. Siekevitz and Palade gave injections of tritiated amino acids to guinea pigs which had been depleted of enzyme by stimulation via feeding. They then fractionated the pancreata and monitored the levels of specific radioactivity for chymotrypsinogen in different fractions of the cell. Peak specific activity was found first in the ribosomes (between 1 to 3 minutes after the injection), next in the rough endoplasmic reticulum (15 minutes), and finally in the zymogen granules (approximately 45 minutes). This evidence, along with morphological evidence which suggested that the Golgi was the precursor of the zymogen granule (Nassonov 1923, Bowen 1926, Hirsch 1932, Sluiter 1944, Sjöstrand and Hanzon 1954, and Ekholm 1962) and biochemical evidence that microsomal fractions were filled earlier than other fractions of the cell (Siekevitz and Palade 1958b) led to the hypothesis of membranous compartments containing enzymes which flowed sequentially from one compartment to another until it finally was released from the cell via exocytosis.

Other systems were used to corroborate and refine the sequence of movement of protein from one compartment to another. An in vitro system was developed by Hokin where tissue slices could be incubated in amino acid containing media and maintain incorporation of amino acids into proteins. Dickman demonstrated that earliest incorporation was seen to associate with the ribosomes. Jamieson and Palade did a number of experiments following the incorporation of amino acid into protein from the

ribosomes to their extrusion into the lumen of the acinus. They utilized the methods of radioautography to follow proteins from the rough endoplasmic reticulum,to the Golgi, to the condensing vacuoles, to the zymogen granules and finally out of the cell. They fractionated tissue in order to separate the rough endoplasmic reticulum from the smooth endoplasmic reticulum. This separation of smooth from rough endoplasmic reticulum was used to suggest the Golgi's role as a distinct entity in the sequence of label incorporation.

This pattern of enzyme secretion, "the membrane packaging model" suggests that enzyme is synthesized on the attached ribosome, it is then transported through the ribosomal membrane at the point of attachment in some undefined manner into the cisternae of the rough endoplasmic reticulum (Sabatini and Blobel 1970) whereupon the secretory proteins are isolated from the cytoplasm of the cell. The secretory proteins are always isolated from cell cytoplasm and are maintained within some sort of a membranous barrier. This barrier at first exists in the endoplasmic reticulum, later in the Golgi cisternae, then in the transitional elements, and finally in the condensing vacuoles and the zymogen granules. This model finally postulates extrusion of granules via reverse pinocytosis or exocytosis. No whole granules are seen over the lumen, suggesting that granules do not move out of the cell as was thought to be the case from light microscopic observations. Membrane fusion, granule membrane to cell membrane, was thought to occur exposing the content of the granule to the external environment and allowing secretory enzymes to diffuse out. Granule membrane was thought to be incorporated into plasma membrane after exocytosis. The work of Meldolesi (Meldolesi et al. 1971a, 1971b, 1971c,

and Meldolesi and Cova 1971, 1972) and coworkers suggest that each membrane has distinct characteristics and separate definable synthetic rates. This suggests that fusion of the zymogen granule membrane to the plasma membrane must be followed by a process of fission wherein zymogen granule membrane is incorporated back into the cell. This prevents membranes from zymogen granules from mixing with plasma membrane, Golgi membrane from mixing with granular membrane, or microsomal membrane from mixing with Golgi membrane. This observation of the identity of membrane being constant, however, developed well after the membrane packaging model of protein secretion was developed.

The membrane packaging model, with its immediate and absolute isolation of newly synthesized protein within membranous compartments and release via bulk exocytosis of granules, was initially suggested (Palade, Siekevitz and Cova, 1962) to describe pancreatic secretion of proteolytic enzymes. Evidence suggesting significant cytoplasmic pools of other digestive enzyme (e.g. -amylase) led Redman and Hokin (1959) to suggest that other enzymes might follow other routes for secretion. This model, over the years, has been generalized to include all protein secretion from the pancreas (Meldolesi and Cova 1971 and 1972; Jamieson 1972) and salivary glands (Schramm 1972 and Castle et al. 1972) and to describe secretion of a host of hydrophillic macromolecules from many tissues (insulin- Orci et al., 1973, catecholamines-Smith et al. 1973, milk proteins- Linzell and Parker 1971, etc.)

II. Granule Properties

The work of Yasuda and Coons (1966) with immunofluorescent antibodies developed in response to digestive enzymes demonstrated with at least three enzymes, chymotrypsinogen, trypsinogen, and amylase, were found to be present in all acinar cells. These experiments suggest that one acinar cell cannot be responsible for secreting one protein alone. The work of Burwen (1972b) and later confirmed by Jamieson (1972a), goes further to suggest that all secretory enzymes are contained in all secretory granules. Burwen's experiments suggested that granules could release almost their entire content of some enzymes and yet solutions containing such depleted granules did not decrease in turbidity. This indicates that some enzyme must be retained in each granule and no granules could contain solely the enzyme lost. Jamieson came to the same conclusion using antibodies to trypsinogen, coupling these antibodies to ferrous ions to make them electron dense, and showing that these electron dense markers became associated with all granules uniformly. This conclusion that enzymes are distributed uniformly throughout all pancreatic cells and apparently throughou all zymogen granules eliminates the possibility of selective movement of enzyme by preferential release of granules or secretion by certain cells.

Hokin (1955) demonstrated that granules are stable between a pH range of 4.5 and 6.5, but solubilize at pH's over 7.5. The solubilization of granules was accompanied both by loss of turbidity in the solution and by the disappearance of sedimentable protein. Other workers have since carried the study of the in vitro granules a great deal further.

The study of the isolated zymogen granules has led to the belief that the zymogen granule is not merely a membrane-filled sac containing digestive enzymes. Instead, there is reason to believe that the granule possesses specific binding sites for proteins. Evidence suggesting this was presented by Rothman (1971). He noted similar granular properties in response to pH changes noted by Hokin, but in addition, he noted that the release patterns for trypsinogen and amylase were distinctly different from one another. Mere indiscriminate lysis of granules could not explain an ordered release pattern, if as most people thought all enzymes were contained in all granules. This nonparallel release of enzyme from granules showed distinct maximal stability points different for each enzyme, and distinct amounts of enzyme release at specific pH for each enzyme. Rothman (1972) went further to demonstrate that zymogen granules lysed at pH 8.5 were capable, when the pH was readjusted to 5.5, of demonstrating reaggregative behavior. Reaggregation could be quite extensive, yielding up to 95% of the protein in the precipitable form. Since neither digestive juices nor solubilized enzymes display significant pH reassociative characteristics without the presence of granular membrane, it was assumed that the granular membrane provided an associative site required to allow reaggregation. This work, along with the work of Burwen, Rothman (1972a) showing that granules do not demonstrate osmotic fragility either in pure water of 0.3 M urea suggested that secretory enzyme is highly ordered within the granule membrane and may exist as a solid state aggregate. Enzyme in solution within granules would in the presence of pure water produce an osmotic force on the granular membrane which should disrupt a normal membrane. The nature of such binding was further investigated by Rothman

(1972). He added exogenous bovine enzyme, α-chymotrypsinogen and trypsinogen, to membrane and found significant association of these enzymes with the aggregate. This association showed specific maxima for both chymotrypsinogen and trypsinogen in relationship to the amount of membrane present. These maxima of association were, however, not competitive between one enzyme and another. Furthermore, the equilibrium constants and the maxima of both enzymes were very distinct suggesting separate binding for each of the two very similar enzymes. The ratio of trypsinogen and chymotrypsinogen at maximal association was surprisingly very similar to the normal ratio found in endogenous derived granules. This suggested that the binding reflects physiological sites and that these sites in a fasted animal may be maximally associated with the endogenous enzyme.

The work of Burwen (1972b) done in parallel to the present work, sugguests that individual granules can partially release their content. Granules placed in 100 millimolar calcium chloride solution almost completely released amylase and chymotrypsinogen (>95%). Release of total protein and lipase was only about 50%. No change was observed in solution turbidity under these conditions. Since turbidity measurements are most strongly affected by the number of granules present, this suggests that the number of particles in the suspension is not changed even though considerable release is observed. Burwen has further demonstrated (1972b) that this process is reversible by dialysis. Since equilibrium is a state function, returning the pellet to its original state will reestablish the old equilibrium. Dialyzing the solutions to remove the calcium ions completely restores the original distribution of enzyme. Restoration

of enzyme in the pellet supports the view that enzyme is in fact in equilibrium and this equilibrium can be affected by many parameters. Some of the parameters that this equilibrium appears to be dependent upon are ionic strength of the solution, specific nature of the ions (divalent vs. monovalent), and the pH of the solution. Equilibrium, however, was thought to be mediated solely by granular properties and not by its environment. Equilibrium in so-called "stable" granules had not been demonstrated. Estimates of normal cellular pH and ionic concentrations would suggest that granules are not in a stable environment in the cell. This has disturbed investigators who do not believe in the existence of a cytoplasmic pool or in the concept that the granules exist in an equilibrium state with enzyme in their environment, where stability only alters the enzyme distribution, but not the condition of the granule.

III. Cytoplasmic Pool

The cisternal packaging model for enzyme secretion provides for the immediate and complete isolation of digestive enzymes from the cytoplasmic environment. It has been suggested (Palade, et. al.1962) that this protects the cells from many of the dangerous digestive enzymes. It is well known that the digestive enzymes, at least the proteolytic ones, are in the proenzyme form, but some investigators have felt that the potential would still exist for a small initial activation of trypsinogen causing a chain reaction of autocatalysis and activation. Isolation of these digestive enzymes within a protective membrane barrier could yield an extra measure of safety. This concept of complete isolation of digestive enzymes was somewhat difficult to reconcile with the observation that certain enzymes in some species are found primarily in the postmicrosomal supernatant fraction, which contains the contents of the cytoplasm. Hokin (1962) showed that for the pigeon pancreas, up to 85% of the amylase is recovered in the soluble postmicrosomal fraction. Others (Hansson 1959) showed that, in some animals, lipase is also primarily associated with this PMS fraction. All enzymes in all species are found to some degree in the postmicrocomal supernatant. The postmicrosomal supernatant fraction represents the soluble cytoplasmic fraction plus artifact derived from disruption of other fractions. This artifact has been suggested by some (Palade et. al. 1962) to account for all of the postmicrosomal supernatant enzyme. This suggestion that all cytoplasmic enzyme is an artifactual derivative of other pools is hard to reconcile with observations made by a number of investigators (Hansson 1959; Laird and Barton 1957, 1958; Morris and Dickman 1960, and

Siekevitz and Palade 1958a, 1958b, 1970) which show incorporation of label into the postmicrosomal fraction with a time course that cannot be attributed to a constant fraction of other pools'specific radioactivity. That is, if the labeled protein in the postmicrosomal supernatant were purely derived from other organelles, the time course of incorporation into PMS should be some additive function of incorporation into these other organelles. This is not the case (at least not for amylase, lipase and ribonuclease). Selective changes in one or another pool in response to certain stimuli without parallel changes in the PMS is further indirect evidence supporting the existence of a functional cytoplasmic pool. If the cytoplasmic pool were truly artifact, one would suppose that the artifactual derivation of this pool would not be affected by application of definite physiological stimuli. Rothman (1970b) showed that by feeding rats trypsin inhibitors the digestive enzyme contant of the glands rose. The increased content however, was found almost exclusively in the postmicrosomal supernatant fraction and very little change was observed in the zymogen granule. This observation suggests that postmicrosomal supernatant fraction is not derived solely artifactually from zymogen granules.

Earlier in the introduction the ability of the pancreas to adjust its secretion selectively in response to different stimuli was discussed. In review, though Pavlov (1888, 1898) initially thought the pancreas could selectively secrete one enzyme or another as needed, the work of Babkin and others suggested that the pancreas could only adapt its secretory function via changes in the rates of synthesis of different enzymes. This theory of release being controlled only by nonspecific means is no longer

teneble. Rothman (1967, 1970) followed secretion of trypsinogen and chymotrypsinogen in the in vitro pancreas in response to cholecystokinin-pancreozymin (CCK-PZ) (Vitrum). Trypsinogen was the dominant product in response to this stimulation. In fact though trypsinogen increased significantly no significant increase in chymotrypsinogen output was noted. This response was a rapid response and due to the very short time course, could not be attributed to changes in the synthetic pattern. More recently Adelson has purified a duodenal peptide different from all known hormones. This peptide has been shown by Adelson and Rothman (1973) to increase protein secretion by the pancreas both in vivo and in vitro. The increase in protein secretion is again not uniform for all enzymes, with chymotrypsinogen increasing very greatly with no apparent change in lipase. In fact, a majority or perhaps even the total increase in protein secretion may be due to the chymotrypsinogen increase. The existence of a selective stimulant for chymotrypsinogen and a predominantly selective stimulant for trypsinogen (CCK-PZ) begins to provide a mechanism by which the fine control over pancreatic enzyme secretion suggested by Pavlov could operate. Rothman (1973) also showed that changes in both the gut and blood amino acid content (i.e. - lysine) could elicit changes in the ratio of enzymes in secretion. These changes were specific showing both positive and negative feedbacks at different concentrations. This end product control suggests a broader physiological role for regulation of this secretion.

Studies investigating changes (Rothman 1970a) in the subcellular distribution of enzymes in response to CCK-PZ also showed marked non-parallel changes. These studies showed a decrease in trypsinogen content in respect to chymotrypsinogen in the postmicrosomal supernatant at an early time course when no

changes were seen in the zymogen granule enzyme content. The fact that trypsinogen decreased in the postmicrosomal supernatant with a concommittent increase in the trypsinogen secretion from the cell, suggested that a transport process of trypsinogen from cytoplasm to duct lumen must be affected by the CCK-PZ. Even not taking into account the short time course, synthetic patterns could not account for this selective release of trypsinogen, since increased synthesis of trypsinogen needed for increased secretion would concommittently produce an increase in all pools of trypsinogen. The decreased pool in the PMS therefore not only rules out zymogen granule origin but also synthetic rate changes being the sole means for altering enzyme ratios in secretion.

Other experiments by Rothman (1967) using methacholine chloride as a stimulant showed that increased protein and enzymatic output could not be accounted for by changes in the zymogen granule fraction alone. However, when changes in the zymogen granule and postmicrosomal supernatant fractions were monitored, these could fully account for the increased secretion. In another observation, Wells and Rothman (1969) showed increased trypsinogen secretion in response to feeding of trypsin inhibitor. In chronic studies using the rat (Rothman 1970), trypsinogen secretion was greatly enhanced, as was total enzyme content of the pancreas, while trypsinogen content in the gland was, at the most, slightly increased. The observed selected increase in trypsinogen secretion along with the decrease in ratio of trypsinogen : total enzyme in the gland, is another example of an inverse change in the ratio of enzymes in the gland and secretion. This suggests that the increased secretion is mediated by a transport process which lowers cellular concentration of enzyme in

response to increased secretion. This demonstration of non-parallel transport of different enzymes in light of the observations that all cells (Yasuda and Coons 1966) and all granules (Burwen, S. 1972b and Jamieson et al. 1972) contain all digestive enzymes makes it very difficult to reconcile selective secretion with all enzymes being secreted via a non-discriminating exocytic mechanism.

The above evidence for selective secretion of one enzyme in preference to others suggests that the cell has the ability to discriminate at the secretory step (post-synthesis) between different enzymes in response to physiological stimuli. Other work by Rothman and Isenman (1973) suggests that a different kind of non-parallel secretion occurs. The above evidence suggests that the cell can selectively secrete one enzyme in preference to another given a certain stimulus. Rothman and Isenman present evidence that in response to a specific stimuli the cell can selectively secrete older enzyme previously sequestered in storage. They showed that shortly after introduction of labeled amino acids into the incubating medium, the specific radioactivity of the postmicrosomal supernatant fraction of the cell was considerably greater than of the zymogen granule fraction, and, furthermore, the specific radioactivity of secretion more closely approximated the postmicrosomal than the granular fraction. This suggests that the immediate precursor of secretion is the postmicrosomal supernatant pool. Furthermore, when a secretory stimulus is applied, the specific radioactivity of amylase in secretion decreases abruptly more closely approaching zymogen granule S.R.A., suggesting that during stimulation the granular pool is a more significant contributory source for secretion. Furthermore, when a cholinergic stimulus was

applied (as opposed to cholecystokinin-pancreozymin), not only was the S.R.A. of secretion decreased, but there was an absolute decrease of label in secretion. An explanation for this observation was that not only does cholinergic stimulation elicit secretion of stored enzyme (similarly to cholecystokinin-pancreozymin), but it also produces competition between old and new enzyme for the secretory pathway. Observations that the cellular source for secretion of one enzyme (i.e. -amylase) can vary under normal physiological control is another independent demonstration that more than one route exists for enzyme secretion.

IV. Aim of the Study.

A model has been presented in the preceding introduction postulating that secretory enzymes are, directly following synthesis, completely isolated from the cytoplasm within membranous compartments and secreted indiscriminantly via exocytosis. Though some have suggested that this intracisternal pathway is the unique route for secretion of protein from the pancreas and possibly other secretory glands, the capability of the pancreas to respond in the manner described above suggests that at least some other pathway for secretion must exist. Reasons for believing that this other pathway involves transit of enzyme through a cytoplasmic pool was also described above. An alternate pathway, as shown in figure 1, involves transit of enzyme directly across membranes, at least plasma membrane. If secretory enzymes do move across membranes, direct observation of such movement should be possible. Such a membrane permeability for secretory enzymes should mediate an equilibrium state on the two sides of the membrane. If such an equilibrium does exist, disturbing this equilibrium should cause a redistribution between enzyme pools. Investigation of the permeability of different secretory membranes to digestive enzymes is a major aim of this work. This will be performed both by directly measuring membrane permeability and by examining redistribution of enzyme in response to disturbances of the enzyme equilibrium. The membranes to be investigated are primarily the zymogen granule membrane and the plasma membrane. Also to be investigated is the possibility that other membranes are permeable to secretory enzymes and that their permeability is contributory to the secretory process. These membranes include the serosal membrane of the pancreas and the membranes present in the gut.

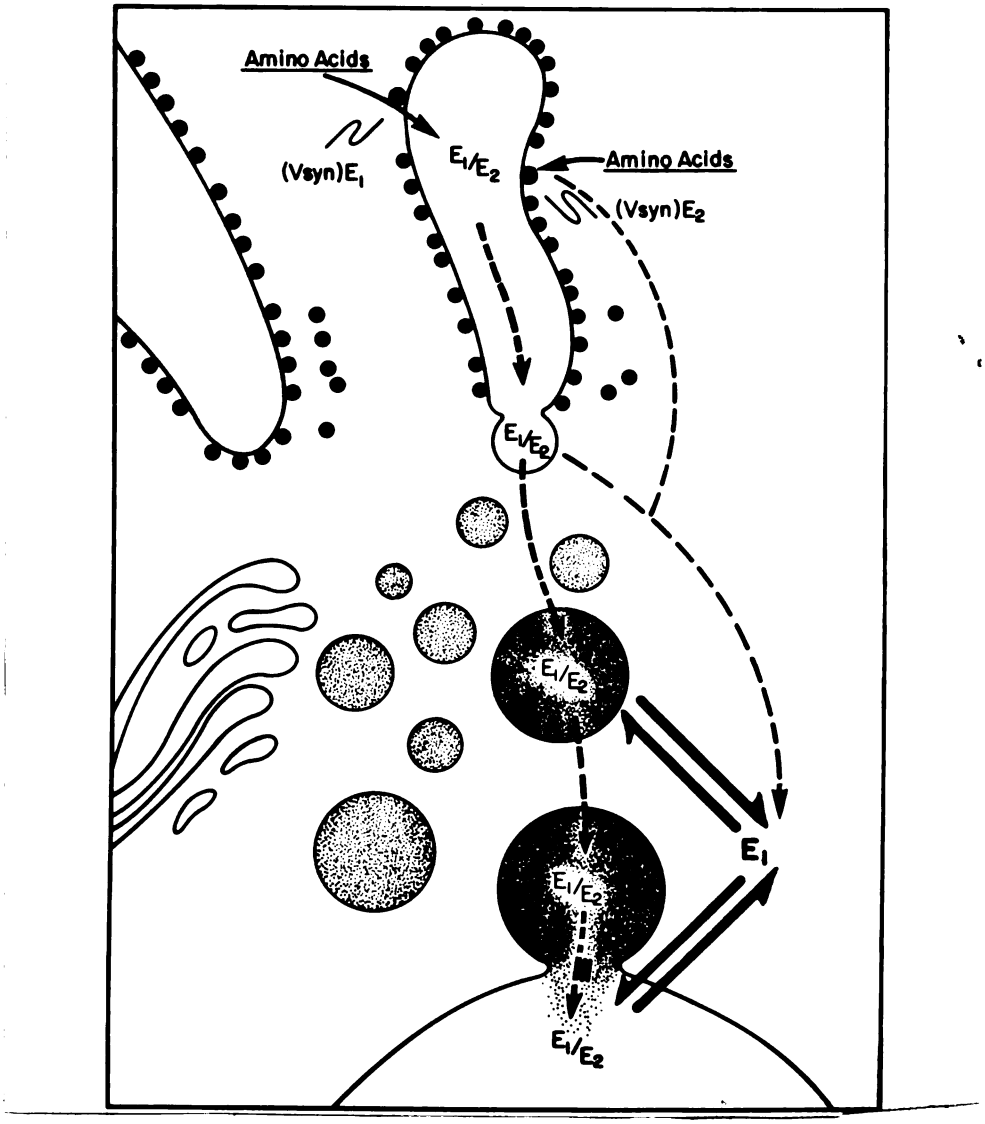


Figure 1. A schematic diagram depicting potential routes for protein secretion from the pancreatic acinar cell. Shown going from top to bottom is the rough surfaced endoplasmic reticulum (RER) on which protein synthesis is thought to occur. This synthesis is either, as depicted for E_1, coupled to translocation into the cisternae of the RER or, (E_2) independent of translocation with enzyme either staying in the cytoplasm, moving from cytoplasm into RER cisternae or moving into some other pool. Each enzyme possesses an independent synthetic rate. According to the cisternal packaging model enzyme moves sequentially to the smooth ER, the zymogen granule, and finally through exocytosis out of the cell. The ratio of enzyme (E_1/E_2) would be fixed by the synthetic rate and would not vary throughout its course. The other potential route involves enzyme being released either from the ribosomes, the RER, the SER or the zymogen granule into the cytoplasm and then into granules or directly our of the cell. This route would allow for varying enzyme ratios but would require exchange of enzyme across granule or cell membranes (shown by heavy equilibrium arrows). This exchange is the subject for study in this thesis.

METHODS

I. General Assay Procedures

Protein was estimated by use of the Folin phenol reagent (Lowry et. al. 1951) using a standard curve made with Bovine albumin. Up to 0.4 ml of sample were used containing less than 0.1 milligram protein. To this, 2 ml of a freshly made mixture of 50 ml 2% Na_2CO_3 in 0.1 N NaOH plus 1 ml of 0.5% $CuSO_4 \cdot 5 H_2O$ and 1% N_a- K tartrate was added. This mixture was shaken and then allowed to stand for between 10 to 15 minutes. After this time 0.1 ml of 2 N phenol reagent was added. This fresh mixture was again shaken and allowed to stand for between 30 to 60 minutes and then read in the spectrophotometer at 750 nm.

Chymotrypsinogen was estimated from the esterase activity of the activated samples in N-acetyl-L-tyrosine ethyl ester (ATEe) solutions. Samples were activated by adding 0.5 ml of a solution of 40 mg enteropeptidase (formerly called enterokinase EC 3348) (Cal. Biochem Grade B) in 100 ml 0.1 M sodium buffer pH 7.4 to 1 ml of an appropriate dilution of sample in the same buffer. This mixture was incubated for 30 minutes in a 37°C water bath. The concentration of enteropeptidase used and the incubation time were found to give optimal activation (Rothman 1970a). The enteropeptidase treated by itself in a similar fashion showed no esterolytic activity. After activation, this total sample was added to 3 ml of a solution of 0.08 M ATEe in 30% methanol. This mixture was placed in a pHstat device whose reaction chamber was maintained at 25°C. The initial rate of hydrolysis was measured by titrating the acid produced with either 0.10 N or 0.02 N NaOH to maintain the pH constant at 7.8. From 0.01 to

0.5 mg of protein were present in the sample. Titrations were initially found to be linear, indicating that the reaction was not substrate limited. Successive dilutions of a sample showed proportionate decrease in the activity recorded and when sample size was plotted against activity an intercept of 0 was found. This confirms the results of others (Rothman 1967), suggesting that neither a reversible or irreversible inhibitor is present in any quantity in these granules. The chymotrypsinogen activity is defined as the total enteropeptidase activatable ATEe esterase activity of the sample. This activity is primarily due to the various chymotrypsinogens present in the cell. The activity is expressed as micromoles substrate split (at 25°C) per milligram protein per minute. Virtually no activity was found in the unactivated mixture, confirming it to be in the proenzyme form.

α-Amylase (α-1, 4 glucan-4-glucano hydrolase, EC 3211) activity was measured by hydrolysis of a starch substrate labeled covalently with Remazol brilliant Blue R (trade name Amylase Azure, Cal Biochem Grade B). Supposedly for each α bond hydrolyzed one dye molecule is liberated into the solution (Rinderknecht 1967). The assay was carried out using the following procedure. 0.5 Ml of an appropriate dilution of sample in 0.1 M sodium phosphate buffer pH 7.4 was added to 4.5 ml of a 2% suspension of Amylase Azure in 0.02 M sodium phosphate buffer pH 7.4 containing 0.05 M NaCl. Care must be taken to keep the suspension uniform since the large starch sediments rapidly. The samples were stirred rapidly by a magnetic stirrer and removed by an automatic syringe. The first few samples from the automatic syringe were returned to the general bath in order to insure uniform sampling. The reaction was terminated after 15 minutes by the addition of

2 ml of 1 N acetic acid. Samples were removed from the waterbath and centrifuged at 20,000 x G_{av} for 15 minutes. This removed the suspended starch. Samples were then carefully transferred to cuvettes with the use of Pasteur pipettes, leaving the bottom layer of liquid untouched in order to prevent disturbing the sedimented dye. The supernatants were then read at 595 nm. These readings were compared to a standard curve. The amount of protein per sample was approximately 0.1 - 0.01 milligrams protein. Samples containing less protein than this amount were incubated for 1/2 hour in 2.25 ml of starch suspension. This increased the sensitivity of the reaction approximately four fold. These results were again compared to a standard curve. Absorbence using both methods was determined to be linearly related to the concentration of amylase in the sample.

Radiation counting was performed using a liquid scintilation counter. Samples were placed in solution in a toluene based mixture containing fluors (Beckman Fluoralloy TLA). Liquid samples were solubilized using Biosolv BBS 3 (Beckman). 1 Ml BBS 3 was added to samples of 1/2 ml or less. 2 Ml of BBS 3 was added to 1 ml liquid samples. This mixture of BBS 3 and sample was shaken in the counting vial. 18 Ml of the toluene fluor mixture was added to the scintilation vial and the vial was then shaken thoroughly. Tissue samples were solubilized in NCS tissue solubilizer (Amersham Searle). Tissue samples of 50 milligrams or less were solubilized in 1 ml of NCS by incubating these samples at 50°C overnight. Such samples then had 18 ml of toluene fluor mixture added to them. Care was taken to cool NCS solubilization products prior to adding the toluene fluor mixture. Gel slices were similarly solubilized using 2 ml of 1 to 10 dilutions of NCS to approximately 0.01 cc of gel. The gel had previously been crushed in order to increase surface area as much as possible.

Counting samples were similarly incubated at 50°C in order to produce solubilization. These samples had external standard ratios between 0.65 and 0.70. Tritium efficiency in this range was between 35 and 40%. ^{131}I showed virtually no change in efficiency over this quench range. ^{14}C showed a very small change in efficiency over this quench region. Due to the small variation in external standard ratios it was possible to directly use counts per minute without a conversion to DPM. All samples were compared to standard samples treated in similar fashion. Separation of different isotopes was performed using variable channel potentiometers. Cross over from tritium to iodine was virtually 0 whereas cross over from iodine to tritium produced counts in the range of 10% of ^{131}I. Tritium was used in all experiments in greater concentration than iodine in order to minimize the effect of this 10% cross over from iodine. ^{3}H bath concentrations were between 5 and 10 fold greater than ^{131}I. This could produce a maximum increase in ^{3}H counts of 2% due to ^{131}I cross over. This was sufficiently small to disregard in calculations.

II. In vitro Granule Experiments

Zymogen granules were obtained from pancreata excised from white male Holtzman rats weighing from between 200 to 450 gm. Rats were fasted for between 18 and 24 hours prior to surgery. Rats were killed by spinal section and exsanguination after light etherization. Most of the fat and connective tissue was dissected from the glandular tissue and the glandular tissue was blotted and weighed. In some experiments glands from different animals were pooled and this pool was treated as one animal. Glands were placed in a glass mortar with 10 times their weight of 0.3 molar sucrose. Homogenization was accomplished using a teflon pestle with 0.13 - 0.15 mm clearance. Five short shearing strokes and two complete shearing strokes were used in order to homogenize the gland. All homogenization was performed in an ice bath in order to keep the temperature of the homogenate low. Granule purification was accomplished by a process of differential centrifugation (Rothman 1971, Hokin 1955, Burwen 1972b). An initial spin of 760 x G_{av} for 10 minutes was used to remove heavy fragments (i.e. whole cells, nuclei and possibly plasma membrane). The supernatant was then recentrifuged at 1,000 x G_{av}* for 10 minutes in order to produce a "crude" granule pellet. This crude pellet was washed with the same low and high speed spins in order to purify the granule fraction. Granules prepared in this way have been shown to contain between 75 and 95% dense profiles or zymogen granules (Jamieson and Palade 1967) and contains little observable chemical contamination (Burwen 1972b). This fraction also contained the highest specific activity of chymotrypsinogen and amylase found in any sub-cellular fraction.

In the dilution experiments pellets of the sort just described were

* Sorvall SS 34 fixed angle rotor.

resuspended in varying volumes of 0.3 M sucrose solutions. Sucrose solutions used were either pH 5.5 or 7.0 as stipulated. pH was adjusted initially using small quantities of 0.1 N sodium hydroxide or hydrochloric acid. In the dilution experiments, the pellets were resuspended and incubated at 37°C for 60 minutes. After this incubation the suspension was centrifuged at 10,000 x G_{av} for 15 minutes. α-Amylase activity, chymotrypsinogen activity and protein were assayed in the supernatant and pellet. The total amount of protein and enzymatic activity remained the same regardless of dilution. Two measurements of release were used; (1) Percentage loss, (100) x (amount in the supernatant)/(the total amount, supernatant plus pellet), or (2) $\frac{S}{P}$, the amount in the supernatant/the amount in the pellet. Each "n" for a given dilution refers to an individual experiment performed on one animal or a group of animals on a given day. Considerably less variation was seen when multiple samples from one animal or one group of animals were analyzed in the same experiment.

The time course of release was determined in a similar fashion using multiple samples at one dilution (1 pancreas/40 ml) for pH 5.5 experiments and another (1 pancreas/8 ml) for pH 7.0 experiments. Samples were incubated for stipulated times and then centrifuged as above. No loss of total enzymatic activity or protein was observed during 120 minute incubations. At 240 minute incubations some experiments showed loss of activity, especially evident for α-amylase. Experiments showing loss were eliminated from the results. 0 Time experiments refer to dilution of the sample with immediate centrifugation. These clearly are not true 0 times because the sample is still in a dilute state during the 15 minute centrifugation procedure (at 0°C). Again each "n" refers to the experiment

performed on an individual animal or group of animals.

Experiments dealing with the temperature sensitivity of the release reaction were performed in a similar fashion. The same dilution was used for 30 minutes incubation at various temperatures. Zero time samples were also taken for this experiment. Zero time release was substracted from the release at each of the specified temperatures giving a measurement of the rate of release at that temperature. Q_{10}s were obtained by comparing this derived rate of release for each temperature. Q_{10} equals the the log of the ratio of release divided by the temperature difference between the two samples. Arrhenius plots were similarly made using the derived rates of release.

Association of tritiated α-chymotrypsinogen (Bovine) was used to show the reverse of the dilution experiments. Granules were depleted to approximately 50% of their chymotrypsinogen content by dilution (1 pancreas/40 ml) for one hour, then centrifuged at 10,000 x G_{av} for 15 minutes, and then resuspended in a sucrose media pH 5.5 containing the exogenous ^{3}H-enzyme and ^{131}I-albumin as a control protein. This suspension was incubated for 60 minutes at 37°C and then recentrifuged for fifteen minutes at 10,000 x G_{av}. Tritium found in the sedimentable portion in excess of the $^{3}H/^{131}I$ bath ratio was considered to represent associated chymotrypsinogen. The time course of this association was determined by varying the time of incubation. Reassociated enzymes were set equal to (sedimentable tritium) - (sedimentable ^{131}I) x ($^{3}H/^{131}I$) bath ratio. Sedimentable ^{131}I was used as a measure of extracellular space of the pellet. Assuming uniform packing of centrifuged granules, this measure of

extracellular space was used as an independent measure of the size of the pellet. In order to normalize the uptake of chymotrypsinogen for granule volume, uptake was divided by intragranular space. Results are expressed as the difference in uptake between ^{3}H at time "t" normalized for extragranular space and uptake ^{3}H at "zero" time.

Reconcentration experiments were performed in a hollow fiber device (Bio Rad Beaker Dialyzer). This device has hollow cellulose fibers which allow molecules up to 5,000 molecular weight to pass. Vacuum pressure of 600 mm Hg was used to reconcentrate suspension from which presumably water and sucrose was removed, leaving protein intact. In fact, no loss of enzyme activity was found within the chamber for at least two hours of incubation. Samples were taken at 15 minute intervals both during the dilution phase and after the reconcentration phase. Samples were not taken during the time of reconcentration. Reconcentration took between 30 minutes and 1 hour to accomplish. Each sample removed from the dialyzer was centrifuged at 10,000 x G_{av} for 15 minutes in order to separate supernatant and pellet. All experiments using the hollow fiber device were performed with 0.3 M sucrose solution kept at pH 5.5.

A final approach used to investigate the rate of release of enzyme from granules utilized a micro-ultrafiltration apparatus (Amicon) with a type XM100 a Dyaflo Filter (Amicon). This filter allows passage of 100,000 MW molecules, allowing proteins such as pancreatic enzymes to pass through the filter but retaining intact granules. A reservoir of 0.3 M sucrose at pH 5.5 or pH 7.0 was pressurized with between 8-20 lbs per in^{2}. Intact granules were placed in the Amicon filtration cell. Excess air in the cell was bled out and the cell was continually stirred.

Temperatures were maintained either at 25°C or at 4°C as stipulated. Samples were collected in a fraction collector for a period of up to 48 hours at intervals ranging between 20 - 60 minutes. Pressures were changed in the reservoir bath in order to change the rate of flow of sucrose solution through the filtration cell. This change in flow allows independent analysis of release as a function of time and as a function of flow. Some samples were reconcentrated in order to increase the amount of enzyme and protein in the solution for assay. This was accomplished by lyophilization and then resuspension. Care had to be taken with Lowry protein assays in this case because in very high concentrations of sucrose, the sucrose reacts with the Lowry reagent.

Reconstituted granule derived material was also diluted to estimate membrane effects. Granules were suspended in a relatively concentrated media containing approximately 1 mg protein/2 ml. Sodium hydroxide was then added to the suspension to raise the pH to 8.5. This suspension was allowed to sit for 30 minutes during which time solubilization of the granules occurred. The resultant solution showed no appreciable light scattering at 540 nm nor contained any appreciable sedimentable protein. It was then, by the addition of hydrochloric acid, readjusted to a pH of 5.5. The suspension became cloudy again and a sedimentable product reappeared. It was allowed to stand for another half hour. The suspension was centrifuged at 10,000 x G_{av} for 15 minutes producing a product referred to as "reconstituted material". The work of Rothman suggests that this reconstituted material has its granular membrane disrupted (Rothman and Ito, 1973). The pellet was then resuspended in a small volume of pH 5.5 sucrose solution. A portion of this suspension was then recentrifuged to determine

the distribution of enzyme and protein in the supernatant and pellet. A volume of sucrose was then added to dilute this suspension to approximately 1 pancreas in 40 ml. Samples were taken at 10-15 minute intervals in order to determine time course of the release phenomenon. Each of these samples was centrifuged at 10,000 x G_{av} for 15 minutes and distribution between supernatant and pellet was determined for protein and enzymatic activity.

III Tissue Uptake Experiments

New Zealand white rabbits weighing between 1.5 to 2.0 kg fasted for 24 to 48 hours prior to experimentation were anesthesized with Dial with urethan (Ciba Pharmaceutical Co., Summit, New Jersey) 0.7 ml/kg body weight. The pancreas with its adjacent intestine was removed. It was stripped from the mesentery, washied in an ice cold bicarbonate Krebs-Ringer (K-R) solution containing 0.2% D-glucose, and then sliced with a razor blade in a chamber specially designed to produce slices not greater than 0.2 mm. Tissue slices totaling approximately 1 gr were then preincubated in 20 ml of a K-R solution with 1 mg per ml α-chymotrypsinogen (Bovine) (Cal. Biochem. Grade A salt-free) at 37°C for 20-30 minutes. The strips were about 1 cm in length and weighed between 100 and 200 mg. After preincubation, 5 ml of K-R solution containing 64 μC/ml ^{3}H-chymotrypsinogen (Worthington Biochem., prepared by exchange tritiation, specific activity 64 μC/mg) and ^{131}I-albumin (iodinated human serum albumin, Abbott, specific activity 500 μC/mg) were added to the bath. Continuous gassing was maintained with 95% O_2 and 5% CO_2 throughout the experiment. Slices were removed from the bath at specified time intervals and washed in 10 ml of ice cold K-R solution containing 1 mg/ml bovine α-chymotrypsinogen. Two 15 second washes were employed for whole tissue experiments and four 30 second washes were employed in the fractionation experiment. The tissue was then blotted on filter paper and weighed.

Slices were immediately homogenized in 0.3 M sucrose solution (pH 5.5, 1:10 w/v) using a motorized teflon pestle (25% ground glass reinforced with 0.11 - 0.13 mm clearance). Five short and 2 long shearing strokes at

4,000 rpm (unloaded) were used keeping the mortar in ice water. Samples not to undergo differential centrifugation were diluted in 0.1 M sodium phosphate buffer, pH 7.4 and aliquots were taken for analysis of ^{3}H and ^{131}I, protein and chymotrypsinogen activity.

Other homogenate samples were fractionated using differential centrifugation. As above centrifugation at 760 x G_{av} for 10 minutes produced sediment containing nuclei and whole cells. The supernatant was spun at 1,000 G_{av} for 10 minutes. The pellet fraction was given a head (760 G_{av}) and tail (1,000 G_{av}) wash. This fraction was shown by our analysis to have the highest chymotrypsinogen specific activity. The supernatant, spun at 20,000 G_{av} (30 minutes) and washed at 8,000 and 20,000 G_{av} is called the mitochondrial fraction. The 20,000 G_{av} supernatant was then centrifuged at 100,000 x G_{av} (60 minutes). The pellet was labeled microsomes and the supernatant, the postmicrosomal supernatant (PMS). These fractions derived by the above procedures have been reasonably well defined by a series of works on the guinea pig (Jamieson and Palade 1967a), the dog (Hokin 1955), and the rat (Rothman 1970b, and Burwen 1972b). The various fractions were resuspended in 0.1 m sodium phosphate buffer pH 7.4. The presence of chymotrypsinogen (s) was estimated, after activation with purified enteropeptidase.

The ^{131}I-Albumin was used as a contamination space estimate for free chymotrypsinogen. Albumin appeared not to be appreciably bound to the tissue (see results) and in experiments using albumin and ^{14}C-sucrose similar results were found for contamination space (Albumin=.75±.05 Sucrose [n=13]).

In slice vs. strip experiments slices were treated as described

above whereas strips were taken and incubated without the slicing procedure. Strips have been shown by Rothman (1969b) to maintain a ductal space compartment for salt secretions. Strips fit a 3 compartment analysis for Na^+ uptake, whereas slices show only the first two compartments. This suggests that the ductal compartment is exposed by the slicing procedure. Ductal paths of approximately 1/2 cm should present an adequate barrier against retrograde enzymatic diffusion into the acinar compartment for a molecule as large as chymotrypsinogen (50% equilibration requires ~100 hrs). Furthermore, since this tissue is secreting fluid, retrograde movement of chymotrypsinogen would have to occur against bulk flow, making it even less of a significant factor. ^{131}I-albumin showed a smaller contamination space per eight of tissue in unsliced preparation than it did in sliced preparations. For unsliced preparations, an albumin space of 0.079 ± 0.008 S.E.M. (n=17) ml/gr tissue was found and for sliced preparations 0.103 ± 0.010 S.E.M. (n=18) ml/gr tissue. Sliced space was therefore greater than unsliced space with $p < 0.05$ and the ratio of unsliced/sliced spaces was 0.76. This indicated a compartment exists, presumably the ductal space from which albumin and chymotrypsinogen are excluded.

IV. In Vitro Pancreas

The in vitro pancreas preparation originally developed by Rothman (Rothman 1964a) has been used by numerous workers to study the pancreas under controlled conditions. Using a procedure similar to that described for the removal of rabbit pancreas for strip or slice preparations, the whole pancreas with attached loops of intestine was removed intact from a rabbit. The intestine was flushed out with K-R solution

and the gland washed in cold K-R solution. The loops were then mounted on a plastic frame with metal hooks placed in the attached intestine. Prior to removal of the pancreas from the animal, a small incision opposite the papilla of Vater in the duodenum was made and the pancreas was cannulated with P.E. 10 polyethylene tubing. The cannula was ligated with 3-0 silk to keep it in place in the duct, and then was then placed through a hole in a lucite chamber and the whole pancreas lowered into the chamber. The chamber was filled with K-R buffer and gassed continually with 95% O_2, 5% CO_2. The mounting of the loop was completed within 15 minutes after its removal from the animal. The bath was stirred continuously and maintained at 30°C by use of an infrared light. A preincubation period of 1/2 hour was utilized in order to standardize conditions. At this point a mixture of labeled and unlabeled bovine α-chymotrypsinogen was added to the bathing medium bringing the total bath concentration up to 0.02 mg chymotrypsinogen per ml (0.8 μM). Later in the experiment when additional chymotrypsinogen was added the new concentration of total chymotrypsinogen was 0.2 mg per ml bath medium (8.0 μM). Samples of secretion were collected at stipulated intervals. Various stimulants were used in different experiments: pancreozymin was used at 6 dog μ/100 ml.; a crude pancreatic stimulatory factor (Amesni-Acid Methanol soluble, Neutral insoluble fraction from Jorpes and Mutt (1962) extraction derived from intestine was used in some samples at 2 mg/100 ml concentration; methacholine chloride was used at 1 mg/100 ml concentration. In some experiments bath medium was changed with fresh K-R solution in order to chase labeled enzyme from the tissue. ^{131}I-albumin was, in two experiments, added to the bath medium along with the chymotrypsinogen as a control protein.

V . Gut Sac Procedure

New Zealand white male rabbits weighing 2.0 - 4.0 kg were sacrificed by air embolism. Animals were immediately opened and selected sections of gut removed. These sections were cut into pieces approximately 3 cm in length. Alternate sections were everted by placing a hemostat through the length of the section fastening on to one end and pulling the hemostat and the end through itself. Sections were tied at one end with 3-0 silk suture and 1 ml of Krebs-Ringer solution which had been previously gassed with 95% O_2 - 5% CO_2 was added to the sac. The far end of the sac was tied with 3-0 silk suture in a fashion similar to the first tie. Tissue weights ranged for duodenum and ileum from 1 to 2.3 gm. The sacs were incubated in a Krebs-Ringer solution containing 1 mg per ml tritiated chymotrypsinogen and trace amounts of ^{131}I-albumin (<4μg/ml). The incubating medium was gassed with 95% O_2 - 5% CO_2, stirred continuously and maintained at 37°C. After one hour incubation (unless otherwise specified) the sacs were removed from the incubating medium, washed in K-R solution (2 washes in 10 ml for 30 seconds) and sliced open with a scalpel. Fluid released after opening was collected and analyzed for ^{3}H, ^{131}I-albumin counts, chymotrypsinogen activity and protein. Water content in the sac was determined by weighing the sac before and after the sac was opened. Concentrations were determined as a function of the difference between these two weights. Unidirection fluxes were considered the amount of material entering the sac divided by the length of incubation. Proteins were precipitated with 20% trichloroacetic acid and the precipitate counted for chymotrypsinogen.

VI . In vivo Experiments

In vivo experiments were performed on New Zealand white rabbits weighing between 2 and 3 kg anesthesized in a similar fashion to in vitro pancreas experiments. Laparotomies were performed on these animals and the gut was tied closed just below the entry of the pancreatic duct. An incision was made opposite the papilla of Vater and the pancreatic duct was cannulated with P.E. 10 tubing. A stipulated amount of ^{3}H-α-chymotrypsinogen (Bovine) and ^{131}I-albumin suspended in K-R solution was injected into the gut below the ligation. Secretion was collected at specified intervals. Secretion was analyzed for ^{131}I, tritium, protein and chymotrypsinogen activity. Some samples of secretions were analyzed by gel electrophoresis to determine if the tritium label remained attached to the original chymotrypsinogen molecule.

VII . Gel Procedure

100 µl samples were layered on top of polyacryamide gels prepared by the method of Jones (1967). Gels were run for 45 minutes at 3.5 Amps/ tube when an Amido Swartz dye was added to the cathode solution in the manner of Ahlroth and Mutt (1970). Electrophoresis was continued for 15 minutes followed by a change of bath. Additional current was passed until free dye was leeched out of the gels. Gels were then scanned for absorbence at 570 nm on an Acta III gel scan. Gels were sliced into 5 or 10 mm sections, crushed by hand and assayed for ^{3}H and ^{131}I in the manner described in the General Assay Section.

RESULTS

I. In vitro Granule Experiments

A. Release as a Function of Dilution:

Results of the dilution experiments indicate increased release in increasing volumes of suspending medium. By sufficient dilution release approached 100% in both pH 5.5 and pH 7.0 suspending media. Release curves for single experiments done on one collection of granules from a group of rats on a single day produced a relatively smooth curve for release as a function of dilution. The result of two such experiments for pH 5.5 and pH 7.0 suspending media is shown in Figure 5. The pooled data (Fig. 6) representing the log of dilution (measured by milliliters/activity of activated chymotrypsinogen) plotted against the log of release (measured by supernatant/pellet distribution of enzyme) produced a relatively straight line. Correlation coefficients of 0.92 were found for both the pH 5.5 and pH 7.0 data. The slope of the pH 7.0 data was found to be approximately 0.9 and for the pH 5.5 data approximately 0.5. No slopes greater than 1.0 for either pooled data or individual experiments were observed. Amylase appeared to be released more readily than chymotrypsinogen. The ratio of (s/p) amylase to (s/p) chymotrypsinogen was 7.1 (using $\log [(s/p)_{amylase} / (s/p)_{chymotrypsinogen}]$ with a 95% confidence limit of 4.8 to 10.4 (n=7). This ratio apparently did not vary with dilution. This supports the suggestion made by Burwen (1972a) that the same type of equilibrium function governs release of amylase and chymotrypsinogen, with amylase merely displaced more toward the soluble compartment. Protein release was generally similar to chymotrypsinogen, showing somewhat greater release at lower dilutions and somewhat less release at higher dilutions (ie. - greater protein release at high concentrations with a

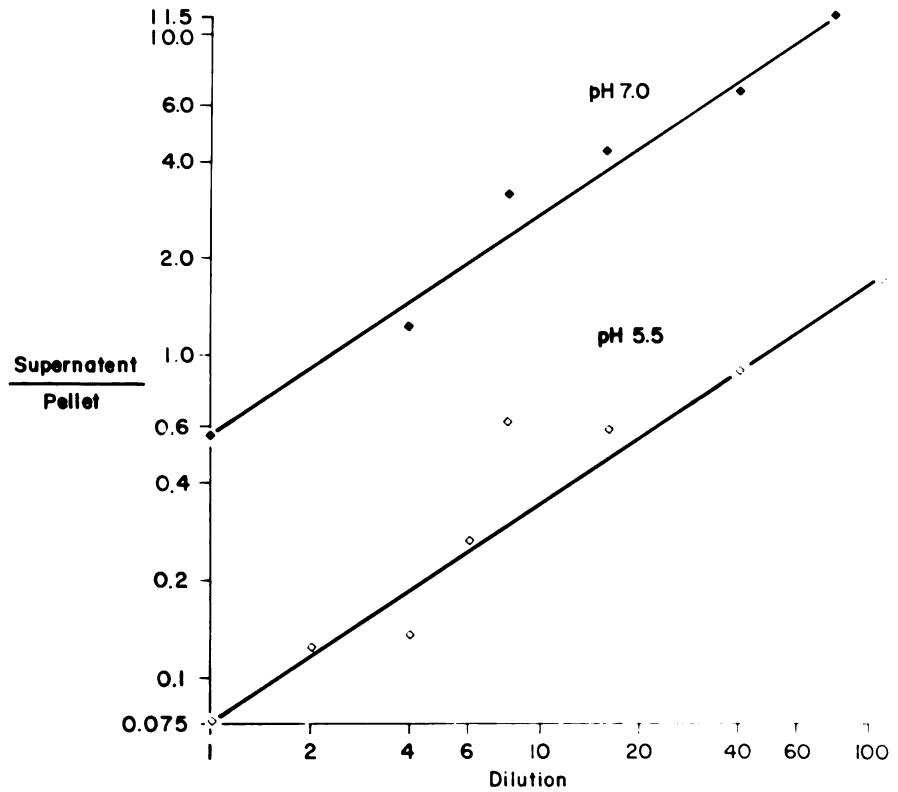


Figure 5. Release as a function of the relative dilutions of granule suspensions. The ratio of log activity of nonsedimenting (supernatant) to sedimenting (pellet) chymotrypsinogen versus log volume per total chymotrypsinogen activity (dilution). ●, pH 7.0; o, pH 5.5.

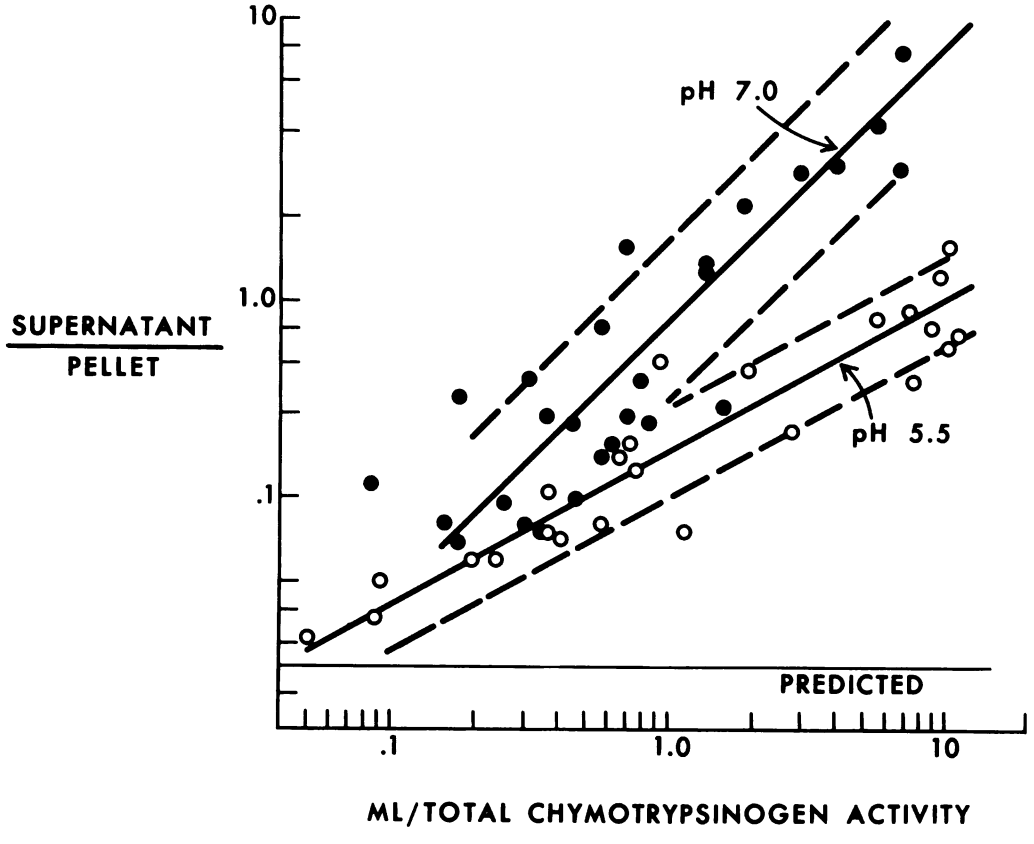


Figure 6. Release as a function of the relative dilutions of granule suspensions. The ratio of log activity of nonsedimenting (supernatant) to sedimenting (pellet) chymotrypsinogen versus log volume per total chymotrypsinogen activity (dilution). ●, pH 7.0; o, pH 5.5. Linear regression lines are drawn the points (pH 7.0 has a correlation coefficient of 0.93, pH 5.5 has one of 0.92). The line labeled "predicted" indicates the absence of dilution dependence as would be observed if granule contents were independent of enzyme content in the surrounding medium. The dashed line indicates the standard error of estimate.

lower slope for release with dilution). The apparent non-linearity of the protein release curve is not surprising since linear s/p ratios as a function of dilution are not linearly additive. The apparent lower release of protein than chymotrypsinogen (see Fig. 7) at higher dilutions along with the much higher release of amylase at all dilutions requires that at least some component of the granule must be much more stable (lower s/p) than chymotrypsinogen. Lipase in the presence of 0.1 M $CaCl_2$ was shown by Burwen (1972b) to be more highly associated than chymotrypsinogen (s/p ratios of lipase were 0.9 and for chymotrypsinogen 7.6). Lipase or some other "tightly" associated enzymes and/or some "structural protein" (estimated at less than 5% of the total granule protein - Keller and Cohen, 1961) could account for this reduction in total protein lability. Single enzymes appear to follow predictable independent release functions. This is consistent with separate associative maxima for each enzyme found by Rothman (1971). Protein release, however, would clearly be dependent on enzyme composition since different enzymes have different release equilibria. For one set of granules where enzyme content is fixed, however, amounts of release at a given dilution are reproducible, as is shown in Figure 5. Variation of release in the cumulative display (Figure 6) may therefore have been partially produced by variations from experiment to experiment in the enzyme composition of granule suspensions. In addition, other causes such as variation in the pH of the suspending media may also have contributed to the variability. I used an unbuffered solution because granules are generally collected in sucrose solutions (Hokin 1955, Siekevitz and Palade 1958a, Rothman, 1971) and are unstable in salt solutions (Burwen 1972a and 1972b). Initial pH measurements

were done using a pH meter Radiometer and were controlled to ±0.2 pH units. pH was noted not to vary be more than ±0.5 pH units during incubations. This observation corroborates Burwen's (1972b) results describing pH stability of granule incubations, but slight variations in pH may have been sufficient to contribute significantly to the variations seen in release patterns.

B. Release as a Function of Time

The absolute rate of release tapered off with time for each enzyme, and release as a function of time fit well with a function approaching equilibrium. Using results from a single experiment and doing an analysis of best fit to a hyperbolic function for release vs. time, half times for equilibration for chymotrypsinogen of 30 minutes at pH 7.0, 37C° and 60 minutes at pH 5.5 were found. In contrast, α-amylase at 7.0 demonstrated a half time of approximately 15 minutes (Figure 7b).

In order to investigate the time course of release for chymotrypsinogen, amylase and protein, I diluted samples of zymogen granule material from three animals independently prepared and treated for specific times. The results are shown in terms of distribution (s/p ratio) (Figure 7a) and absolute amount of release (% release) (Figure 7b) (Total protein and activity remained constant in all experiments). Amylase had a higher concentration in the bath than chymotrypsinogen or protein. Protein release showed a somewhat different pattern than either of the enzymes; protein has a higher initial release and lower rate of release than amylase and chymotrypsinogen. The higher initial release may be due to contaminating nonassociating proteins in the preparation. A cumulative release function, derived from a number of individual functions (for individual enzymes - e.g. amylase, chymotrypsinogen, etc.), some being rapid and others slow, could, except for the initial, presently inexplicable, period in the protein release curve displaying granule uptake of protein, resemble the protein release curve.

Granule material reconstituted from alkaline solubilized granules by readjusting pH to 5.5 was diluted in a manner similar to that described for native granules. The release in response to such dilution is shown

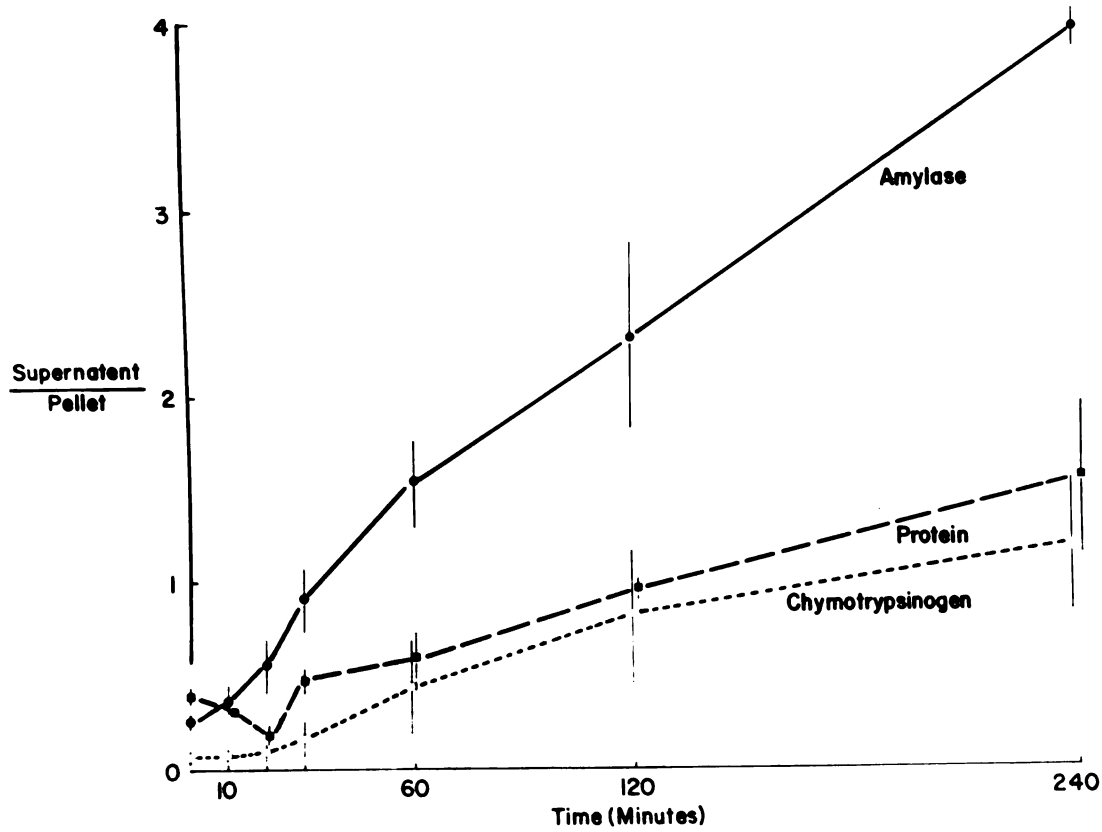


Figure 7a. Release as a function of time. Plotted is the change in distribution between pellet and supernatant with time after diluting a suspension of granules. _____ ●, represents amylase; _ _ _ _ ■, represents protein and ----o, represents chymotrypsinogen. Error bars represent the Standard Error of the mean. 3 Trials for each point.

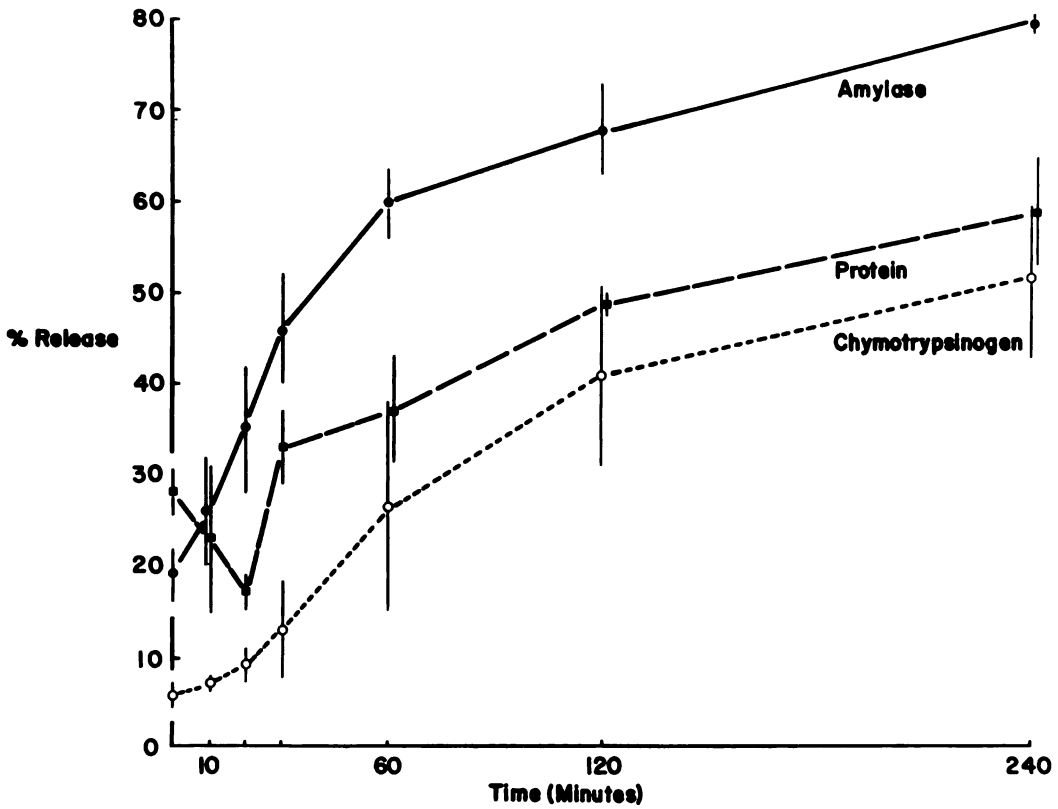

80
70
60
50
40
30
20
10
0
% Release
10
60
120
240
Time (Minutes)
Amylase
Protein
Chymotrypsinogen

Figure 7b.

in Figure 8 for one experiment and in Table I for the cumulative results of 4 experiments. These results indicate that equilibrium is reached within the first ten minute period and that the enzyme distribution does not change thereafter. Table I shows, also, that lowering the temperature from 37°C to 25°C does not alter the equilibrium distribution. Though different initial dilutions were used in the experiment shown in Table I (3 fold range), the effect of an 8 fold dilution produced a uniform change in the ratio $[(s/p)_{\text{pre-dilution}}/(s/p)_{\text{60-post dilution}}]$ independent of the initial state of dilution. The uniformity is seen by comparing the standard deviation (S.E.M. $x\sqrt{n}$ with $n = 4$) of the ratio of (s/p) before dilution vs. 60 minutes after dilution with the standard deviations of the ratio of release at other times after dilution and 60 minutes after dilution release. Since the variance of the release ratios depends on measurement variation and variation due to dilution changes, since 10, 30 and 60 minute-cold incubations are at steady state, and since the standard deviation of predilution and post-dilution release were the same, then a constant dilution caused a constant variation in s/p ratio regardless of initial state of release and the dilution itself did not add to the variation. This is not so for percentage loss. The standard deviation of % loss is increased by diluting the sample. This suggests that % loss is not a linear function with dilution, whereas s/p is.

$$\frac{(s/p)_x \,/\, (s/p)_{60}}{}$$

	undiluted / diluted 60	diluted 10 / diluted 60	diluted 30 / diluted 60	diluted/cold / diluted 60
Mean	0.503	0.975	1.009	0.976
σ	0.156	0.208	0.196	0.184
S.E.M.	0.078	0.104	0.098	0.092

$$\frac{(\sigma)_{0/60}}{(\text{Mean } \sigma)_{10/60,\ 30/60,\ C/60}} = 0.80$$

$$\frac{(\%\ \text{loss})_x \,/\, (\%\ \text{loss})_{60}}{}$$

Mean	0.627	0.986	0.994	0.999
σ	0.160	0.124	0.099	0.091
S.E.M.	0.080	0.062	0.050	0.045

$$\frac{(\sigma)_{0/60}}{(\text{Mean } \sigma)_{10/60,\ 30/60,\ C/60}} = 1.53$$

Table 1. Release of chymotrypsinogen from reconstituted material. Presented here is the ratio of release at 60 minutes after dilution and either predilution release, 10 or 30 minutes after dilution, or 60 minute cold (25°C) incubation (in all but the cold situation incubation was at 37°C). All calculations were made using 4 separate preparations from animals treated independently, initially suspended as 1 pancreas/1 ml, and then diluted 8 fold. The yield from each pancreas (chymotrypsinogen recovered per pancreas) was not identical producing variability in the initial concentration of granules.

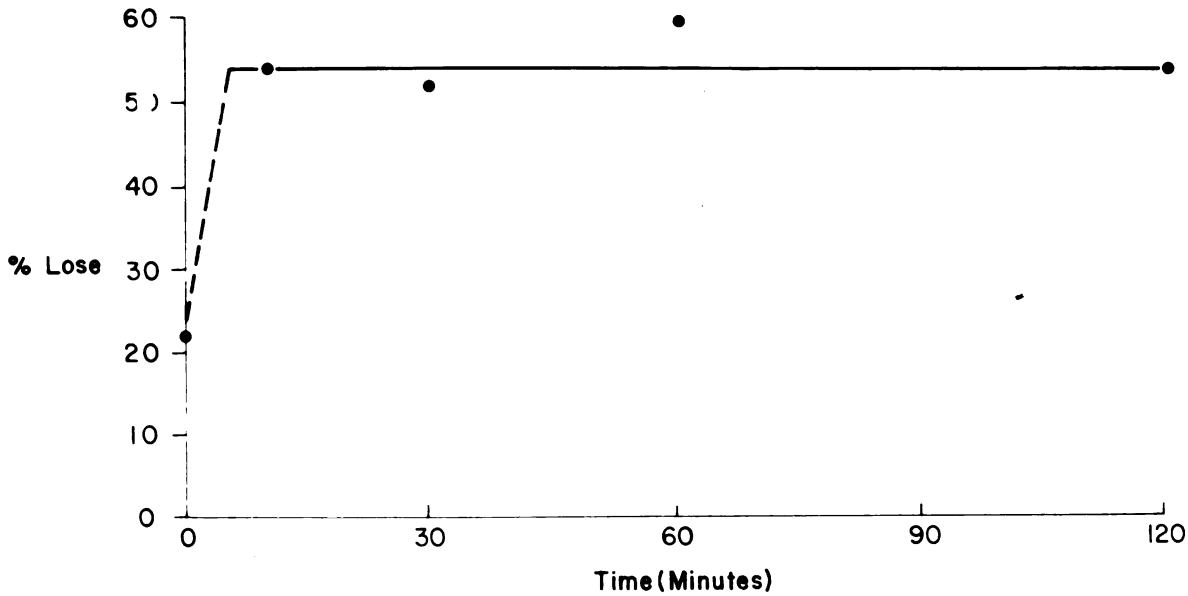


Figure 8. Release from reconstituted material in response to dilution as a function of time. The distribution of sedimentable and non-sedimentable protein is shown in this graph. o time represents a pre-dilution sample. After sampling at zero time, the suspension is diluted. Sequential samples were then analyzed.

C. Temperature Sensitivity of Release:

The rate of release of proteins was very sensitive to changes in temperature. Q_{10}s were determined using multiple samples derived from the same collected granules and by comparing the change in release at 0 time to release after 20 minutes incubation at either 20°C or 30°C. The difference in release between unincubated samples (0 time) and samples incubated at 20°C - 30°C was used to calculate the Q_{10}. The observed Q_{10}, over 5 for chymotrypsinogen and protein and considerably less for amylase (as shown in Figure 9) can only be taken as an accurate determination of Q_{10} if, during this 20 minute interval, release rates remained at initial rate. This was probably not so for α-amylase, and perhaps for the other enzymes as well. Furthermore, these Q_{10}s are also only applicable to the 20°C to 30°C span. Estimates of reaction rate sensitivity to temperature were also made comparing a group of animals treated separately whose conditions were standardized by using zymogen granules derived from 1 pancreas from a rat weighing 200 - 300 gms diluted in 40 ml. From these results Q_{10}s greater than 20 were found. The most sensitive temperature region appeared to be between 20° and 25° and the least sensitive temperature region appeared to be between 30° and 37° (Figure 10). An Arrhenius plot is shown in Figure 11. The nonlinearity in the plot suggests that temperature dependence is a complex function and not merely dependent on a single energy of activation. The temperature dependence may likely be due to a change in protein configuration.

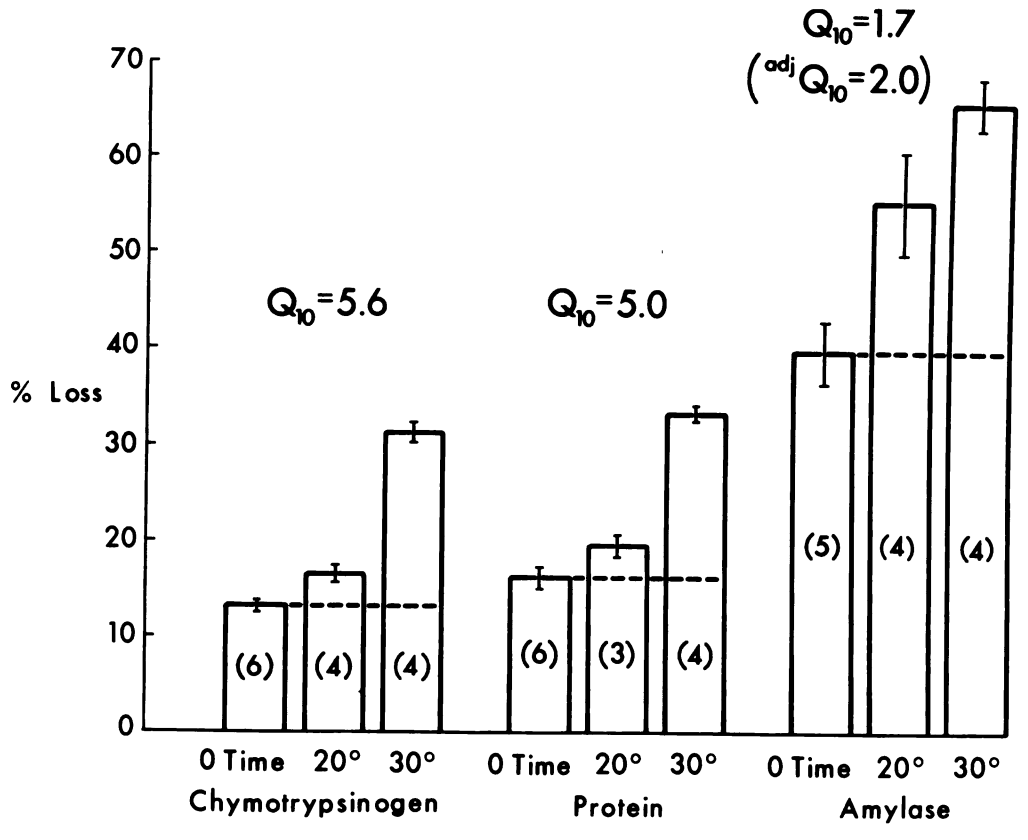


Figure 9. Q_{10} determination for chymotrypsinogen, protein, and amylase. "0 Time" refers to release prior to incubation. "20°" and "30°" refer to release after 20 min incubation in 20° or 30°C baths. The ratio of release during 30°C incubations (30°C release minus 0 Time release, 30°C column above dashed line) to that released during 20°C incubations was used to determine the Q_{10}. ( ) = Number of trials; I = s.e.m. Adjusted Q_{10} for amylase derived from supernatant/pellet ratio rather than % loss to compensate for the close approach to 100% loss.

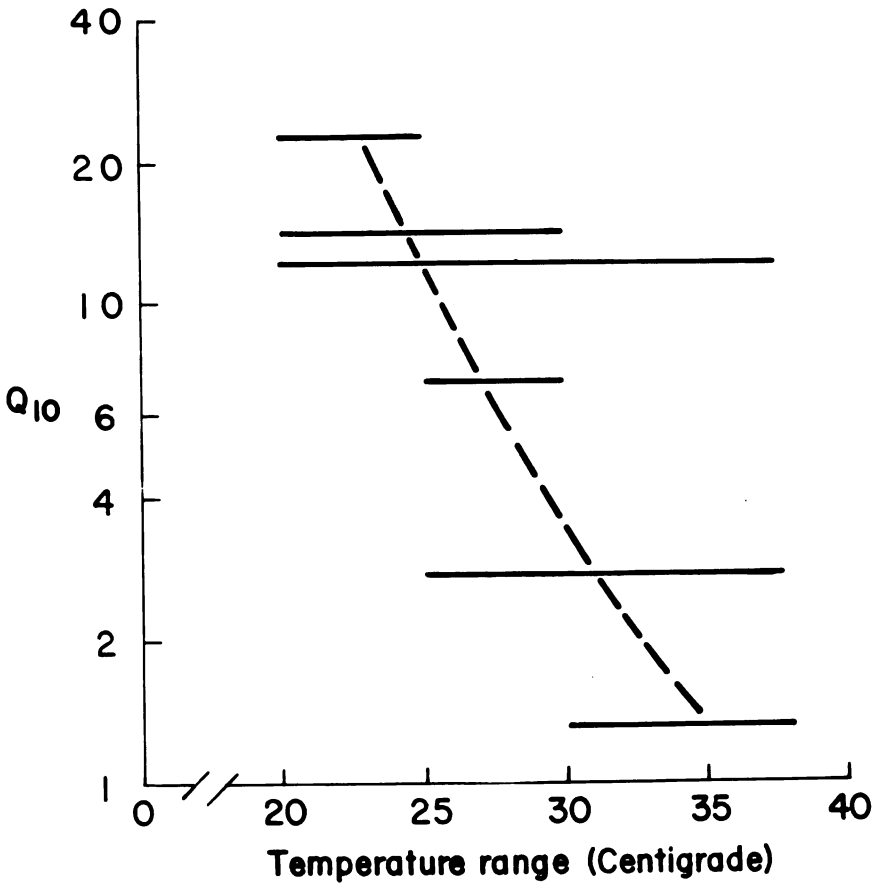


Figure 10. Q_{10}s computed for a range of temperatures. Shown on the ordinate are the values of the Q_{10}s found, and on the abscissa is the corresponding range of temperatures. Each Q_{10} was found in a manner similar to the computations made in Figure 9, except that 30 minute release times were used. Zero time release and release at the stipulated temperatures were computed from 4 samples each. The dashed line is an approximation showing the decrease in temperature sensitivity of the reaction going from 20 to 37°C.

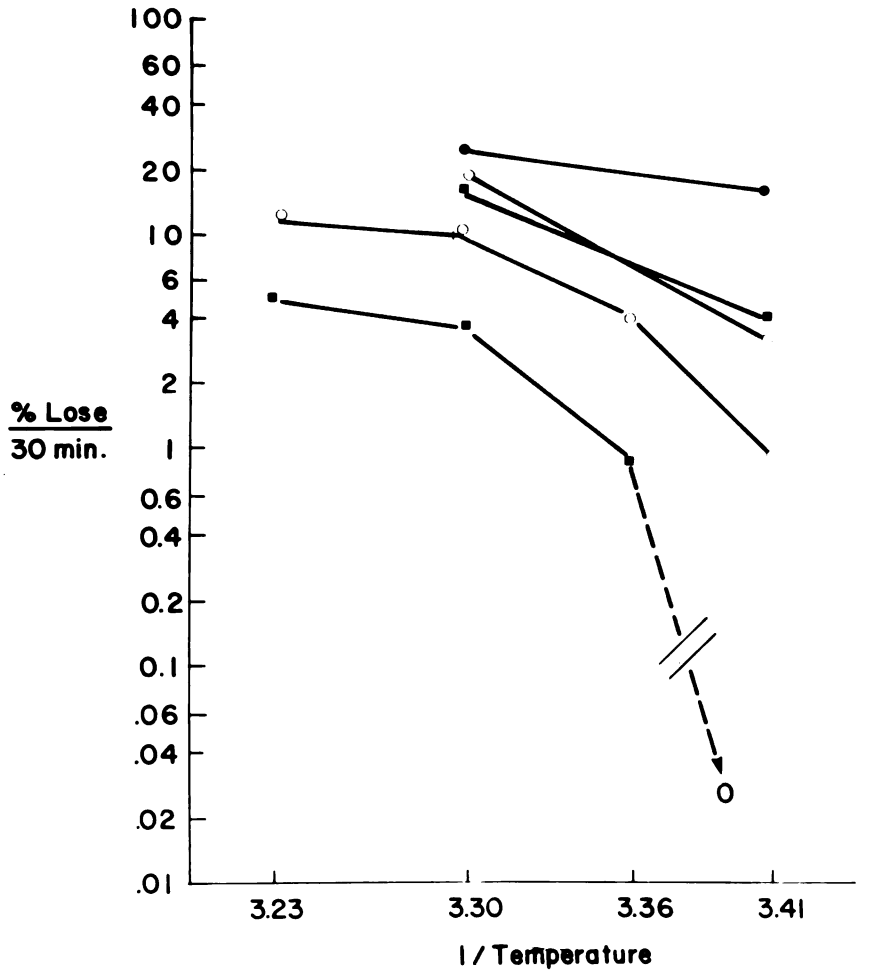


Figure 11. An Arrhenius plot for specific release from granules. The ordinate represents the difference between release at a specified temperature and zero time release (normalized to 30 minute release) displayed on a logarithmic scale. The abscissa represents the inverse of temperature (left to right - 37, 30, 25 and 20°C). The lines connecting two points represent the results shown in Figure 9, and the other is from the data in Figure 10. All points were determined with 4 samples (all zero times release were determined from 6 trials). 0 represents chymotrypsinogen, ● amylase and ■ protein. The upper lines represent release as computed from 20 minute release experiments and the lower two represent 30 minute release experiments.

D. Reversal of Release

Reversal of the release process was demonstrated in a number of ways. One method examined the association of exogenous tritiated α-chymotrypsinogen (Bovine) with granules previously partially depleted of enzyme. Chymotrypsinogen uptake appears to occur and the uptake process has a time course of the same order of magnitude as the release phenomenon (Fig. 12).

Another method for demonstrating reversibility of the release reaction involved reconcentrating suspensions of depleted granules with their endogenous enzymes still present by hollow fiber dialysis. Reincorporation of these enzymes also indicates reversal of release. Release during dilution in the dialysis chamber was similar both in magnitude (s/p vs. dilution) and the time course of release for chymotrypsinogen determined by individual granule incubations (pH 5.5 suspensions had s/p ratios of ~1 at 1 pancreas/40 ml). The dialysis process itself (to return volume to initial volume) in the beaker dialyzer required at least 30 minutes. This prevented me from applying a "step-function" reconcentration change and thereby made it difficult to investigate the time course of the reassociation process. Reassociation occurred in response to reconcentration as is shown by a dialysis experiment in Figure 13. Furthermore, reconcentration returned the bath:pellet ratio back to the original predilution values. This indicates complete reversibility of the system. In response to dilution, a similar ratio of change in s/p ratios occurred in both chymotrypsinogen and amylase, as shown by equal magnitudinal deviation on a semi-log plot (Figure 13).

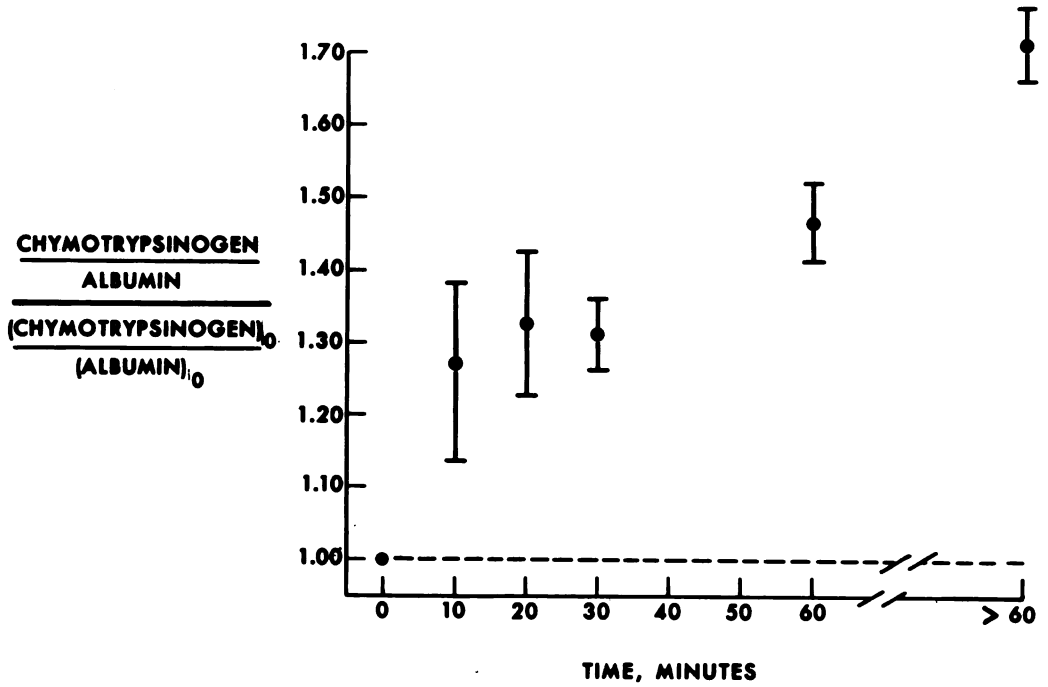


Figure 12. The change in the ratio of exogenously added ^{3}H α-chymotrypsinogen (Bovine) to ^{131}I-albumin (human) with time in granules previously about 50% depleted of chymotrypsinogen. Increase in the ratio indicates chymotrypsinogen uptake by granules. "0 Time" after separation is $^{3}H/^{131}I = 1.0$ by definition. Dashed line indicates no uptake. Moving out from 0 Time, n = 9, 4, 5, 5 and 6. >60 represents pooled data (1 at 75 min, 1 at 90 min, and 2 at 120 min).

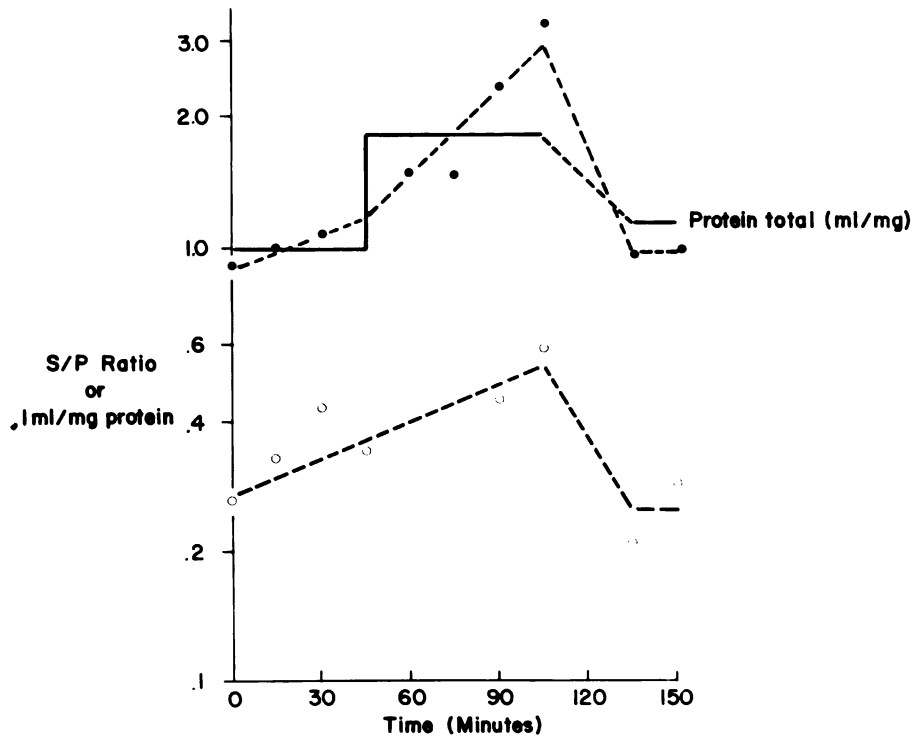


Figure 13. Endogenous reassociation. Shown here is the response in supernatant/pellet ratio (plotted on a logarithmic scale) with time to changes in granule suspension concentration. ● represents amylase points, and o represents chymotrypsinogen determinations. The solid line represents changes in concentration as measured by total protein concentration (0.1 mg/ml on the ordinate scale). The dashed part of the solid line represents the reconcentrating phase carried out by ultrafiltration dialysis during which time concentration was not measured.

E. Initial Rate Release

A preliminary attempt to quantify characteristics of the release reaction was made using an ultrafiltration apparatus (Amicon). With this system it appeared that we might potentially be able to separate enzyme release as a function of time and as a function of dilution. Theoretically, in the two extreme cases,that is at 0 flow and at infinite flow, release should be dependent in each case on only one of these variables. That is, at low flow, equilibrium should be reached and time should not be a variable, only flow rate (dilution). At infinite flow, with no backflux at all, the functional relationship of dilution should be eliminated and release should be purely a function of time. In an attempt to see if these conditions could in practice be reached, I set up criteria for attainment of these functional relationships. If flow could be varied without varying the total amount of release, then release would no longer be a function of flow and would therefore be purely a function of time. If flow could be varied without varying the emzymatic or protein concentration of the ultrafiltrate, then the ultrafiltrate would be in an equilibrium state with the granules. Release would therefore be purely a function of dilution. These two conditions were in fact approached, although not met. Using the filtration apparatus described in Methods, with granules for 10 rat pancreata at 25°C, flow was varied with very little variation seen in the concentration of enzyme. This result is shown in Figure 14b. By running the same chamber with the granules derived from only one rat pancreas in a cold room maintained at approximately 3°C (the temperature was lowered in order to reduce release rate because flow could not be sufficiently increased), flow was varied sufficiently to produce marked changes in the enzymatic concentration of the solution, but there was only a slight variation in the

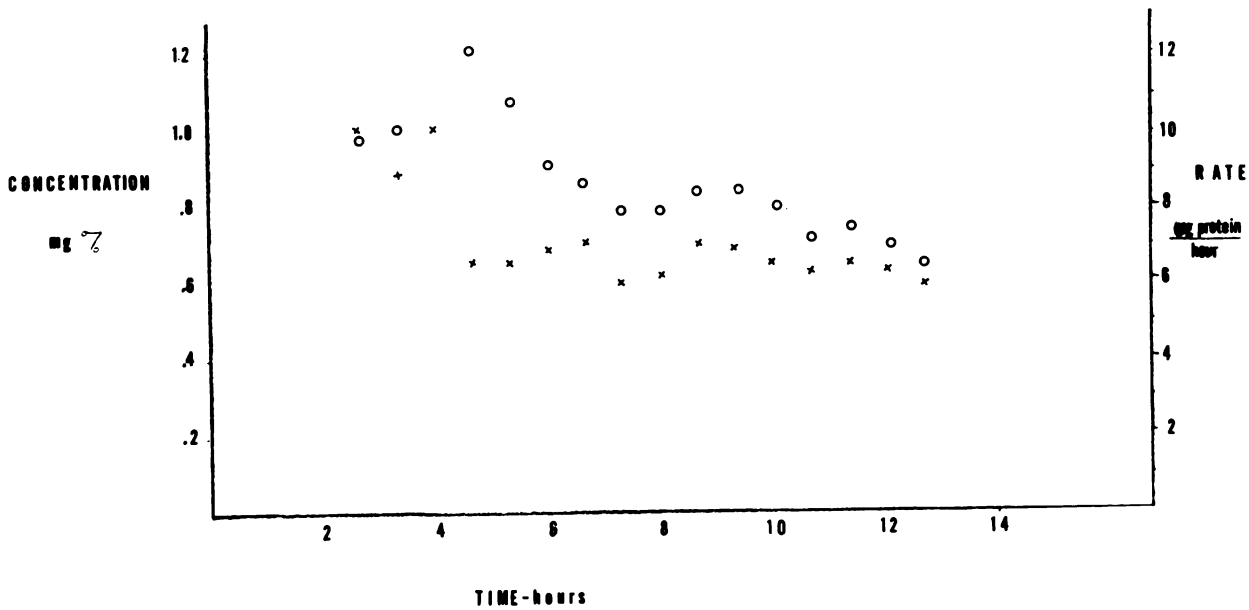


Figure 14a. Steady state release. Shown is the concentration (X-mg%) of protein in the effluent of the Amicon filtration device with granules derived from 1 rat pancreas filtered at 4°C. Also shown is rate of release (0-mg protein/hour) calculated by multiplying concentration times amount of effluent. At 4 hours, filtration pressure was increased from 8 to 24 lbs. This produced a sudden decrease in protein concentration, but a change in rate of release for only one period. This one period change was probably related to wash out of dead space.

	Concentration (mg%)			Rate of Release (μg/hr)		
	Before	After	After/Before	Before	After	After/Before
10 Rats - 25°C	7.7	7.7	1.00	534	627	1.17
1 Rat - 2°C	.96	.67	.80	101	108	1.07
1 Rat - 2°C	.25	.20	.80	36	36	1.00

Figure 14b. Effect of flow change on release in the Amicon Filtration apparatus. Concentration refers to the concentration of protein in the effluent of the filtration cell. Rate of release refers to the amount of protein in the effluent. "Before" refers to the mean value for 3 periods before the filtration pressure was changed (8 lbs./in^2 to 24 lbs./in^2) causing a change in flow rate. "After" refers to the mean value for 3 periods after the pressure was changed excluding the first collection period. This period was excluded to avoid contamination from dead space. The "After/Before" ratio is a measure of change due to change in flow rate. In the first experiment granules from 10 rats were filtered in the cell at 25°C. In the other two experiments, granules from 1 rat were filtered at 2°C. A change in rate of release (a ratio greater than 1.0) indicates a relationship dependent on flow rate, whereas no change (ratio = 1.0) indicates independence of flow rate. No change in concentration (ratio= 1.0) indicates that release in independent of time. The first experiment therefore indicates independence of time, and the others approach independence of flow.

total amount of release due to changes in flow rate (Figure 14a & b). This system indicates that release as a function of time and release as a function of flow may be separable by varying conditions, and that this system may allow one to explore a particular parameter of initial rate release in the future. Also, this system shows that the release-volume relationship is not merely an artifact of the centrifugation procedure, since no centrifugation is required here.

II. Granule Size Determination

The determination of the variation of granule size using techniques described in Methods yielded a number of estimates. An examination of the population r (slice radius) for $\Delta \to 0$ (halo size) [$r \to R$ (sphere radius)] shows a range of r less than two fold, and an observed standard deviation (S) from $\bar{r}$ of ± 16%. This provides a realistic limit for the standard deviation of the whole population. Using an estimate of T (slice thickness), a distribution of R (sphere radius) was determined for the whole population of granule sections (Figure 15). The observed standard deviation of R for the whole population determined this way was found to be ±19%. Choosing a T value anywhere in the possible range .25 to .20 units (see footnote 3 to convert to ~thickness) did not alter the standard deviation by more than 5%. This observed standard deviation (s) (19 + less than 5%) includes variations caused by a number of other factors other than the true granule size variation. Error in measuring r and Δ, distortion in fixation and sectioning, distortion in the process of granule isolation, and of course true variation in R, are all components which together cause the observed standard deviation. The error in R caused by errors in the measurement of r and Δ will increase (for constant r and

Δ measurement error) as Δ increases. Since this error increases throughout the range of Δ, the error will be smallest as $\Delta \to 0$. This range of Δ · therefore, gives the best approximation of the true variation of R. For small Δ, R[f(Δ, r, T)] was seen to vary with an observed standard deviation (S) of 14% (the worst T between .25 and .20 would not increase this more than 0.5%. For larger Δ, the standard deviation was seen to range up to 27%. Various estimates for E_r (error produced by S error in r measurement) and E_Δ (error produced by S error in Δ measurement were made). Each however was dependent on a number of assumptions (such as the ratio of δ for E_r to δ for E_Δ), and varied accordingly with these assumptions. Most methods estimated that measurement error accounted for about one third of our observed deviation (ie., S = 14%, σ (true variation) $\simeq$10%). This figure cannot presently be fixed with our data and methods, but the true standard deviation must be less than the observed standard deviation (σ<S).

In order to test the null hypothesis that the granule size does not vary, the observed Δ frequency should be compared to a cosine function. A correlation coefficient of .975 was found between the observed Δ frequency and cos function. This high correlation should be taken as evidence that the sphere sizes are narrowly distributed, but not necessarily that they are identical, since a bimodal distribution of R $\pm$ 10% would also produce a correlation coefficient of .975. The standard error of the correlation coefficient was too large ($\simeq$0.1) to test the validity of our null hypothesis, but it is independent evidence supporting the conclusion that the size distribution of granules probably varies with a σ within the approximate range of 10%.

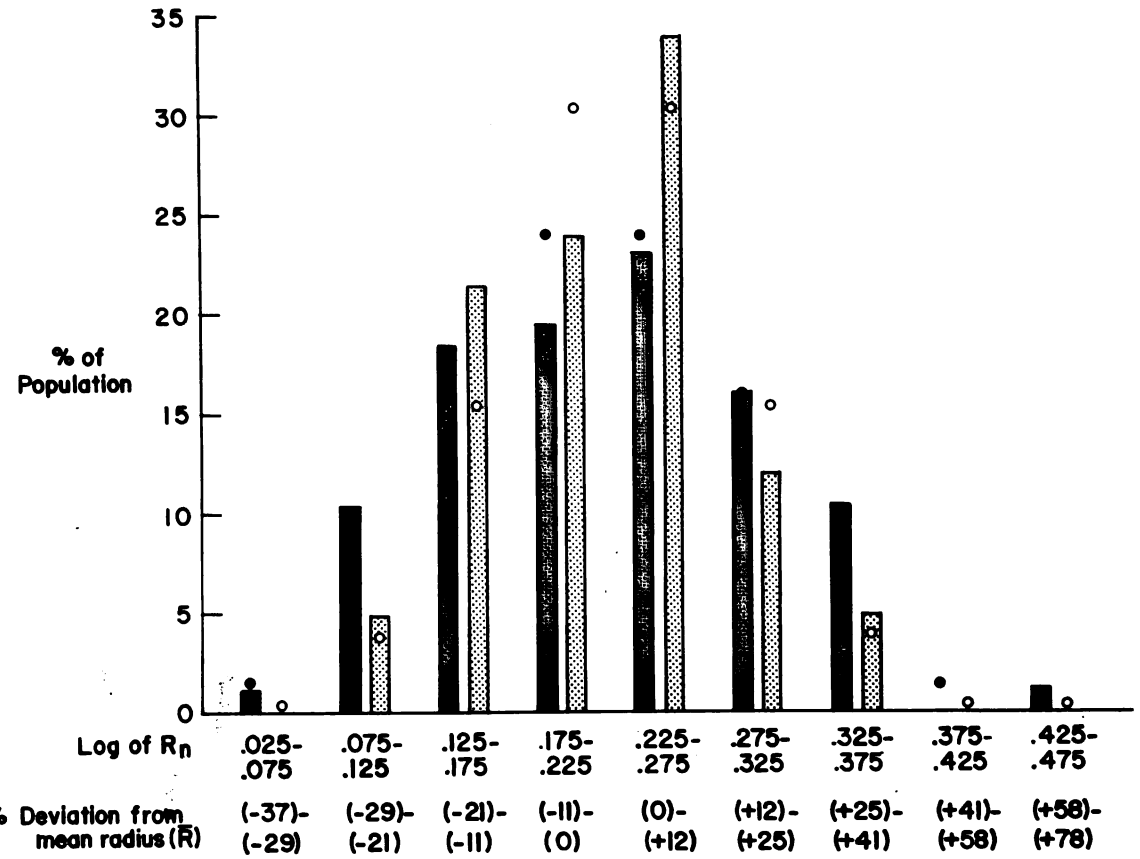


Figure 15. The distribution of the size of zymogen granules from granules isolated by differential centrifugation. The intervals represent equal log intervals with the corresponding percentage deviation from the mean shown below. The solid bars represent the distribution over the whole population (n=87). The stippled bars represent the distribution for sections with small halos ($<\Delta<.20$ units) (n=66). The circles represent the frequency of a normal distribution with the same deviation as that of the observed population.

Since this analysis was performed on a pellet of zymogen granules collected from 10 rats, it could be argued that the limitation in size variation shown is not a limitation on granule size *in vivo*, but is an artifact produced by our collection method (viz., a function of the depth in the pellet). To consider this, we analyzed the granules within a single cell in tissue sliced from a rat pancreas. The difficulty of analyzing tissue slices lies in the lower granule density *in vivo* (number of granules per volume) than in the pellet, and the greater difficulty in resolving granule halos when the suspending medium (the cytoplasm, *in vivo*) varies in density. A standard deviation for the whole population (from one cell) was 15% (as compared to 19% *in vitro*), and for small Δ was 11% (14% *in vitro*). Despite the technical problems a similar *in situ* constraint on sphere size could therefore be shown.

III. Tissue Uptake Experiments

Tissue slices of rabbit pancreas incubated for 30 minutes at 37°C showed selective uptake of tritiated chymotrypsinogen relative to ^{131}I-albumin. This was demonstrated by comparing the ratios of tritium counts to iodine counts in the tissue homogenate and the bathing medium. Any increase above a ratio of 1.0 would indicate the selective association of chymotrypsinogen with tissue (accepting the fact that albumin distributes uniformly and non-selectively in the extracellular space in a generally similar fashion to free chymotrypsinogen, see Methods section). Ratios (Table 2) of 3.0 were observed without washing the tissue. In the methods section I discussed that albumin was predominantly unbound because of the similarity between albumin space and sucrose space, and if tritiated chymotrypsinogen is in part "tightly associated" with the tissue then the ratio would be greatly increased by additional washes. Two 30 second washes in ice cold Krebs-Ringer solution increased the ratio to 7.8 to 1 and four 30 second washes increased it to 17.0 to 1. In Table 3, the release of exogenous chymotrypsinogen (^{3}H) and albumin (^{131}I) from tissue is shown for successive washings. More rapid release of albumin is evident, with retention of a majority of the chymotrypsinogen.

The selective association of tritium can be explained in three ways: (1) chymotrypsinogen binds to the cell surface or to elements in the extracellular space; (2) tritium dissociated from the chymotrypsinogen either as a free ion or as a small molecule, such as an amino acid and either due to its larger space of distribution or due to selective uptake is concentrated in the tissue; or (3) tritiated chymotrypsinogen enters the cell. The addition of cold chymotrypsinogen (40 μM ≡ 100 mg %) to the last two washes

did not significantly alter the final ratio of ^{3}H chymotrypsinogen to ^{131}I albumin or the ^{3}H chymotrypsinogen content of the tissue (3.3 ug exogenous chymotrypsinogen/gr tissue protein without "chasing" with "cold" chymotrypsinogen compared to 3.0 with "chase" - not significantly different) (Table 2). This absence of release of associated chymotrypsinogen in response to cold chymotrypsinogen chase suggests that selective uptake of ^{3}H is not due to a freely exchangeable surface bound pool.

Label, therefore, apparently enters the cell eliminating (1), but whether it is free (2) or with chymotrypsinogen (3) must be determined. The tritium apparently remained associated with the exogenous chymotrypsinogen since washing the granule fraction with 2 ml of 20% TCA precipitated 95.7% (±0.7% SEM, n=4) of the tritium. Measurements of the time course of incorporation of labeled amino acids into protein in the zymogen granule indicate that newly synthesized protein could not account for ^{3}H in the granules at this time. (Jamieson and Palade 1967 b). Furthermore, the label was treated as secretory enzyme by the cell and accumulated in an intracellular compartment of the cell, the zymogen granule fraction. After 5 minutes incubation with labeled enzyme, the specific radioactivity (SRA Cpm/mg protein) of the zymogen granule fraction was 13.7 (± 1.0 SEM, n=8) times the SRA of the homogenate (see Figure 17a). ^{3}H was 150 (±10 SEM) times more concentrated in this fraction than ^{131}I (the extracellular marker) as compared to 17 times more concentrated in the washed homogenate. This shows that exogenous chymotrypsinogen enters the cell (3) and is treated as endogenous material and accumulated in the zymogen granule.

Uptake of exogenous chymotrypsinogen continued for a period of at least 30 to 60 minutes, as is shown in Figure 16. The initial rate of uptake into tissue was approximately 1.0 micromole (25 milligrams).

# of rinses (30 sec. Krebs-Ringer solution at 0°C.)	$\frac{(^3H/^{131}I)Tissue}{(^3H/^{131}I)Bath}$
0	3.0±0.3 (n=9)
2	7.8±0.6 (n=26)
4	17.0±1.9 (n=4)
2 Krebs-Ringer+2 cold 1 mg/ml Chymotrypsinogen in Krebs-Ringer in chase	20.0±1.6 (n=4)

Table 2. Rinse Effects on Tissue Slices. Tissue increase in ^3H-chymotrypsinogen/^{131}I albumin ratio over bathing medium. Successive washes increase this ratio as shown. N represents the number of experiments. SEM is given.

# of Rinses (30 sec. Krebs-Ringer solution at 0°C.)	% Total Exogenous ^{3}H-Chymotrypsinogen in wash	% Total ^{131}I Albumin in wash
1	22	57
2	8	20
3	4	10
4	2	3
% remaining with tissue	65	10

Table 3. Distribution of Chymotrypsinogen - Shown here is the distribution of exogenous chymotrypsinogen and albumin in successive washes (10 ml 0°C Krebs Ringer solution per approximately 100 mg tissue sample). Also shown is the % remaining in the tissue after 4 washes.

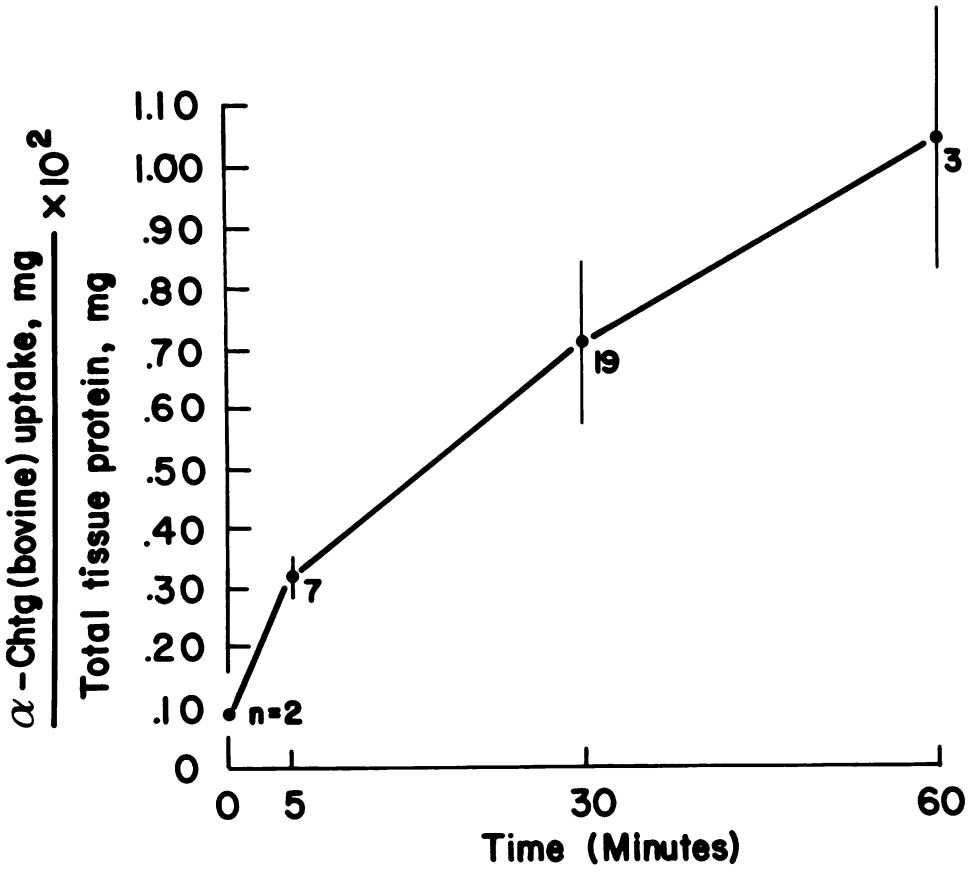
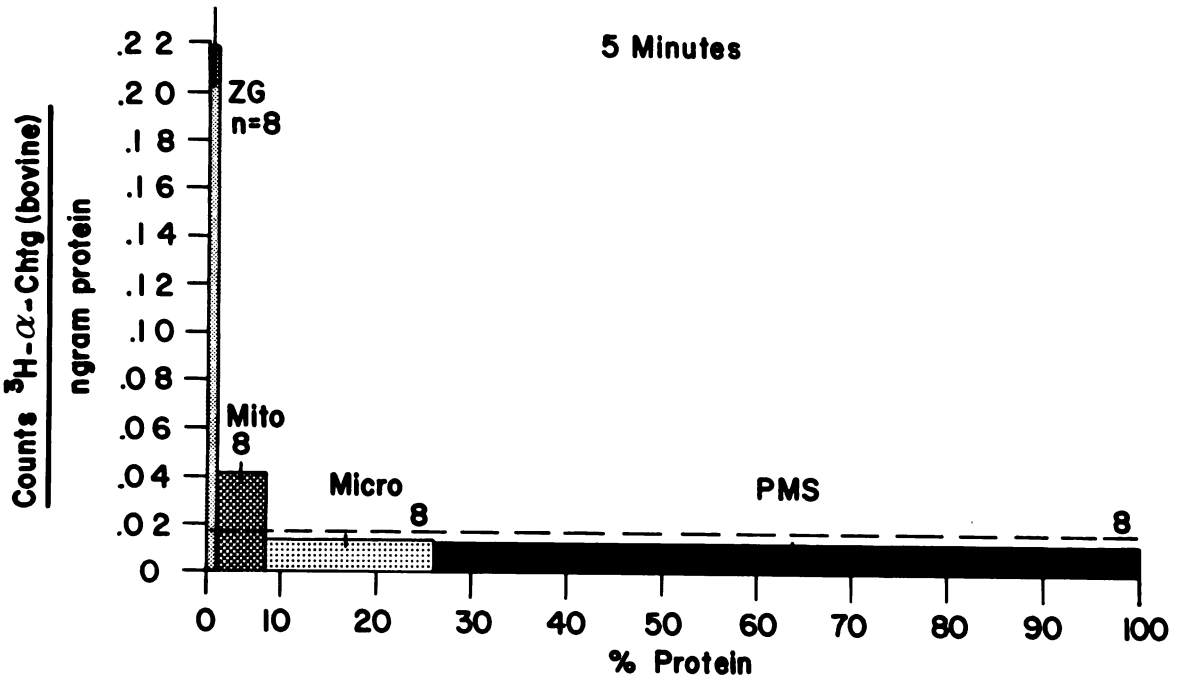


Figure 16. Uptake of exogenous enzyme into whole tissue vs. time Uptake of exogenous chymotrypsinogen is determined as a function of ^{3}H counts minus equivalent counts in ^{131}I-albumin space. Uptake is normalized to quantity of tissue by dividing by total tissue protein. Uptake is plotted against the duration of incubation with labeled proteins at 37°C. Numbers refer to number of experiments and error bars represent standard error of the mean.

chymotrypsinogen per gram tissue protein per hour (±0.1 SEM, n=7) (as determined from tissue concentrations after 5 minute incubation). The rate of uptake appears to decrease with time. In the 30-60 minute period, the rate of uptake is approximately 1/4 that found for the initial period. It is not clear from this finding whether the system is approaching equilibrium as a whole, or if only some pool is.

Entry into different pools of the cell was studied by monitoring either 5 or 60 minutes incubation and washing 4 times so that all rapidly exchanging chymotrypsinogen was lost, zymogen granules (ZG), heavy mitochondria (M), microsomes (ER) and postmicrosomal supernatant (PMS) fractions were separated. Specific radioactivity (counts representing exogenous enzyme per nanogram protein) is shown in Figure 17a for 5 minutes and in 17b for 60 minutes incubation. On this basis exogenous enzyme shows the greatest concentration in the granule fraction and least in the postmicrosomal supernatant and microsomes. The exogenous chymotrypsinogen is distributed similarly to endogenous chymotrypsinogen in the sense that it is concentrated in the zymogen granules, (see Figure 18).

To determine pool equilibration the approach to equilibrium of tritiated chymotrypsinogen with total chymotrypsinogen content was examined. This is shown in Figures 19a and b comparing tritium counts [representing exogenous α-chymotrypsinogen (Bovine)] per millimole ATEe split per minute in activated samples (chymotrypsinogen activity) in different pools. Activated chymotryptic activity includes both exogenous and endogenous chymotrypsinogen, whereas bath activity includes exogenous enzyme almost exclusively. The approach of a certain pool's specific radioactivity to the bath's specific radioactivity conotes tracer equilibration. After 60



- 17a-

Figure 17. The specific (protein) radioactivity of different cell fractions. Shown here is ^{3}H counts (representing α-chymotrypsinogen (Bovine)) per ng tissue protein after 4 tissue washes. Fractions are: ZG (zymogen granule fraction); mitochondria; microsomes; and PMS (postmicrosomal supernatant fraction). Each specific radioactivity is plotted against % total recovered protein (after washes) represented by these fractions. The dashed line represents the sum of the counts collected in these fractions divided by the sum of the protein collected. The numbers represent the number of experiments from which the determination was made. Error bars are the standard error of the mean. Figure 17a is fractionation after 5 minutes incubation and Figure 17b after 60 minutes incubation.

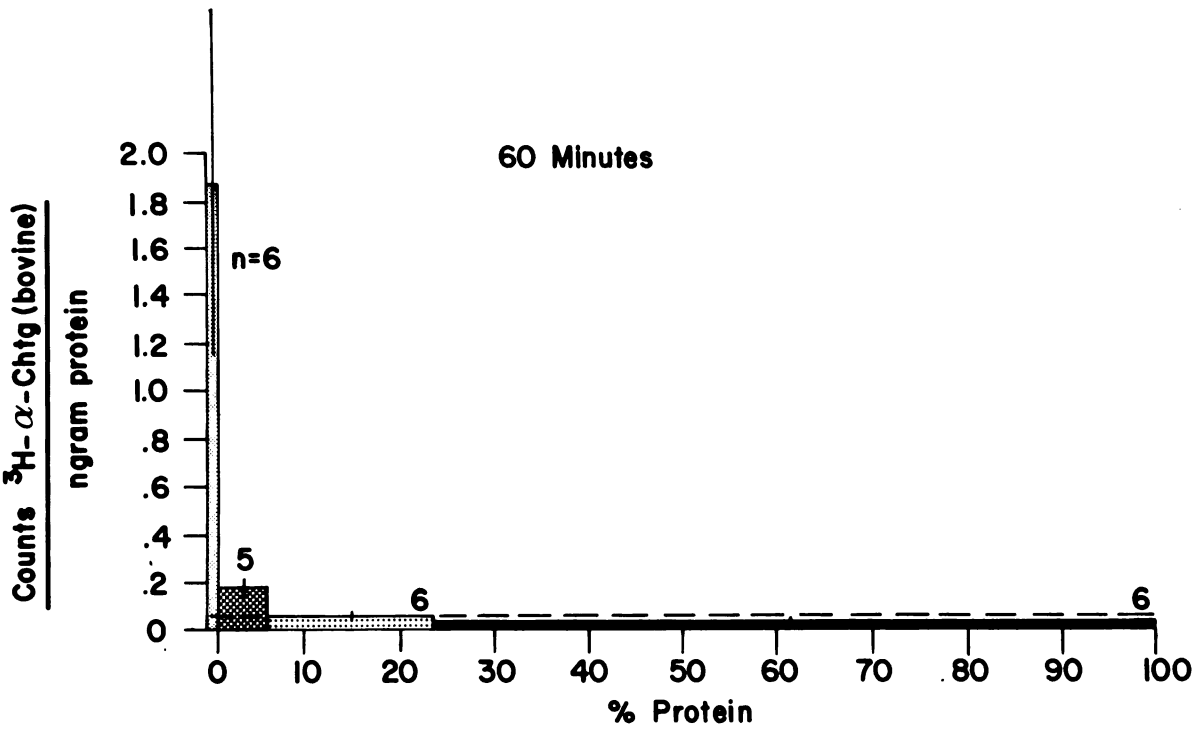

60 Minutes
n=6
5
6
6
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
0
10
20
30
40
50
60
70
80
90
100
% Protein
Counts ^{3}H-α-Chtg (bovine)
ngram protein

Figure 17b

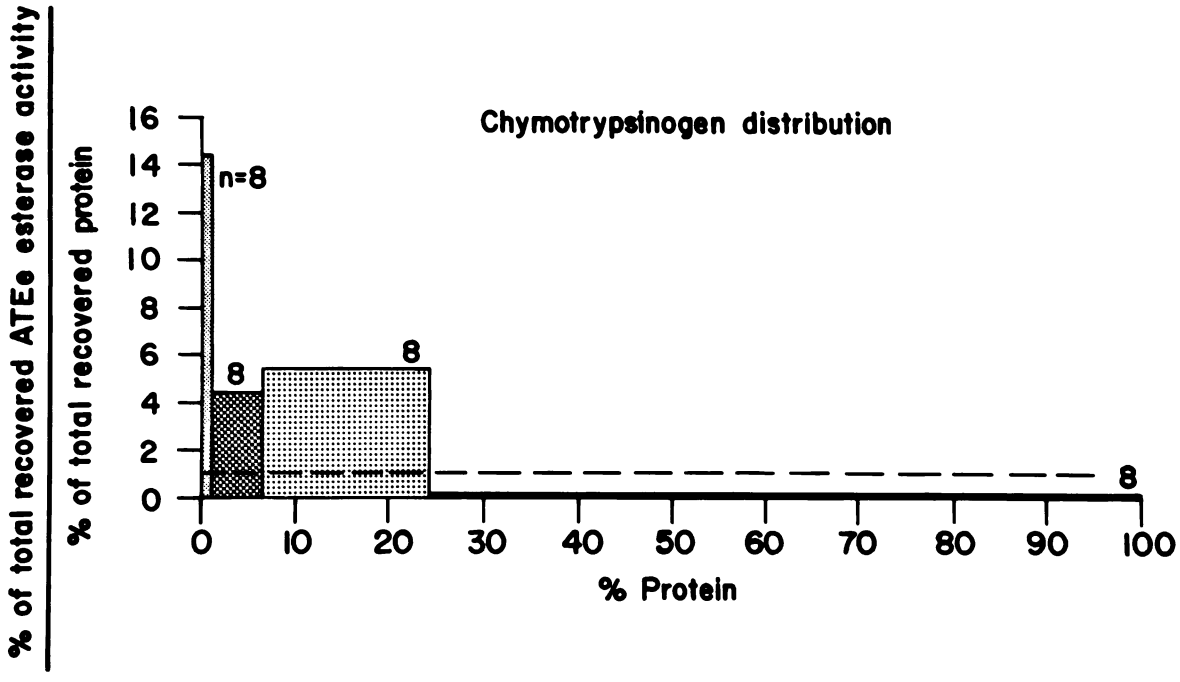


Figure 18. Chymotrypsinogen distribution. This graph is similar in form to Figure 17. It represents specific chymotryptic activity, (in terms of protein). Percentage of recovered ATEe esterase activity divided by percentage of recovered protein is plotted against percentage of recovered protein found in the specific fraction. Fractions are again arranged from left to right: zymogen granule; mitochondria; microsome; and postmicrosomal supernatant. Numbers represent the number of experiments. The dashed line represents the mean specific activity.

$(1 \equiv \frac{100\% \text{ ATEe activity recovered}}{100\% \text{ protein recovered}})$.

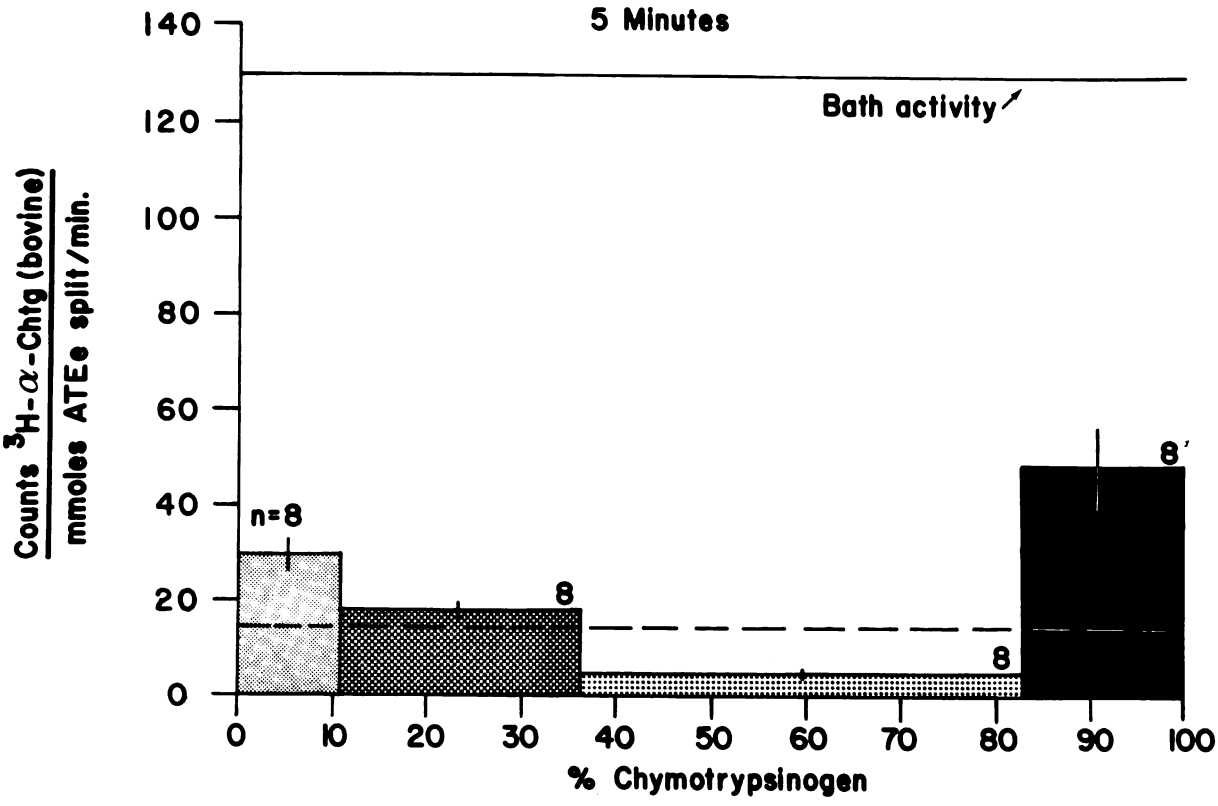


Figure 19 a.

Figure 19. The specific (chymotryptic) radioactivity of different cell fractions. Fractions are again from left to right: zymogen granule; mitochondria; microsome; and postmicrosomal supernatant. ^{3}H counts (representing α-chymotrypsinogen (Bovine) or exogenous chymotrypsinogen) per ATEe esterase activity (total chymotrypsinogen) is plotted against % esterase activity found in each fraction. The dashed line represents the specific radioactivity of the sum of the represented fractions. The numbers are the number of experiments and the error bars are the standar d error of the mean. The solid line represents the bath, or trace, specific activity of chymotrypsinogen. Figure 19a is for 5-minute incubations and 19b is 60-minute incubations.

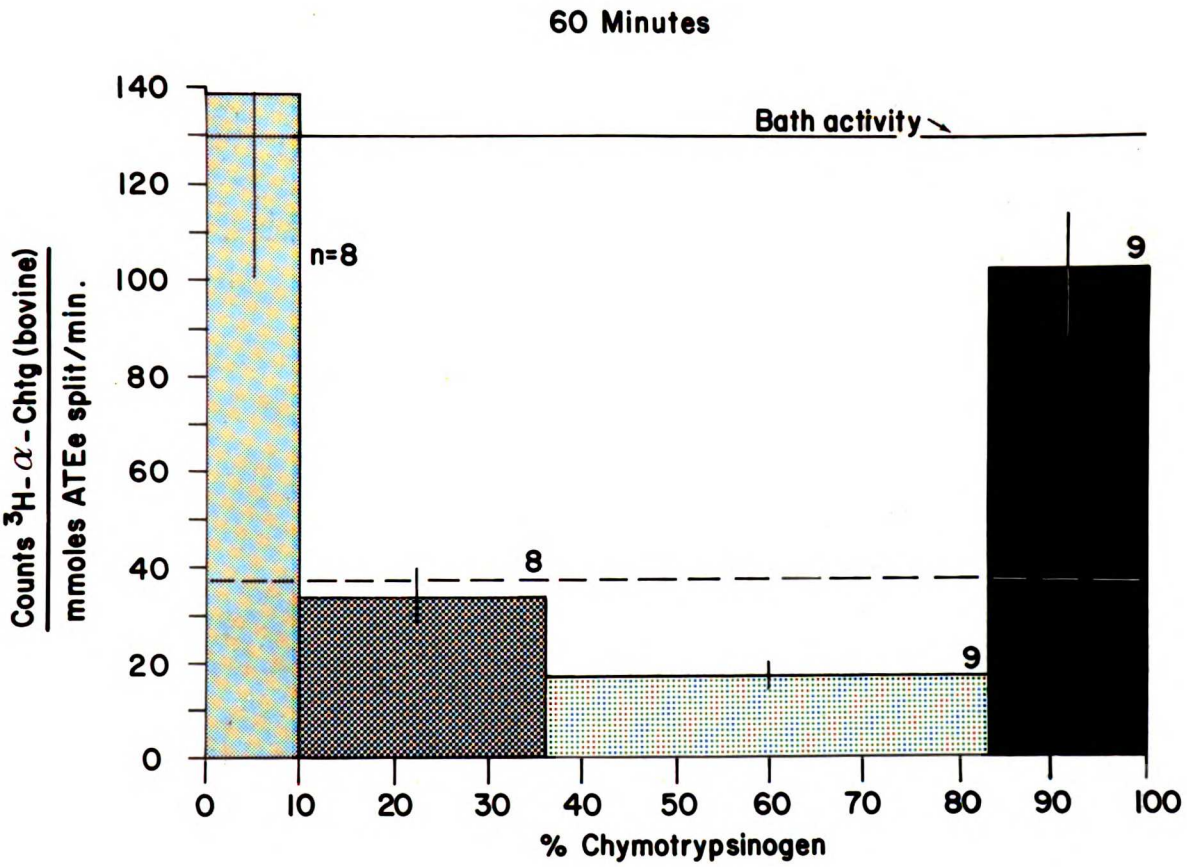

60 Minutes
Bath activity
n=8
8
9
9
Counts ^{3}H-α-Chtg (bovine)
mmoles ATEe split/min.
% Chymotrypsinogen
0
20
40
60
80
100
120
140
0
10
20
30
40
50
60
70
80
90
100

Figure 19b

minute incubation, the postmicrosomal pool and the zymogen granule pool both have specific radioactivities that are statistically indistinguishable from one another or from the bath. This demonstration of pool equilibration also indicates extensive exchange between pools since multiple turnover of enzyme is required to accomplish this. Furthermore, since equilibration occurs with respect to chymotrypsinogen activity instead of with respect to protein content or secretory enzymes in general, this suggests that ^{3}H truly remains associated predominantly with chymotrypsinogen. Lastly pool equilibration indicates that all enzyme in the pool is free to exchange with exogenous enzyme. Any portion of a pool maintained purely from endogenous sources would reduce the specific radioactivity accordingly.

As is shown in Figure 19a, the 5 minute SRA in the PMS is significantly greater than in the ZG ($p<.005$) and both are significantly higher than either of the other two fractions (mitochondria and microsomes) ($p<.001$). The rate of uptake falls off with time. In the first 5 minute period, an uptake rate into the cytoplasmic fraction of 9.5 ± 1.5 SEM (n=8) counts/(μM ATEe split/minute) / minute was found. Over the next 55 minute period the average rate of net uptake fell to 1.0 ± 0.2 SEM (n=9). This decrease in the rate of net uptake suggests pool saturation. A comparison of pool saturation can be made between postmicrosomal supernatant and zymogen granule fractions by comparing the ratio of net uptake into the two fractions for 5 and 60 minutes. The ratio of specific radioactivities of the zymogen granule fraction to the postmicrosomal fraction at 60 minutes was significantly greater than at 5 minutes ($p<0.01$). The 60 minutes ZG/PMS ratio was 4.1 times the 5 minute

ratio. This indicates that the PMS fraction displays saturation considerably before the zymogen granule fraction.

IV. Slice vs. Strip Uptake of Exogenous Enzyme

In experiments where paired pieces of tissue were incubated in similar fashion with the only difference being that one sample was sliced and the other sample kept as an intact strip, sliced tissue did not pick up significantly more chymotrypsinogen per gram of tissue wet weight than did unsliced samples (Sliced = 0.313 ± 0.044 SEM mg exogenous chymotrypsinogen/gr tissue weight/30 minute [n=19] vs unsliced 0.257 ± 0.072 [n=16]). The difference in chymotrypsinogen uptake per gram of tissue was approximately 20% more in the sliced preparations.

Since albumin space gives an estimate of extracellular space that is accessible to protein it also indicates the amount of surface area in the tissue. When tritium uptake is measured per unit surface area no difference at all between sliced an unsliced preparations is seen (see Table 4). The increased chymotrypsinogen uptake in sliced tissue is therefore presumably directly a function of increased surface area for uptake. The absorption of tritiated chymotrypsinogen is proportional to surface area independent of slicing and this suggests that the serosal border of the tissue is capable of considerable enzyme uptake.

	Sliced	Unsliced
Chtg Uptake: $\left(\frac{\text{mg exogenous Chtg}}{\text{gr tissue wet weight x 30 minutes}}\right)$	.313±.044 (n=19)	.257±.072 (n=16)
Albumin Uptake: $\frac{^{131}I \text{ tissue}/^{131}I \text{ bath}}{\text{gr tissue wet weight x 30 minutes}}$	.103±.010 (n=18)	.079±.007 (n=17)
Ratio: $\frac{^{3}H/^{131}I_{tissue}}{^{3}H/^{131}I_{bath}}$	4.69± .52 (n=19)	4.40± .69 (n=18)

Table 4. Sliced vs. Unsliced Tissue. The association of chymotrypsinogen and albumin with tissue as a function of the slicing procedure. Chymotrypsinogen uptake after two washes is expressed as mg exogenous chymotrypsinogen/gr tissue wet weight/30 minutes. Albumin uptake is expressed as equivalent bath volume of ^{131}I taken up per gr tissue weight/30 minutes. The chymotrypsinogen: albumin concentration (relative to bath) is shown in the bottom line. Variation is expressed as S.E.M.

V. In Vitro Pancreas

These experiments (above) suggest that the serosal membrane of pancreatic cells is capable of enzyme uptake. Furthermore, this serosal membrane appears to be approximately as permeable as the ductal membrane. If chymotrypsinogen can enter the pancreatic acinar cell via the serosal border and, since the cell is capable of enzyme secretion, then the pancreas may possess the capability of mediating a transpancreatic flow of chymotrypsinogen and perhaps other enzymes. Labeled chymotrypsinogen and ^{131}I-albumin were added to the bathing medium of a whole *in vitro* pancreas preparation. In this preparation ductal cannulae lead outside the bathing solution isolating the ductal fluid from the bathing medium. Radioactive label added to the bath appeared in secretion in significant quantities within 10 minutes. The appearance of exogenous tritiated α-chymotrypsinogen in secretion is shown in Figure 20. The pancreas was ~20 x more permeable to exogenous chymotrypsinogen than albumin. The length of time required to approach a steady state exogenous enzyme transport is of the order of 75 minutes. Counts however, were seen in secretion within 5 minutes of the addition of label. Addition of cold chymotrypsinogen to the bath produced a transitory decrease in the amount of label transported, but the amount of label rapidly reapproached the previous secretory rate. The transient decrease in secretion of ^{3}H-chymotrypsinogen with no decrease in ^{131}I-albumin secretion suggests that the cold chymotrypsinogen may be competing with labeled material for transport (the initial chymotrypsinogen bath concentration was 2 mg % and after addition of chymotrypsinogen it was 20 mg % (0.8 μM and 8 μM)). This therefore, suggests that pancreatic permeability to chymotrypsinogen is a selective event mediated by a reaction dependent process. The transient decrease

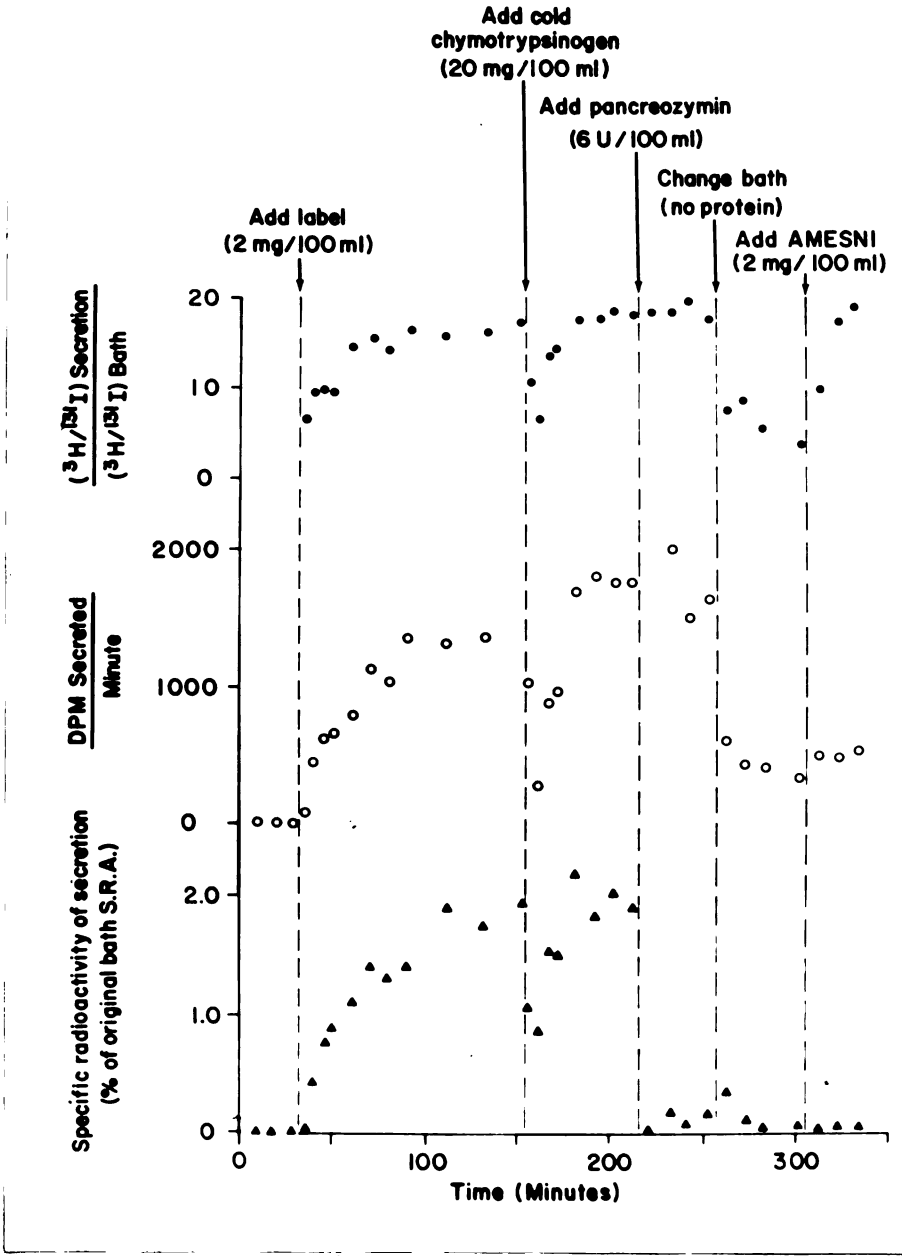


Figure 20. Transpancreatic movement of exogenous enzyme. The permeability of the pancreas to ^{3}H-chymotrypsinogen related to ^{131}I (top), a direct measurement of the rate of secretion of exogenous enzyme (middle), and the specific radioactivity of the secretion in terms of protein (bottom) over time in response to changing stimuli. During the run of the experiment label was added to the bath (2 mg Chtg/100 ml bathing media), cold chymotrypsinogen was added reducing the specific radioactivity of the bathing media 11 fold, pancreozymin (GIH Laboratory) was added 6 Ivy dog units/100 ml bathing media), the bath was changed with no enzyme or label in the bath, and finally, during the wash out phase, AMeSNI (acid methanol soluble, neutral insoluble fraction of the Jorpes and Mutt (1962) pancreozymin-secretion purification) a fraction known to stimulate chymotrypsinogen secretion (Adelson and Rothman 1973) was added. Enzyme is seen to approach 20 fold greater permeability than albumin. This same 20 fold limit (without AMeSNI) has been seen in two other trials - greater than 20 x ratio is only seen after AMeSNI stimulation.

		AMeSNI			PANCREOZYMIN		
	#	Before	After	% Change	Before	After	% Change
	1	0.79	1.43	+81	5.00	5.40	+ 8
Cpm/min:	2	16.9	27.4	+62	28.3	29.0	+ 2
	1	.023	.035	+52	.950	.079	- 92
SRA:	2	7.25	8.23	+14	8.23	3.15	- 62

Table 5. Effects of stimulants on transpancreatic movement of enzyme. Shown here is the transpancreatic flux (Cpm/min of exogenous ^{3}H chymotrypsinogen coming out in secretion) and specific radioactivity of secretion of in vitro rabbit pancreas preparations in response to the addition of stimulant to the bathing medium of the preparations. Stimulants were added in the concentration 2 mg% AMeSNI or 6 Ivy dog units/ 100 ml. Results presented are the mean of the two periods preceding the addition of the stimulant, and the mean of the two periods collected 1/2 hour after addition. The delay was provided in order to give the stimulant a sufficient time to maximize its effect. AMeSNI increased transpancreatic flux of enzyme, whereas pancreozymin did not. Both AMeSNI and pancreozymin increased protein secretion, but pancreozymin had a much more pronounced effect (pancreozymin increased protein output by 836%, AMeSNI it by 57%). Experiment 1 here refers to the experiment shown on the preceding page, with pancreozymin addition first and AMeSNI addition later. Experiment 2 had this sequence reversed. Also, the experiment 1 AMeSNI administration was given after the removal of the label from the bath (during the wash out phase).

and rebound of exogenous chymotrypsinogen secretion suggests that a middle compartment (ie. - the cell) exists in the secretory process. This compartment may exhibit exchange diffusion with the bath causing a transient absolute decrease in label content in the cell. Reequilibration after the addition of cold chymotrypsinogen took approximately 25 minutes. When chymotrypsinogen was removed from the bath, the counts in secretion fell rapidly with over 50% reduction seen within 5 minutes of removal of the label. AMeSNI, a mixture of duodenal peptides shown (Adelson and Rothman 1973) to increase chymotrypsinogen secretion, increased transpancreatic flux of chymotrypsinogen (Table 5). Pancreozymin, known to increase protein secretion but not chymotrypsinogen secretion, did not significantly affect transpancreatic flux of chymotrypsinogen though it did enhance protein secretion. These are preliminary results requiring further experimentation.

VI. Gut Sacs

Transport across gut sacs and everted gut sacs was studied. Tri tiated α-chymotrypsinogen (Bovine) and ^{131}I-albumin entered sacs. Sacs were more permeable to chymotrypsinogen than to albumin 7.1 ± 0.6 (n=12) times. The amount of chymotrypsinogen entering the sacs during the 60 minute incubation is shown for different levels of the gut in Table 6. Transmural fluxes were observed in both directions, but the mucosal-serosal flux was, for all areas studied, greater than the serosal-mucosal fluxes. The flux ratio was greatest in the upper ileum where it approached 10. Duodenal M-S fluxes were similar to ileal M-S fluxes, but in the duodenum an almost comparable S-M flux was seen. Hence net flux was smaller. M-S fluxes in the upper ileum were 0.24 micrograms/hr for a gut section approximately 1 cm long. An entire rabbit ileum (approximately 25 cm long) could transport at the same rate 6 micrograms of chymotrypsinogen/hr. 42% of the tritium in everted sacs was TCA precipitable as opposed to only 12% in noneverted sacs. The label could have entered intact and then dissociated or the dissociated label itself could have entered the sacs. Even if all free label in everted sacs is discounted, and bound label in everted sacs is compared to total label in noneverted sacs, the maximum bias, a flux ratio of 4 is still found.

	$J_{m \to s}$	$J_{s \to m}$	Net Flux	$J_{m \to s}/J_{s \to m}$
DUODENUM	.225±.142(3)	.120±.017(3)	.105	1.9
ILEUM	.244±0.63(3)	.025±.004(3)	.219	9.8

	% of Tritium Precipitated by TCA
Everted Sacs	42±10(6)
Noneverted	12±3(6)

Table 6. Gut Transport of Chymotrypsinogen. The undirectional fluxes of tritiated chymotrypsinogen at different levels of the gut. The units are expressed as μg chymotrypsinogen transported per hour per gr tissue weight. Variability is expressed as Standard Error of the Mean. The number of experiments are shown in parenthesis. The flux ratio is the ratio of the two means. All flux measurements were determined from 1 hour incubations in solutions containing 1 mg/ml chymotrypsinogen. % of tritium precipitated by TCA is given for pooled everted and noneverted sacs since it did not appear to vary with the section of the gut.

VII. Whole Animal Circulation

Since the two necessary steps in enzyme circulation appear to exist; transpancreatic movement of chymotrypsinogen (In vitro Pancreas) and chymotrypsinogen absorption from the gut (Gut Sacs), I tested to see if chymotrypsinogen could circulate from gut, to blood, to pancreatic secretion, and finally back to gut. The gut was tied just below the papilla of Vater and 5 mg of labeled chymotrypsinogen (SRA) was injected into the gut just below the tie. Pancreatic secretion was collected and after 2 hours, a total of approximately 1.5 micrograms of labeled chymotrypsinogen appeared in secretion. The rate of label secretion, however, was still increasing rapidly at the end of this time period (Table 7). 50% of the counts appeared in the last 20 minutes, giving a rate for this period of approximately 1.2 μg chymotrypsinogen secreted per hour.

The possibility that ^{3}H was no longer part of the original enzyme was examined by gel electrophoresis of samples of the secretory product. The exogenous α-chymotrypsinogen (Bovine) has a migratory peak very different from all of the endogenous enzymes (Figure 21). This allowed for relatively complete separation of exogenous chymotrypsinogen from endogenous enzymes. Slicing the gel at 0.5 or 1 cm intervals and counting tritium content in the gels showed 67% of the label in the area where the exogenous peaks would be. This peak represents at most a few percent of total protein seen in secretion. Specific radioactivity of protein with migratory characteristics similar to exogenous chymotrypsinogen is several orders of magnitude greater than in the area of endogenous protein secretion.

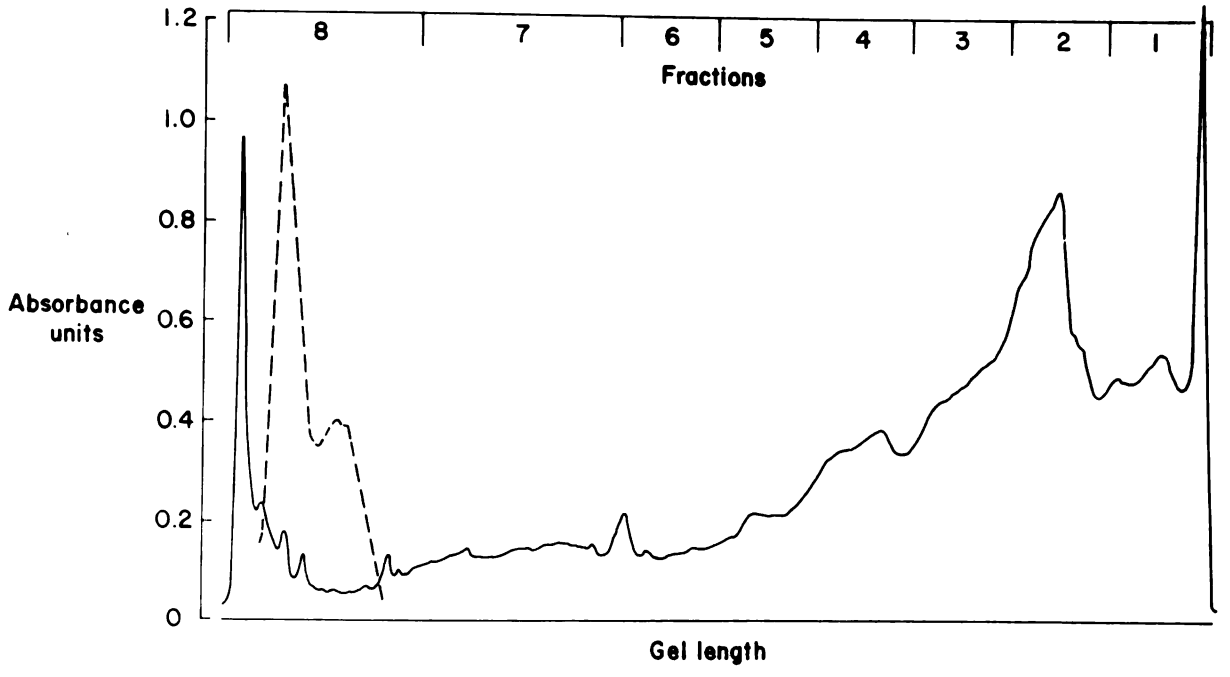


Figure 21. Gel scan of secretion after administration of ^{3}H-chymotrypsinogen (Bovine) into the rabbit gut. The solid line represents a scan of a gel prepared in the manner of Jones (1967) and stained by the procedure of Ahlroth and Mutt (1962) on which 25 µl of secretion collected 1 hr after the injection of labeled enzyme into the gut was layered. The gel was run for 25 minutes at 3.5 amps/tube. The dashed line represents a scan of 10 µl of exogenous enzyme run in the same way at the same time. The number at the top represent the dividing line for gel Fractions analyzed for tritium content. Fraction 8, the section of a gel of exogenous enzyme containing >70% of the label, in secretion contained 67% of the label although only a few percent of the total protein.

Period	Secretion Rate-µg/hr
1	.09
2	.27
3	.89
4	1.23

Table 7. Enteropancreatic circulation of enzyme. Rate of pancreatic secretion of exogenous chymotrypsinogen is shown in response to an injection of 5 mg exogenous enzyme into the duodenum of rabbit below the pancreatic duct. These are 20 minute collections begun 40 minutes after the injection.

DISCUSSION

The results presented cover many diverse topics. They demonstrate (1) the in vitro permeability of isolated zymogen granules to secretory proteins, (2) a size constraint placed upon these granules, (3) cell membrane permeability to exogenous enzyme, (4) permeability of both the serosal side and mucosal side of acinar cells, (5) transpancreatic flux of pancreatic digestive enzymes in vitro and, finally, (6) that transpancreatic movement of enzyme may be part of a system in which whole, intact enzymatic proteins are cycled through the digestive tract and resecreted by the pancreas. These diverse observations fit with many of the observations ennumerated in the introduction and suggest that pancreatic protein secretion may not occur as is now thought. I shall try in the following section to relate the observations in the literature (see Introduction) and the present observations in such a way as to supply a new picture for pancreatic function.

I. In vitro Granule Permeability

The zymogen granule has been shown to function as part of the secretory process emptying upon stimulation of the pancreas and then disappearing from view. This in vivo response of the zymogen granules along with in vitro observations demonstrating high concentrations of secretory proteins in zymogen granules suggests that they serve as stores for pancreatic enzymes. In vitro properties of the zymogen granule discussed in the introduction suggest they are in some sort of solid state formed so that highly concentrated proteins are not osmotically active (Burwen 1972a). Some preliminary results of other investigators suggest that granules need

not completely lose their identity while losing or releasing enzyme (Burwen, 1972b) and, therefore, the granular membrane, at least in vitro, may not present an absolute barrier to protein movement. My in vitro granule experiments support this view by demonstrating that granular enzyme is in equilibrium with its surroundings and that release of enzyme may be mediated by a specific process. The specificity of the process itself suggests that release is not merely a function of enzyme diffusion through holes developed in the isolation process but is mediated by a specific system..

1. Intragranular Enzyme is in Equilibrium with Enzyme in the Surrounding Solution:

The dilution experiments provide evidence that the granule enzyme is in equilibrium with its surroundings. Rothman's work (1971) with reconstituted granules suggests a means by which an equilibrium could be maintained between granules and their environment. Workers, however, have felt that granule membranes normally provide an absolute barrier to protein movement and that the binding characteristic functions possibly so the granule can concentrate enzyme. It is felt that it does not imply any sort of physiological equilibrium distribution. This is true even though Burwen (1972b) has presented evidence suggesting that partial release of enzyme is possible. Partial release indicates that enzyme can move out of the in vitro collected granules without the granule losing its particulate identity. Dilution experiments try to combine the observations of binding potential in the granule and partial loss of enzyme from isolated granules. They try to show that granules normally, at least in the in vitro isolated condition, are in equilibrium with their

environment. Release of native endogenous enzyme occurs apparently in response to decrease of this enzyme concentration in the surrounding environment. Addition of exogenous enzyme (as is seen in the labeled chymotrypsinogen additions to granule suspension) or increasing endogenous concentrations outside the granule both apparently reverse the release of enzyme. This behavior is based on the fact that non-granular enzymatic concentration will vary inversely with dilution, whereas granular enzymatic activity (concentration x activity coefficient) will not vary with dilution due to the fact that dilution does not vary granular volume.

In review, the evidence for enzyme existing in equilibrium with enzyme in the bathing medium is: (1) Increasing the volume of suspending media (at least at low concentrations of granule suspension) causes increased release of enzyme from granules (dilution experiments). (2) This release is a continuous function of dilution suggesting that enzyme release is not caused by decreasing the content of some stabilizing factor. If this were the cause, then granules should all become unstable at a specific dilution producing a pronounced sigmoidal curve. (3) The reaction is reversible by increasing bath content of enzyme, either endogenous or exogenous. Burwen has recently shown (1972b) that enzyme reagregation also occurs in response to removal of ions by dialysis. (4) Continuous release of enzyme is possible in response to flow of 0.3 M sucrose. The ultrafiltration experiments suggest that this release is dependent on time ($K_{release}$) and on flow ($K_{release}/K_{uptake}$). This response is consistent with the enzyme being in equilibrium between bath and granule. The equilibrium that exists was not greatly affected by changes in temperature. This is shown in the reaggregated release experiment (Table 1) showing the

same s/p ratio at 60 minute incubation (equilibrium) at 37°C and 25°C demonstrating an insensitivty to temperature change.

If enzyme is in equilibrium, the "activity" ("activity" in the physical instead of the enzymatic sense) of enzyme in granules could vary two ways with release. Either release would lower granule enzymatic concentration and thereby lower activity or the enzyme itself may be in a solid state form where its activity is a constant even if the granular concentracion were to change. These two possibilities for granular enzyme behavior would alter the form of dilution dependent release. The reason for this alteration would be that for the former (enzymatic "activity" in the granule varies with enzymatic concentration) granular activity would fall with loss of enzyme from the granules, but in the latter (solid state form - enzymatic activity is a constant) decreases in enzymatic content in the granules would, up to a point, not alter the enzymatic activity in the granules. Doubling suspension volumes halves the external concentration of enzyme which could only be compensated for by release of enzyme from the granules (Figure 22). If granule enzyme activity were a function of granule enzyme content, then the magnitude of enzyme release would not have to equal previous enzymatic supernatant concentrations since the granules themselves would be depleted and the supernatant enzyme concentration would not have to be restored to its previous value. If granule enzyme activity were proportional to concentration, then the supernatant/pellet ratio would have to be inversely related to suspending volumes. This is because supernatant enzymatic concentration ([S]) would equal supernatant enzyme amount (S) divided by suspending volume (V) and granular enzymatic concentration ([P]) would equal pellet enzyme amount (P)

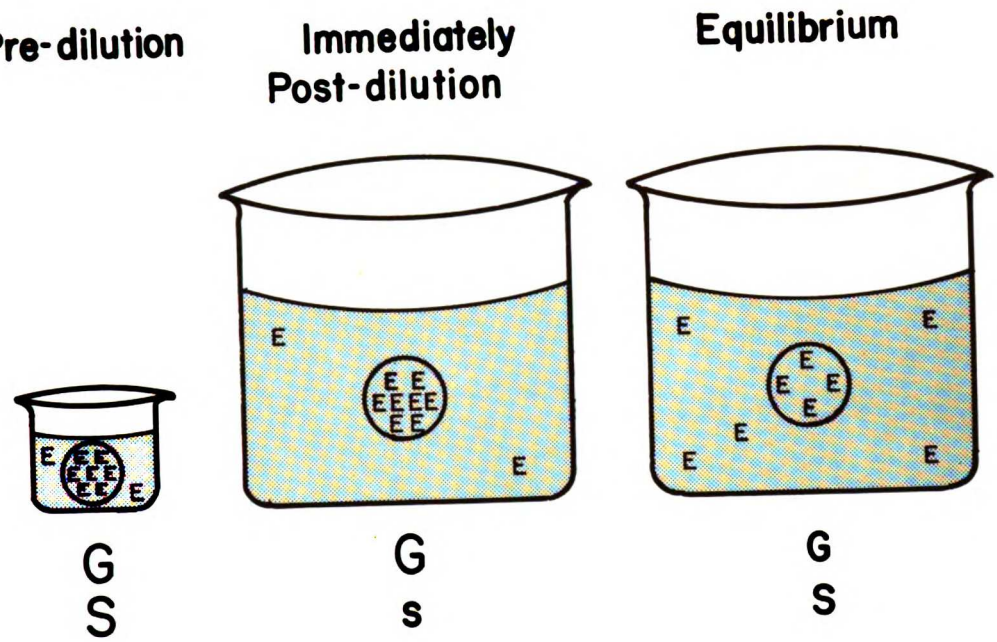


Figure 22. A schematic representation of the dilution effects. Three states, predilution, immediately after dilution before redistribution can take place, and the final equilibrium state after dilution. The "G" and "S" at the base of each diagram represent the concentration of enzyme in the granule and supernatant respectively by the size of the letters. Immediately post dilution, concentration in the suspending media was reduced with the amount there staying the same, and the concentration and amount in the granule was unchanged. After equilibration the concentration in the granule fell along with the amount in the granule, but the concentration and the amount in the media increased.

divided by granular volume (P_V). Unless the granule volume changes [$P_V = K_1$] the granular concentration is proportional to the pellet enzyme [P] = (P)/K_1. This means that if [S] remains proportional to [P], then (S)/V α (P) $\therefore$ S/P (supernatant to pellet ratio) α V or the distribution varies inversely with the dilution. If the granules do behave as solid state aggregates whose activity is a constant, not a function of volume, then doubling the suspending volume should require compensatory doubling of release. That is that solid state aggregate granules would require the percentage release to vary inversely and linearly with dilution (percentage loss = constant x dilution), whereas non-solid state behavior suggests (as described) that $\frac{s}{p}$ ratios would be a linear function of dilution. A log-log slope of 1 indicates a linear relationship. If % loss = constant x dilution, then when s/p is plotted against dilution, a log-log slope > 1 would be seen for all dilutions, the slope increasing with release (Fig. 23). In dilution experiments, no log-log slopes > 1 were ever recorded for individual experiments. For pH 7.0 slopes approached 1 but did not excede it. Slopes for chymotrypsinogen release at pH 5.5 at 60 minutes, a state shown not to be at equilibrium, were below 1.

The significance of a linear s/p variation with dilution is that the effective tendency to release enzyme molecules from a granule would be proportional to the concentration of enzyme in that granule (as discussed above). If enzyme exists in large polymers inside the granule, then only terminal molecules (either surface molecules in a globular polymer or end-chain molecules in a linear polymer) would be free to exchange. The

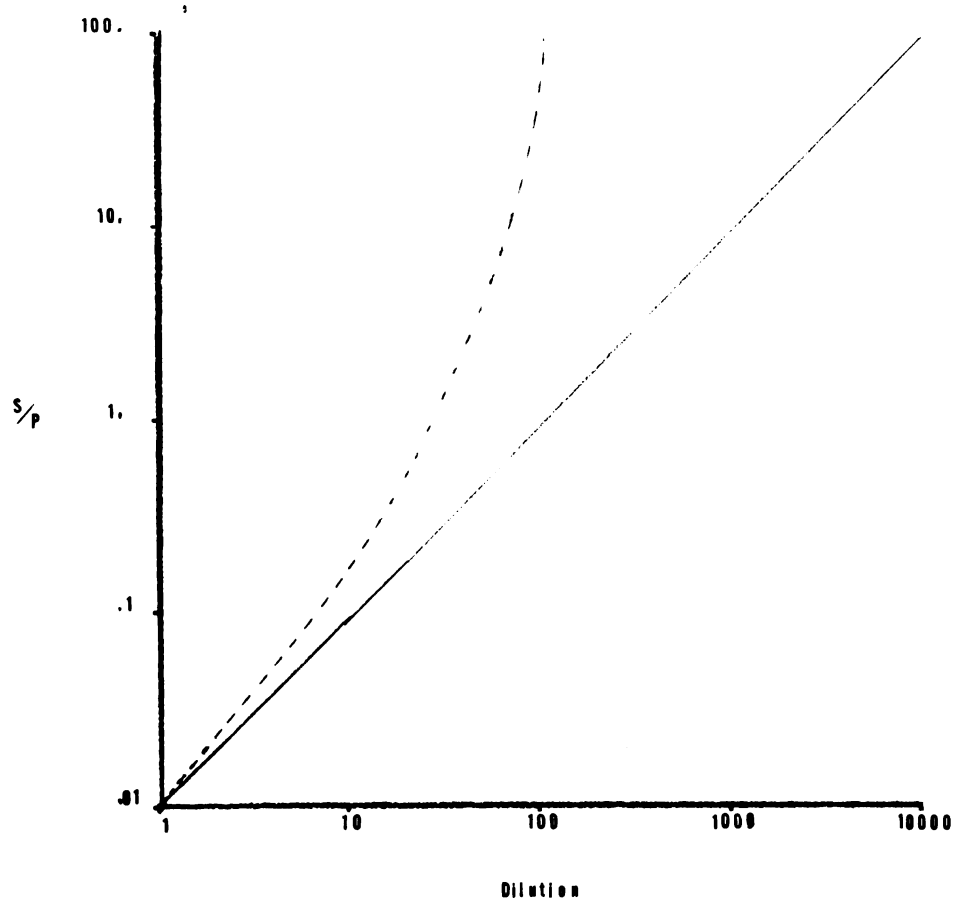


Figure 23. Theoretical variation of s/p with dilution. Plotted on a log-log plot is the theoretical value of s/p plotted against dilution. A dilution (volume) of 1 is set to equal an s/p distribution of 0.01. The solid line represents the relationship if s/p is proportional to dilution. This line has a slope of 1.0. The broken line represents the plot if % loss is proportional to dilution ($\frac{s}{s+p} \propto V$). This line has a slope always greater than 1.0.

above results suggest that all molecules are free to exchange ($flux_{out} \alpha$ concentration)^4, then this suggests that polymers, if they exist, must be of very limited size. Globular polymers could not exist of much more than 27 molecules and still maintain almost all molecules on the surface. Linear (chain) polymers as suggested by Rothman (1971) virtually could not exist. Furthermore, with the elimination of long polymers (polymers that could originate on the surface of the granule and extend to the granule's center) as a possibility, this effectively states that release of enzyme from the granule must follow kinetics of a three compartment system. These compartments are: (1) The external environment separated by the granule membrane from (2) free enzyme inside the granule which exchanges with (3) bound enzyme inside the granule. Two separate exchange processes must therefore occur: (1) one across the granule membrane and (2) the other between free and bound enzyme. The reaggregation studies (discussed below) are an attempt to separate these two processes.

2. Digestive Enzyme Mediation of Equilibrium:

Equilibrium behavior could be mediated by some granule factor other than granule enzyme, such as lipid, ATP, Calcium or some other molecule, but this is unlikely due to observations made in the reversal experiments. The addition of exogenous enzyme showed substantial recombination with the granules, suggesting that no outside mediating factor is the limiting factor for reaggregation. The dialysis experiments, where the suspensions are reconcentrated using dialysis, show that if there were a hypothetical stabilizing factor it could not pass through the dialyzing membranes. If it did pass through the dialyzing membranes then it would be readily lost and no reconstitution would be seen (in fact dilysis should cause increased

release of enzyme, which it does not). The dialyzing membranes are made of cellulose acetate with molecular pass weights of approximately 5,000. This indicates that lipids could not be the stabilizing factor since they would pass right through the cellulose acetate. Since ATP is smaller than this molecular weight and should therefore be lost also, it could not be the stabilizing molecule. Calcium would also be lost through the dialysis. This result suggests that if any of these molecules are necessary for association with the granule, they must be relatively tightly bound to protein even after release and therefore not lost in the dialysis procedure. Again the exogenous addition of chymotrypsinogen causing reassociation suggests that these factors could not, in any event, be the limiting factor in the normal aggregate.

3. Membrane Mediation of Equilibrium:

These experiments show a slow time course for release suggesting that there is significant factor delaying the approach to equilibrium. This factor can be either (1) a slow associative and disassociative rate for the binding reaction in the granule with the membrane producing no barrier, (2) a diffusional membrane barrier caused by small and infrequent holes appearing in the granule due to the <u>in vitro</u> collection process which impedes diffusion of molecules, or (3) a rate limiting transport process existing in the granule membrane by which secretory enzymes cross this membrane. Comparison of the pattern of enzyme release from granules with the pattern of enzyme release from pH reassociated material suggests that an additional barrier exists for release from whole granules. pH reassociated material is obtained by collecting granules in the manner described (Methods) and then raising the pH from the stable 5.5 level to

pH 8.6. At this alkaline pH suspensions of granules lose their turbidity and centrifugation fails to produce any significant precipitate, indicating release and solubilization of the granule content. When the pH is then returned to 5.5, solutions begin to regain their turbidity and a precipitate forms. On examination of this precipitate (S.S. Rothman and S. Ito, Personal Communication), it was found that granule membranes appear to be disrupted and protein aggregates appear to be associated with filamentous elements. This filamentous distribution of proteins suggests that the granule's vesicular structure has been disrupted and that the protein aggregates are now exposed directly to the outside environment. The reaggregated material provides a means of examining the associative behavior of the secretory proteins with the granule structure without a membrane permeability barrier. Upon dilution of the reaggregated suspensions, redistribution of protein from granule to supernatant appeared to be complete within 10 minutes and appeared to be stable throughout the examined time course thereafter. (Table 1-1/2 time for release of chymotrypsinogen from native granules was approximately 1 hour). The granule pH solubilization and reaggregation process eliminated the rate limiting step in release of enzyme from intact granules. This rate limiting step is most probably the transit across the membrane and removal of the delay is consistent, then, with the apparent (by electron micrographic evidence) removal of the membrane. If, therefore, the membrane does present a barrier, then one of the last two explanations must be true.

The possibility that "holes" are created in the granule membrane during isolation allowing for slow diffusion of enzyme out through these holes, but impeding the rapid approach to equilibrium seen in the reassociated

material was exmined by viewing the specificity of the release and reuptake kinetics. As shown in results, release of all proteins does not occur in a parallel fashion either following absolute amounts of release or in half time of approach to equilibration. Of the two proteins examined specifically amylase and chymotrypsinogen, amylase appears to be released more rapidly and more completely than was chymotrypsinogen. Half times for amylase release were significantly less than were the half times for chymotrypsinogen release, and the amounts of amylase release were at all points viewed considerably greater than was release of chymotrypsinogen. This demonstration of selective and preferential release of amylase suggests that size constraints alone produced by physical holes in membranes could not account for this discrepancy in release pattern. This is so because amylase is a considerably larger molecule than is chymotrypsinogen having a molecular weight of 64,000, whereas chymotrypsinogen's is approximately 25,000 (both have the same approximate coefficient of eccentrictly [1.13] showing that correspondingly their radii must be proportional to the cube root of their mass). More rapid release of the larger molecule suggests that if the limiting factor for release were holes in the membrane the smaller chymotrypsinogen should be released more rapidly than the larger amylase molecule, which is seen not to be the case. Furthermore, if the rate limiting step for release of enzyme from the granule were a simple diffusional barrier presented by the existence of holes in granules, then this simple diffusional barrier should behave in kinetic fashion as other simple diffusional barriers. Simple diffusion is rather unaffected by changes in temperature, that is it presents normally a Q_{10} of only slightly above 1. Q_{10}s are minimally above 5 over a broad

temperature range and for certain temperature ranges (ie. the temperature range around 20-25°C) possibly a great deal higher than this. This high Q_{10} suggests that a very significant energy plateau, or, more probably, a distinct protein configuration (dependent on temperature) is necessary for discharge of digestive enzymes from the granules. These requirements are not consistent with a simple diffusional barrier. In summary then, the selectivity of the process is not dependent upon physical size, and the existence of a high Q_{10} for release both suggest that this release process is reaction dependent. This is consistent with the view that enzyme moves across granule membranes, rather than through holes in the membrane, and that such movement is accomplished by a "membrane transport" of these proteins.

To recapitulate then, eliminating the first two possibilities as a cause for the delay in the approach to equilibrium, that is (1) the membrane is not a barrier and the dissociative reaction is the rate limiting step, and (2) the membrane is a non-selective barrier due to "artifactual" holes produced by the separation procedure, leaves the final possibility that there is a definite membrane transport system existing within a granule membrane barrier and that this is the rate limiting step for release of enzyme from the granule. In vivo experiments discussed later lend support to the conclusion that these results are not artifactual, and that they have an in vivo counterpart.

II. Zymogen Granule Size

Zymogen granules collected from a group of rats were found to vary in size over only a relatively narrow range. The total observed variation (S) for our best estimate of the standard deviation (σ) was 14% of the mean (11% in a single cell). The distribution was similar to a normal distribution (Figure 19), fits well with a unimodal distribution, and would be impossible to reconcile with a multimodal distribution where the peaks were significantly separated. If size were a function of content this finding would make homogenous enzyme content in each granule appear less likely, since it is unlikely that the properties of different enzymes would act to produce granules of such similar size. The observed variation is made up of true granule size variation, measurement error, and variation due to possible distortion during fixation, sectioning and handling. This means that the true standard deviation for size of zymogen granules must be less than the observed 14% of the mean size.

The size of some intracellular vacuoles can vary greatly, for example, as fat vacuoles vary with the nutritional state of the animal. Such vacuoles vary greatly in size from animal to animal, as well as from cell to cell. Their size appears to be limited only by the size of the cell itself. Secretory granules, such as the zymogen granule are always much smaller than the cell (approximately one order of magnitude smaller in radius), and their range of size is rather limited. Some factor or factors must control the size of the developing secretory granules in order to produce this narrow range. The control appears to be rather precise for each cell, and furthermore, does not vary significantly even from animal to animal of the same species. Control mechanisms of many types can

be envisioned, but the uniformity of size, particularly between animals, suggests that the control itself may be a function of the structure of the zymogen granule. This view leads me to suggest two possible models for zymogen granules organization which would of themselves control granule size.

Certain properties of the zymogen granule suggest an interpretation of its structure and a potential control mechanism for determining granule size. Zymogen granules are very resistant to osmotic forces (Burwen 1972a & b); are disrupted in the presence of even relatively low ionic strength solutions; show instability in a pH-dependent manner (Hokin 1955); and their contents apparently passively reaggregate (Rothman 1971). Taken together these characteristics suggest that granule contents are probably not in a free water phase, but in bound form. Furthermore, maturation of zymogen granules in situ is not dependent on energy sources (Jamieson & Palade, 1971), suggesting an energetically favorable event or configuation. This view of the zymogen granule as a solid state aggregate is consistent with a self regulating system.

If some of the digestive enzyme contents are in the solid state within the mature granule, then an equilibrium would exist between these proteins and like species in the solution. Since the activity of the polymeric or solid material is independent of the quantity present, then the concentration of soluble protein and the pressure within the system solely determines the equilibrium state. At equilibrium, if the concentration of soluble protein is uniform (the cytoplasmic concentration), then the pressure must assume a unique value. The difference between this

value and atmospheric pressure is the pressure across the granule membrane. It is inversely proportional to the radius of the granule by the Laplace relationship $P = K/R$ (assuming membrane surface tension independent of granule size). Therefore, the concentration of soluble protein in the cytoplasm determines granule size ($P = f(C)$, $R = g(P)$; $R = h(C)$.

The other model bases size control on a requirement of a specific surface to volume ratio for each granule. Evidence has been given for site-binding of digestive enzymes within zymogen granules (Rothman 1972) probably on granule membrane. Such sites would exist with a characteristic frequency (membrane : site ratio). Reaggregation experiments suggest they have a definite maximum capacity and therefore a site : capacity (volume) relationship. From this a specific membrane (surface) : volume ratio is determined. Once the surface : volume ratio is set, only one radius can possibly be produced. Therefore, the enzyme binding characteristics in a granule in equilibrium with external enzyme would, of themselves, control granule size.

III. Tissue Uptake Studies

There are a number of observations in the literature which suggest that a cytoplasmic enzyme pool may be a direct source of secretion. These observations include: (1) a high percentage of certain enzymes in some species is found in the PMS portion of tissue homogenates (Hokin 1962 and Hansson 1959); (2) certain newly labeled enzymes are recovered in the PMS as a distinct early peak (Laird and Barton 1958); (3) continued rapid secretion of proteins is observed in the near absence of granules (Rothman-Personal Communication); (4) selective enzyme secretion elicited by pancreozymin is accompanied by complementary changes in the enzyme content of the PMS with no changes observed in the zymogen granules during this initial phase (Rothman 1970a); (5) in the early periods after administration of labeled amino acid, enzyme in secretion more closely mirrors the higher specific (enzyme) radioactivity of the postmicrosomal supernatant than of the zymogen granules (Rothman and Isenman 1973b); (6) stimulation of secretion by pancreozymin or cholinergic drugs causes a depression in specific radioactivity toward granule SRA (Rothman and Isenman 1973a); and (7) cholinergic stimulation (as opposed to pancreozymin) causes an absolute decrease in the rate of counts secreted, suggesting competition between older (less radioactive) and newer (more radioactive) protein for secretion (Rothman and Isenman 1973a)

In light of these ideas and the present observations, suggesting that zymogen granule membranes may not present an absolute barrier to the movement of individual proteins, the possibility that membrane transport exists for digestive enzyme between granule contents and cytoplasm and between cytoplasm and duct was studied. These tissue studies demonstrated

(1) a specific permeability of the cell membrane to chymotrypsinogen; (2) that exogenous enzyme enters the cytoplasm directly and in all likelihood then crosses from cytoplasm into the zymogen granules across the granule membrane; and (3) the transport process appears to have a relatively large capacity (~1% of cell protein exchanged/hr).

The time course of association of exogenous enzyme with tissue slices is slow and consistent with a transport process rather than surface binding [it would require a very slow reaction to be explained as a surface binding phenomenon ($t_{1/2}$ = 1 hr)]. Furthermore, the lack of a significant cold chymotrypsinogen chase effect (Table 2) suggests that uptake could not be mediated by a freely exchanging surface binding site. Finally and most clearly, if surface binding were responsible for uptake, concentration in a purely intracellular compartment (zymogen granules) would not result, nor could equilibrium between intracellular pools and bath label occur. Without exchange, the endogenous pool would remain intact and total pool equilibration with the bath would not occur as it does. This suggests that exogenous and endogenous enzyme behave similarly in the cell and that pool mixing is truly occurring. Uptake, therefore, represents a finite entry which is specific for chymotrypsinogen, excluding albumin.

The initial rate of entry of exogenous ^{3}H-α-chymotrypsinogen (Bovine) diminishes after 5-30 minutes of incubation. At 5 minutes entry into the cytoplasmic pool (PMS of homogenates) was at approximately 40% of equilibrium (bath) SRA while movement into the zymogen granules occurred more slowly (25% of equilibrium SRA), and microsomal equilibration

was very slow (4% of equilibrium SRA). Movement into the zymogen granule fraction is most probably via the cytoplasm rather than directly from the duct via reverse exocytosis because of the slower time course of entry into zymogen granules relative to the cytoplasm and by the very high specificity demonstrated by the $^{3}H/^{131}I$ ratio in this fraction. The zymogen granule has by far the highest chymotrypsinogen/albumin ratio in the cell (~150 : 1), virtually excluding albumin.

40% equilibration of the cytoplasmic pool with exogenous enzyme in 5 minutes and essentially complete equilibration of both cytoplasmic and granule pools in an hour suggests that these pools must have exchanged a number of times with the bath pool in order to "dilute out" endogenous enzyme . Furthermore, exogenous entry into these pools must be considerably more than endogenous entry from synthesis since the pool/bath SRA ratio→ 1 (therefore endogenous flux/exogenous flux → 0). Equilibration, therefore, indicates that this route is of significant proportions as determined by a rapid exchange time and a low endogenous flux relative to the exogenous flux.

When the initial rate of chymotrypsinogen uptake (approximately 25 mg chymotrypsinogen/gr tissue/hr) is compared to the basal rate of secretion from an <u>in vitro</u> pancreas (of the order of 5 mg total protein/gr tissue/hr) the observed uptake rate is clearly of considerable magnitude relative to the physiological process. Actually, chymotrypsinogen comprises only a portion of total secreted protein, and therefore, the unstimulated uptake rate of chymotrysinogen in the presence of 4×10^{-5} Molar chymotrypsinogen is of a comparable order of magnitude to maximally stimulated secretion of

chymotrypsinogen (in the order of magnitude of 5 mg chymotrypsinogen/gr tissue).

The observation that the cytoplasmic pool equilibrates most rapidly with labeled exogenous enzyme supports the observations discussed at the beginning of this section and suggest that the cytoplasm is a precursor pool for secretion. Newly synthesized protein (labeled) always appears rapidly in the PMS (Laird and Barton 1958) suggesting that the cytoplasmic pool may also be a recipient of newly formed enzyme. This is consistent with (see below) the view that at least some enzyme can travel from its synthetic site of origin through the cytoplasm to the duct lumen. The delay in filling zymogen granules from exogenous sources (relative to the PMS) along with the apparent permeability of isolated zymogen granules to enzymes suggests an in situ equilibrium or steady-state between granule enzyme and cytoplasmic enzyme in which the granule may, for at least a portion of secretion, serve solely a capacitive function and not its usually implied transport role (Figure 24). This view is consistent with (1) sequential labeling of cell fractions with incorporated amino acids; (2) the existence of a cytoplasmic pool which acts in part as a precursor to secretion; (3) the selective or "nonparallel" transport of digestive enzyme; (4) granule disappearance in response to augmented secretion (depletion of storage); and (5) the existence of an enzyme transport mechanism in the zymogen granule membrane.

It should be remembered that these uptake measurements were made using tissue slices in which the "whole" surface of the cell is available, whereas exocrine secretion occurs only across the apical membrane and

hence these uptake measurements do not represent a reversal of a secretory flux. Moreover, this enzyme flux may not be via the only secretory route. Nevertheless, capacity for transport appears to be sufficiently large so that it still would appear to be a process of potential physiological significance.

IV. Circulation Experiments

In running controls for the tissue uptake experiments, I used tissue "strips" (not sliced) without the acinar lumen exposed in order to show changes in the permeability of these tissues as a function of exposure of apical membrane. Much to my surprise, I found that tissue strips still demonstrated a significant uptake of tritiated α-chymotrypsinogen (Bovine). This could not be accounted for by diffusion up the intact tubular elements since this represents a major diffusional pathway too long to be covered by a molecule of chymotrypsinogen's size in this time (~1/2 cm duct length has diffusion time in the order of days) and in addition there is significant flow of liquid down this tube which would essentially prevent back diffusion (Geuze and Poort (1973) showed no ferritin in acini of similar preparations). The uptake suggested that the serosal membrane of the pancreatic acinar cell was permeable to digestive enzymes. In fact, increased surface area as determined by albumin space totally accounted for increased permeability. This new surface area includes both serosal and mucosal surface. This suggests that serosal permeability cannot be significantly less than mucosal, at least in the bath to cell flux direction. In considering the existence of a serosal permeability, it was difficult to imagine, teleologically, a function for the permeability of this membrane to a molecule to which it is not normally exposed. The only possibility that I could think of was that maybe there was endogenous enzyme exposed to the serosal border of the pancreas in vivo and that exchange of enzyme from blood to pancreatic tissue or vice versa normally occurred. This suggested among other possibilities, the possibility that enzyme after secretion into the gut might be reabsorbed intact, circulated through

the blood stream to the pancreas, where transport from blood to pancreatic duct again occurred producing cycling and reuse of enzyme.

There is some suggestion in the literature that a circulatory system for digestive enzymes may exist. Heidenhein (1875) and others have shown that depletion of granules and enzyme content of the pancreas could occur completely only by removal of ductal secretion from the body (external fistula) and preventing ductal secretion from reentering the gut. This suggests that possibly enzyme in the gut can prevent the depletion of pancreatic stores of enzymes. The amount of enzyme in the gut may even be a more explicit controlling factor in the secretion of enzyme from the pancreas. Green and Lyman (1972) showed that introduction of enzyme into the gut reduced enzymatic secretion from the pancreas. This type of control is consistent with a circulatory system.

Activity normally similar to that displayed by digestive enzymes is normally found in the blood and variations in blood levels of enzyme occur in certain pancreatic diseases (Rao et al., 1972). Blood enzymes assayed clinically are generally amylase and "kallikreinogens". Kallikreins are measured by p-toluene sulfonyl-L-arginine methyl ester · HCl (TAMe) esterase activity, the same substrate used to monitor trypsin activities, and hence by their measurements alone is indistinguishable from trypsin. Though both blood enzymes monitored are generally accepted not to be of pancreatic origin, no evidence exists showing that this is so. These enzymes are elevated in concentration in the blood during pancreatic diseases such as pancreatitis and cystic fibrosis (Rao et al., 1972) and also in response to intestinal obstruction. Though these observations by

themselves certainly do not indicate enteropancreatic circulation of enzymes, they are explicable in these terms.

Other observations suggest more directly that circulation of digestive enzymes occurs. Ambrus et al., (1967) showed a selective increase in blood specific esterase activity upon ingestion of the corresponding exogenous enzyme (trypsin or chymotrypsin). This suggests some passage of intact enzyme from gut to blood. Upon direct stimulation of secretion by pancreozymin, however, they found no corresponding increase in blood esterase levels. They concluded that this meant exogenous enzyme was taken up intact whereas endognous enzyme was not. Another interpretation of this observation might be that pancreozymin increases pancreatic reabsorption from the blood thereby eliminating a rise in blood esterase levels.

Pelot and Grossman (1962) found that under certain conditions the activity of trypsin, chymotrypsin and lipase injected into the gut disappeared rapidly. They ascribed this disappearance of activity to inactivation of enzyme. Disappearance, however, did not follow a predictable inactivation course. In vitro (37°C) inactivation of trypsin was an order of magnitude slower than in situ disappearance of activity. Furthermore, the higher the enzyme concentration in the gut, the slower was the proportional loss of enzyme activity. The proportional rate (1/2 life) of inactivation normally increases with higher concentrations of enzyme. This apparent anomalous behavior can be explained by enzyme transport from the intestine. The proportional rate of a specific transport process normally decreases with increased activity due to transport competition as Pelot and Grossman observed.

Studies of other systems suggest not only the possibility of protein transport across organs but the possibility of enzyme circulation. Neonates of some species appear to be capable of absorbing ingested antibodies intact. Lysozymes which appear to circulate in the blood are normally filtered by the kidneys and yet are transported back to the blood stream intact by the kidney tissue. In fact, the lysozyme system in the kidney appears in many ways to handle proteins in a similar fashion to the pancreas. The work of Maack et al., (1972) suggests that lysozymes exist normally free in the cytoplasm of kidney cells. In situations, however, where the kidney is exposed to excessive lysozymal loads, a maximum concentration of lysozymes develop in the cytoplasm and they then begin to appear in the granular pool. Lysozymes in the cytoplasm appear to remain intact and to be transported out from the serosal border of the cell back into the general circulation. Lysozymes in the granules, however, appear to be degraded. An equilibrium is suggested for lysozymes (a protein of approximately 14,000 M.W.) between pools in the kidney duct, cytoplasm, granules and blood. This equilibrium is analogous to the equilibrium which I discuss below for chymotrypsinogen (and, by inference, other digestive enzymes) between blood, cytoplasm, zymogen granules and pancreatic duct.

In light of evidence suggesting serosal membrane permeability to enzyme, and the above observations, I decided to investigate directly the possibility of entero-pancreatic circulation of enzyme. Protein circulation would involve the transport of enzymes from blood to duct across the pancreatic cells and the transport of enzymes from inside the gut lumen to the blood stream across the digestive tube. Investigation into enzyme

circulation was therefore divided into examination of these two steps in the circulatory pathway (ie., but-blood, blood-secretion) and confirmation of the whole circulatory pathway was carried out in vivo.

1. Transpancreatic Flux:

Tritiated α-chymotrypsinogen (Bovine) placed in the bath of the in vitro pancreas was secreted at a significant rate. With 20 mg% chymotrypsinogen in the bath, up to 20% of the protein in unstimulated secretion was exogenous chymotrypsinogen. Exogenous chymotrypsinogen was concentrated 20 fold over albumin in secretion. A peptide in AMeSNI, a micture of duodenal peptides from hog duodenum apparently has the ability to selectively stimulate the secretion of chymotrypsinogen from rabbit in vitro and in vivo (Adelson and Rothman 1973). When AMeSNI is added to the bath, it stimulates the transpancreatic movement of chymotrypsinogen (Table 7). It did not affect the movement of albumin. Pancreozymin does not significantly increase the secretory rate of chymotrypsinogen in this system (Rothman 1973), but it does very significantly increase the rate of total protein secretion. The present experiments show that pancreozymin does not affect the transpancreatic flux of exogenous chymotrypsinogen either. This observation in conjunction with AMeSNI stimulation, suggests that transpancreatic flux of chymotrypsinogen behaves in a physiologically similar fashion to secretion of chymotrypsinogen (AMeSNI increases both transpancreatic flux and secretion of chymotrypsinogen whereas pancreozymin does not). Changing concentration of chymotrypsinogen (but no altering that of label) in the bath solution from 2 mg % to 20 mg % produced a transient decrease in the secretory rate of tritium from the pancreatic duct. This transient decrease is consistent with a three comparment model

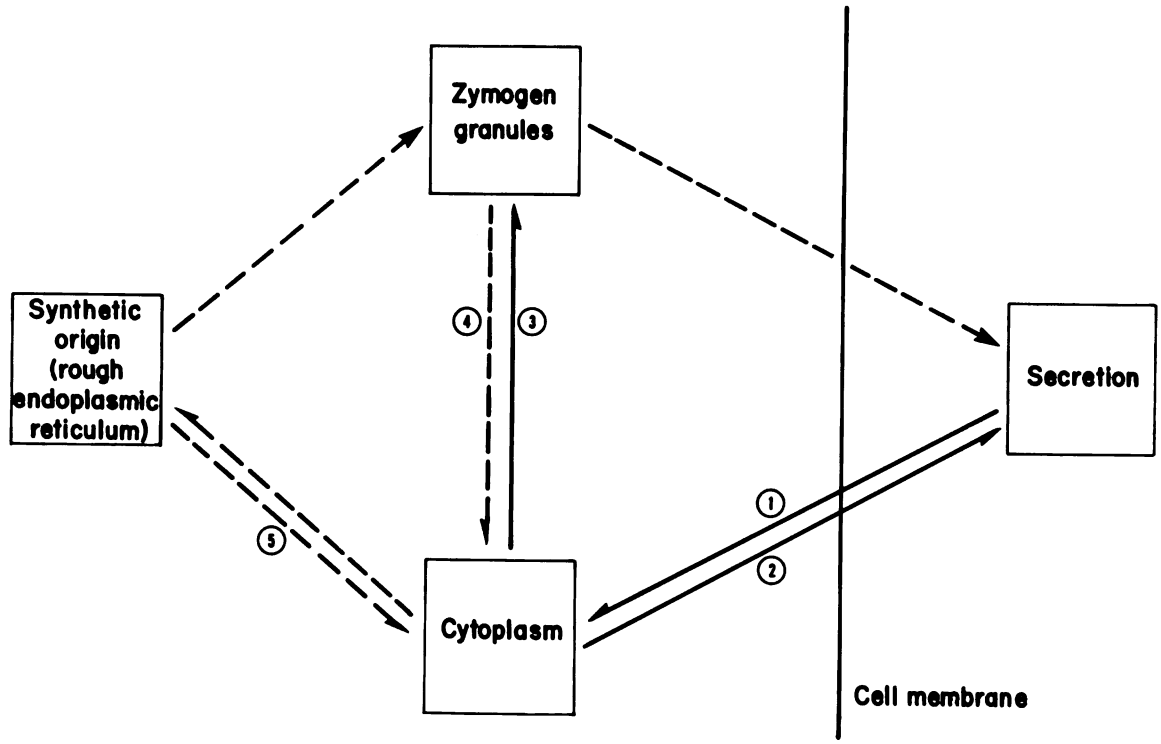


Figure 24. A model system for describing intracellular routes for secretion of pancreatic enzymes. Exchange between pools is either equilibrium dependent (bidirectional arrows) or unidirectional (single arrows) mediated by movement of packaging elements in the exocytic process. Where direct evidence exists for movement over a specific pathway, solid arrows are drawn. Evidence for specific routes (corresponding to numbers in diagram) if: (1) uptake studies in this paper showing initial filling of cytoplasm from extracellular compartment; (2) selective pancreozymin elicited secretion derived from the cytoplasm; (3) secondary filling of zymogen granule pool (shown here); (4) in vitro release of enzyme from zymogen granules suggesting a similar in vivo capability; and (5) early rise in cytoplasm SRA after addition of labeled amino acids with distinct peaks of incorporation observed in this pool for certain enzymes.

(Figure 24) for transport, wherein the specific activity of the second compartment is markedly decreased by the addition of cold chymotrypsinogen. This can be explained by an exchange diffusion between cold and hot chymotrypsinogen at the serosal surface similar to the exchange seen between cold and hot glucose in cells transporting glucose.

2. Intestinal Absorption of Exogenous Chymotrypsinogen:

The second step in investigating the existence of an enteropancreatic circulation of enzyme involved studying the possibilities of gut transport of digestive enzymes. In these experiments, I observed movement of tritiated α-chymotrypsinogen (Bovine) across the gut wall. Chymotrypsinogen was over 5 times more permeable than albumin to the sacs. A minimal estimate of flux ratio of 4 : 1 (J_{ms}/J_{sm}) was observed in the ileum suggesting that transport at this level is directional and active. The prime gut transport site showing maximal net flux was in the ileum. This made sense since by this point enzyme would have performed its digestive function and should now be ready for recovery.

3. Demonstration of Circulation in the Intact Animal:

With the demonstration of two individual steps required for circulation I went on to investigate whether this process could occur <u>in vivo</u>. Exogenous ^{3}H-α-chymotrypsinogen (Bovine) injected in the gut appeared in secretion. The secreted tritium was shown by gel electrophoresis to be primarily the exogenous enzyme. The ratio of secretion of exogenous enzyme was 1.4 μg/hr. at the end of the one experiment (2 hours after injection of enzyme into the gut) and it had not yet reached steady state. Transport of enzyme from the entire gut appears to be approximately 2

micrograms per hour for the total transport from an entire whole intestine (calculated from in vitro gut segment incubations). The intact animal transport rate was slightly higher, but was still magnitudinally consistent with, the gut sac observations. This rate of circulation is small in comparison to estimated maximal secretory rates in the rabbit and therefore, suggests either: (1) circulation occurs only for a small portion of secreted enzyme and is probably of importance only as a control mechanism; or (2) some stimulant for reuptake of enzyme is lacking in these preparations giving an artificially small value for circulation. The suggestion that circulation acts as a control is in agreement with the observations of others (Green and Lyman 1972) that enzyme in the gut has an inhibiting effect on protein secretion from the pancreas, and I observed an apparent decrease in protein output in in vitro preparations upon the addition of exogenous chymotrypsinogen to the bath as well.

The existence of such a control mechanism does not mean, however, that circulation does not act also as a mechanism of conservation. Man secretes between 10 and 30 gms of enzyme per day, an amount apparently far in excess of the amount required to fulfill the digestive function. Certain digestive enzymes appear to be relatively protected against degradation by proteolytic enzymes, at least as compared with the rate of degredation of "unprotected" proteins (in vitro incubated of protein and proteases still display significant protease activity after almost all substrate is hydrolyzed). Salvaging some of these 10-30 gms of enzyme intact could yield an increased efficiency to the digestive process. Reabsorption of enzymes could be dependent upon some stimulus that is absent or in small amounts in our preparation such as a neural stimulus

disrupted by surgery or the absence of a hormonal stimulus. Many digestive hormones are found in the duodenum, including cholecystokinin-pancreozymin and chymodenin (Adelson & Rothman 1973). These hormones stimulate protein secretion from the pancreas and, if protein secretion is mediated by protein transport, then these hormones may stimulate protein transport in the pancreas. Hormonal stimulation of protein transport is consistent with the absence of an increase in blood esterase activity in response to pancreozymin (Rao et al., 1972). This, however, is speculative and requires direct investigation to determine if such a system in fact exists.

The most glaring objection to the recirculation theory is that though the pancreas secretes proenzymes and these observations have been made using exogenous proenzymes, the proenzymes must be converted to active enzymes in the gut in order to function. How, then, can this system function with gut catalytic activation of proenzymes? A few models can be built to get around this problem.

A simple explanation involves such a system as a control mechanism only. If only trace amounts of enzymes are normally resorbed, there need only be trace amounts of enzyme which escape activation. This small pool of inactive enzyme might then be the recirculated enzyme and the control factor as well.

A second and similar explanation involves larger quantities of enzymes remaining inactive and being resorbed intact. It is not clear as to how much enzyme, in absolute terms, is activated in the gut and how rapidly. (Studies have been made of the level of activity in the gut,

but these have not adequately defined 100% activation or measurements of activation vs. degradation vs. reabsorption). A pool of inactive enzyme may be maintained in the gut in order to rapidly control digestion by regulating activation. This inactive pool may be the pool that is resorbed. Maintenance of an inactive pool is consistent with the idea that much more digestive enzyme is in fact secreted than is required for the process of digestion.

The third and most fascinating possibility for explaining this process involves the reinactivation of previously activated enzyme. A mechanism to accomplish activation seems difficult to envision even for enzymes which lose little in terms of amino acids from their actual structure during activation, but almost impossible when discussing trypsinogen which loses a sizable peptide (6 amino acids). Selective reversal of mass action laws might allow for inactivation of some activated enzyme through the use of trypsin as a synthetase. The digestive protein may in its own structure maintain the requirements for inactivation back to the original proenzyme. The synthetase capability of trypsin is suggested by experiments showing trypsin capable of mediating non-specific protein synthesis in the presence of high concentrations of amino acids. Hence, bonds specifically split by trypsin during activation might, by trypsin, under the proper conditions, potentially be reformed. For trypsin, however, it is hard to imagine how a peptide could be specifically reintroduced. Enteropeptidase is required in very small quantities near the papilla of Vater to start the catalytic change of trypsinogen to trypsin. Once this process is started, it is autocatalytic. This enzyme is found in large quantities quite a distance distal to the entry site of

digestive enzymes. The enzyme is very specific only for trypsinogen, and only for cleavage of the inactivating segment. The amount of enzyme, the specificity, and the location suggest that possibly, under favorable conditions,enteropeptidase might selectively reattach the inactivating segment to trypsin.

The determination of the physiological importance of enteropancreatic circulation, as well as how it relates to the activation and possible inactivation of digestive enzymes must, however, await further investigation.

SUMMARY

The work presented in this thesis appears at first glance to be diverse in nature. On closer examination, however, the relationships between experiments become apparent and lend strength to the whole work as a unit. Two major themes were investigated: (1) the nature of zymogen granules studied *in vitro* which suggest that they may act as secretory capacitors, taking up and discharging enzyme into the cytoplasm via a specific membrane transport system and (2) the capability of tissue to take up chymotrypsinogen through the cytoplasm. Two other concepts were also explored: (1) the existence of a constraint on zymogen granule size, and (2) an apparent enteropancreatic circulation of enzyme. Many of the observations presented in this dissertation reinforce one another.

Granule permeability studies indicate the existence of membrane transport of enzyme and, therefore, the existence of a cytoplasmic pool. The tissue uptake experiments reinforce the idea of membrane permeability to enzyme and, further, also demonstrate the existence of a cytoplasmic pool. Hence, both experiments are self reinforcing.

The tissue studies also demonstrated serosal permeability to digestive enzymes. This permeability, along with the known capability of pancreatic acinar cells to secrete enzyme into the lumen, indicates that enzyme molecules are capable of transpancreatic movement. Such movement of enzyme was observed directly in the *in vitro* pancreas experiments. The functional response of this system to material that stimulate specific secretion suggests that transpancreatic permeability has physiological relevance. Active transport in the gut reinforces the suggestion that a physiological role for transpancreatic transport of enzymes exists.

Furthermore, it is another demonstration of membrane permeability to a digestive enzyme. The in situ observation of enteropancreatic circulation of enzyme reinforces the separate observations (ie. - gut and pancreatic movement) both of which suggest this capability.

The works as a whole are not only interrelated and reinforcing, but often provide independent observations of the same process or phenomenon. For example, absorption is a potential artifact in the uptake of exogenous enzyme into granules or cells, but this artifact cannot explain transpancreatic movement, enteropancreatic movement, movement into intracellular compartments or complete release of endogenous enzyme from suspended granules. Tritium movement dissociated from enzyme might be consistent with movement of label into and across cells, but it is not consistent with concentrating label in granules both in vitro and in situ, equilibration of labeled chymotrypsinogen activity with chymotrypsinogen in different pools of the cell, competition in transfer with purified enzyme, and selective stimulation of pancreatic secretion, nor can it have any relationship to release of endogenous enzyme from granules.

Enzyme movement into and across cells might be explained by pinocytosis, but if this process is very selective, susceptible to competition, capable of mediating directional transport and very temperature sensitive, then the distinction between pinocytosis and membrane transport blurs kinetically, if not anatomically. Furthermore, with apparent initial entry of enzyme directly into the cytoplasm, or at least directly into the postmicrosomal supernatant fraction, pinocytosis appears less likely to be the mediating event. Finally, though evidence may exist demonstrating the

capability of pinocytosis in some cells, not only does no such evidence exist for pinocytosis in granules, but no pinocytotic indentations have been seen in membranes of granules in suspensions.

The evidence presented, therefore, suggests that certain membranes (e.g. - pancreatic cell membranes, zymogen granule membranes, membranes involved in the enteric endothelial barrier) are permeable to large globular proteins (ie. - chymotrypsinogen). This permeability, furthermore, is mediated by a"membrane transport" system. This conclusion puts some constraints on models for membrane structure. The contention arising from much independent evidence that proteins can extend completely across membranes is supported by these observations (a protein, the size of the membrane, crossing the membrane, must extend across it for at least an instant). The thought that protein extends across membrane, furthermore, suggests a mechanism by which proteins could cross membrane. If protein incorporation into membrane could occur from either side of the membrane, and this reaction were reversible, then protein movement across membranes could be accomplished merely by protein incorporation on one side of the membrane and discharge on the other. This may provide a new insight into the interaction between proteins and other membrane components.

Once membrane transport of protein is established, a parallel can be drawn between enteropancreatic circulation of digestive proteins and other systems. Certainly the enterohepatic circulation of bile salts can be compared to secretory protein circulation. Circulation of a protein, lysozyme, with filtration and recapture by the kidney, also appears in some ways to mirror pancreatic handling of digestive enzyme. Lysozyme

appears to cross cell membrane, enter the cytoplasm, possibly enter granules, and finally be transported directionally across the cell. Enteric circulation of antibodies may also follow the pattern of entrance into the gut with secretion from salivary glands, resorption from the lower portions of the gut, and, finally, resecretion. The general pattern of circulation and recapture, and the specific patterns of protein circulation, seem to be much utilized by the body. Secretory protein circulation may follow this pattern.

APPENDIX

I Granule Size

Electron micrographs were obtained from thin sections cut from zymogen granule pellets and whole pancreas. The isolated zymogen granules were collected from pancreata excised from 10 fasted male Holtzman rats (300-450 mg/body weight) by a method previously described (Rothman 1971). Samples were fixed in 1.3% paraformaldahyde, 0.3% potassium dichromate and 2% glutaraldehyde and stained with 1% uranyl acetate followed by 0.02% lead citrate (Venable and Coggeshall, 1965). Micrographs of granules and tissue are shown in figures 2a and 2b.

Electron micrographic magnification of the order of 10^4 was used and slice thickness was approximately 0.1 μ. Measurements[1] were made of the diameter[2] of the outer (labeled d_{outer}) and inner (labeled d_{inner}) circles comprising the halo of the granule. These circles correspond to the larger and smaller circles formed by the intersection between the surface of the granule sphere and the plane of the slice, one closer to the center of the sphere, and the other further from the center. This is shown in Figure 3.

Calculations of the diameter of the sphere (D) were made from measurements of d_{outer} and d_{inner} and an assumption of slice thickness (T). The calculations were derived as follows:

(1) $d_{outer} - d_{inner} \equiv 2\Delta$, where Δ is the thickness of the halo

(2) $\frac{d_{outer} + d_{inner}}{2} \equiv 2r$, where r is the average radius of the section

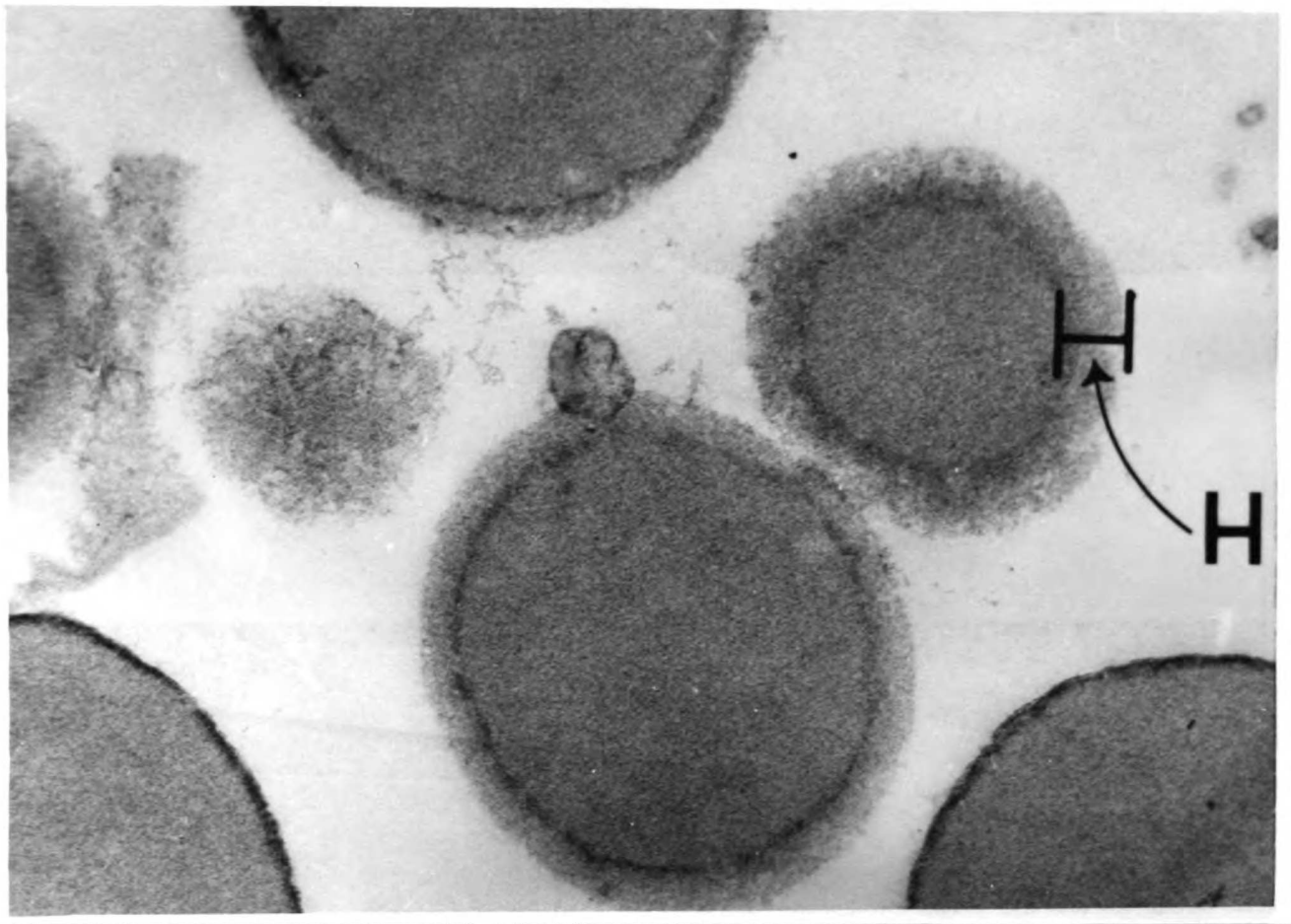


Figure 2a. Electron micrograph of a zymogen granule pellet. Shown here are sections of zymogen granules demonstrating "halos". The inner boundry of the halo can be seen to be a well defined area. The halo is bracketed and referred to as "H".

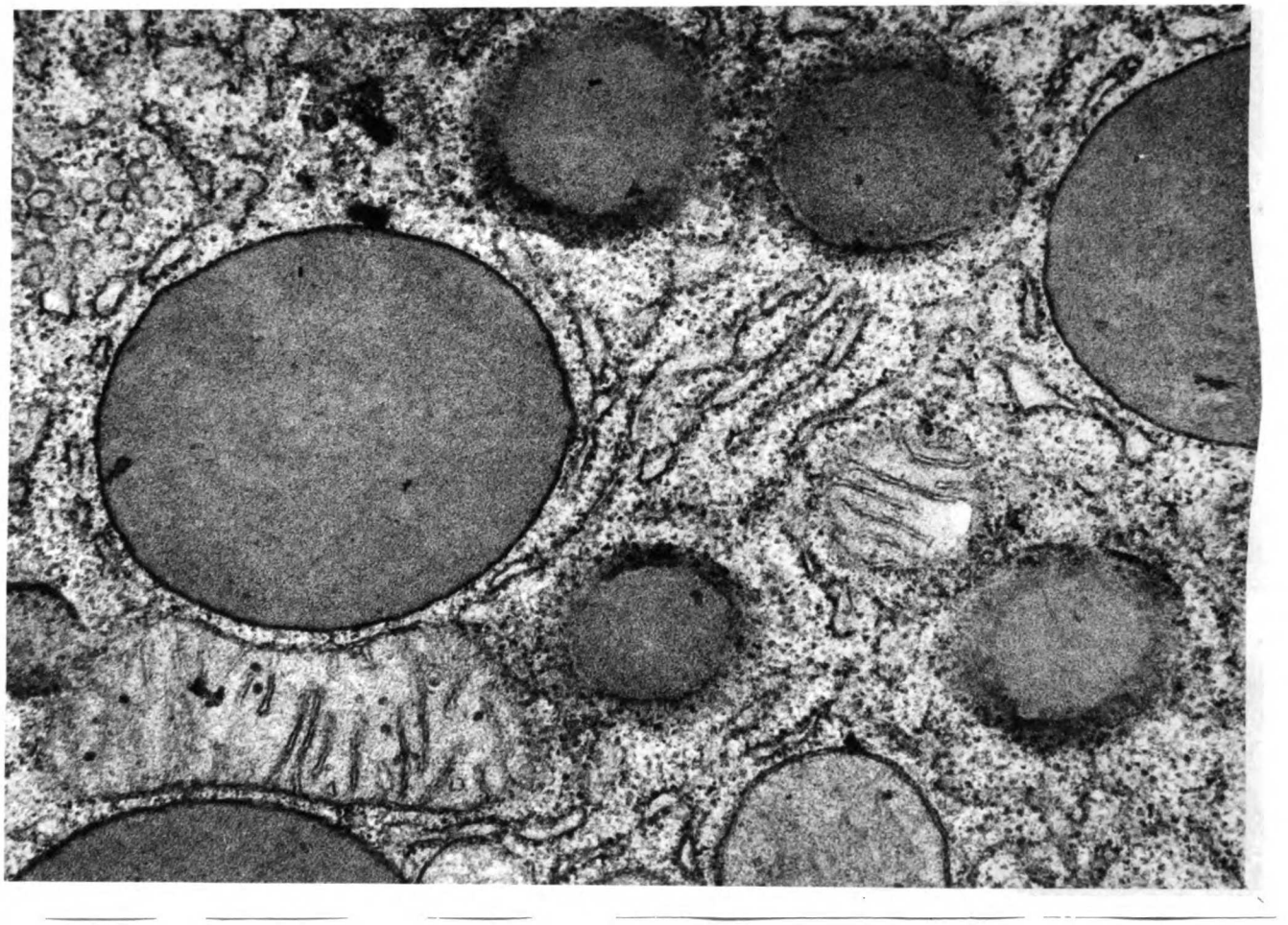


Figure 2b. Electron micrograph of a section of pancreatic acinar cell. Shown are zymogen granules with halos ranging from nonexistent to large halos. Also visible are mitochondria and rough surfaced endoplasmic reticulum.

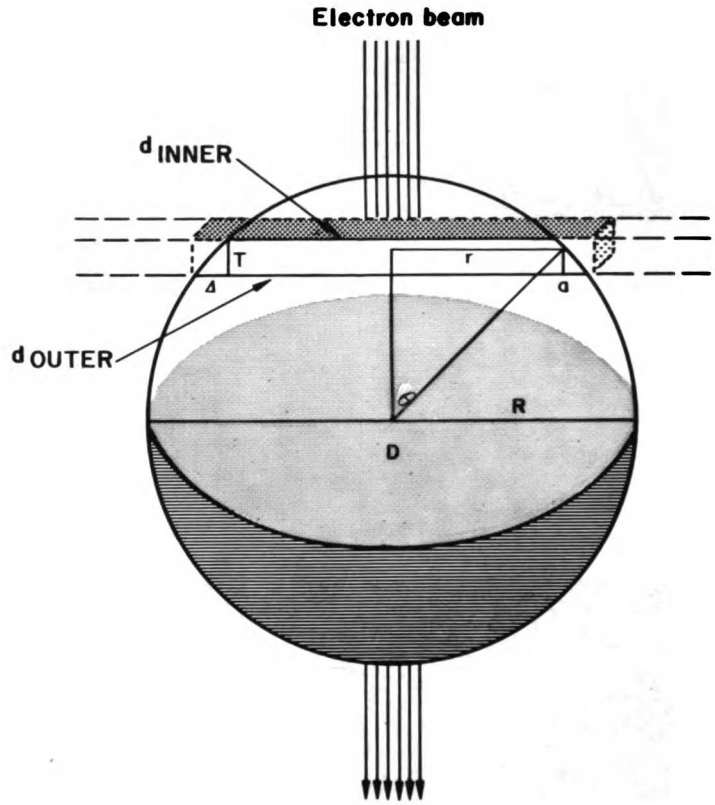


Figure 3. Diagram of a sphere through which a section is drawn representing section viewed through the electron microscope. "D" represents sphere diameter; "R", sphere radius; "d_{outer}", the diameter of the plane of the section closest to the center of the sphere; "d_{inner}", the diameter of the plane of the section furthest away from the center of the sphere (the circle inside the halo) (Fig. 2); and "T", the thickness of the section. The diameter of the sphere is drawn perpendicular to the electron beam. "Δ" is the thickness of the halo. "a" is the point on d_{outer} bisecting Δ. The projection of "a" parallel to the electron beam onto the sphere surface is used to define the average section "r". A radius of the sphere is drawn to this point on the sphere surface. The angle formed between this radius and the electron beam is "θ".

From figure 3 $\frac{T}{\Delta}$ = slope of chord from d_{outer} to d_{inner} and assuming the average slope of a small arc being approximately equal to the slope near the center of the arc, then:

(3) $\tan\Theta \simeq \frac{T}{\Delta}$, where Θ is formed by a plane parallel to the electron beam and the family of radii of the sphere drawn to the line of intersection of the electron beam which passes through (a), and the sphere surface (figure 3). (a) is the line bisecting Δ.

It is also the case that:

(4) $R \sin = r$, where R is the radius of the sphere

(5) $\sin\Theta = \frac{r}{R}$

(6) $\tan\Theta = \frac{\sin\Theta}{\cos\Theta} = \frac{T}{\Delta}$

(7) dividing (5) by (6), $\cos\Theta = \frac{\Delta r}{TR}$

(8) summing the squares of equations (7) and (5),
$\sin^2\Theta + \cos^2\Theta = \frac{r^2}{R^2} + \frac{\Delta^2 r^2}{T^2 R^2}$

(9) $\frac{r^2}{R^2}(1 + \frac{\Delta^2}{T^2}) = 1$, since $\sin^2\Theta + \cos^2\Theta = 1$

Solving equation (9) for R yields:

(10) $R = r\,(1 + \frac{\Delta^2}{T^2})^{1/2}$

If r, Δ and T are known, equation (10) can determine the radius of each granule sphere. r and Δ measurements are obtained directly from micrographs, but T must be determined independently. Though there are a

number of methods for accurately measuring T, in this work we did not attempt that measurement.

Equation 10 tells us that R can be approximated by r if Δ is small; that is, $r \to R$ as $\Delta \to 0$. For the limit of this case, at $\Delta = 0$, $r = R$. This explains the reasoning behind the microscopists method of taking granules with sharp outline ($\Delta = 0$) and accepting this sharp outline as evidence that the viewed section of the sphere comes from the center of the sphere, and therefore, $r = R$. The practical validity of this approximation can be tested using the relationship that as $\Delta \to 0$, $r \to R$ in a smooth, continous fashion; and furthermore, the standard deviation of r should also be a smooth, continuous function as Δ approaches and equals 0. The results show that at $\Delta = 0$ these functions are discontinuous $\bar{r}_{\Delta=0}$ is smaller than $\bar{r}_{\Delta\to0}$ (8.5% smaller, significant at 0.05) and has a larger observed standard deviation (21% vs. 16%). An explanation for this

$$\left[\text{i.e.,} \quad (\bar{r}_{\Delta=0} < \bar{r}_{\Delta\to0}), \left(\sum_{i=1}^{n} \frac{(r_{i\Delta=0} - \bar{r}_{\Delta\to0})^2}{n_{\Delta=0}} > \frac{(r_{i\Delta\to0} - \bar{r}_{\Delta\to0})^2}{n_{\Delta\to0}} \right) \right]$$

could be that for some sections, the halo is indistinct, not detected, and the sections are improperly labeled $\Delta=0$. The results of relying on $r = R$ at $\Delta=0$ indicate that in practice r for $\Delta=0$ and $\Delta\neq0$ (improperly labeled $\Delta=0$) are pooled, biasing determinations of R and providing a smaller mean size and larger standard deviation than actually exists. If sections with $\Delta=0$ could be detected without mixing $\Delta\neq0$, then this technique would be valid. Since this is not practical, another technique must be used. A better method for R estimation uses $r \to R$ as $\Delta \to 0$. Here, since all halos are seen, there is less possibility of r being used with large halos.

As mentioned above, the thickness of the slice must be known in order to determine R from equation (10). If spheres of one size were sliced randomly and r and Δ combinations measured, the use of only one T, the actual T to calculate R, would allow all r and Δ combinations to yield a single unique R when fit into equation (10). Furthermore, an inaccurate assumption of T causes a predictable distortion in R as a function of Δ. This is shown in figure 4. Here, R is calculated from equation (10) for r and Δ combinations derived from spheres of 1.71 units^3 diameter sliced .225 units thick. Different T are assumed and the deviations of the calculated R from the true one can be seen. Only T = .225 will produce $dR/d\Delta = 0$; other T values will produce $dR/d\Delta$ always greater or always less than 0. Therefore, if all spheres are the same size, the true T can be found by choosing T to minimize $\Sigma \frac{dR}{d\Delta}$. This is the method which I used to estimate T so that we could determine all R for individual granule profiles. This method which is rigorously applicable if all spheres are the same size, is not if the spheres have a finite range of size. The standard deviation of R for rat zymogen granules was shown to be within ±16% by use of $r_{\Delta \to 0}$. If we estimate this population by a bimodal distribution separated by ±16% this estimate would produce only a small deviation of $dR/d\Delta$ from 0. This deviation is represented by the dashed line in figure 4. Considering all Δ values except those approaching Δ maximum, $\Sigma \frac{dR}{d\Delta} = 0$ is a good approximation. Furthermore, T values of .25 units and .20 units completely encompass all $dR/d\Delta$ changes produced by the population range. Therefore, our method of T determination (set T so $\Sigma \frac{dR}{d\Delta}$ is minimized) cannot bias our results by more than the bias produced by changing T to any value from .25 to .20.

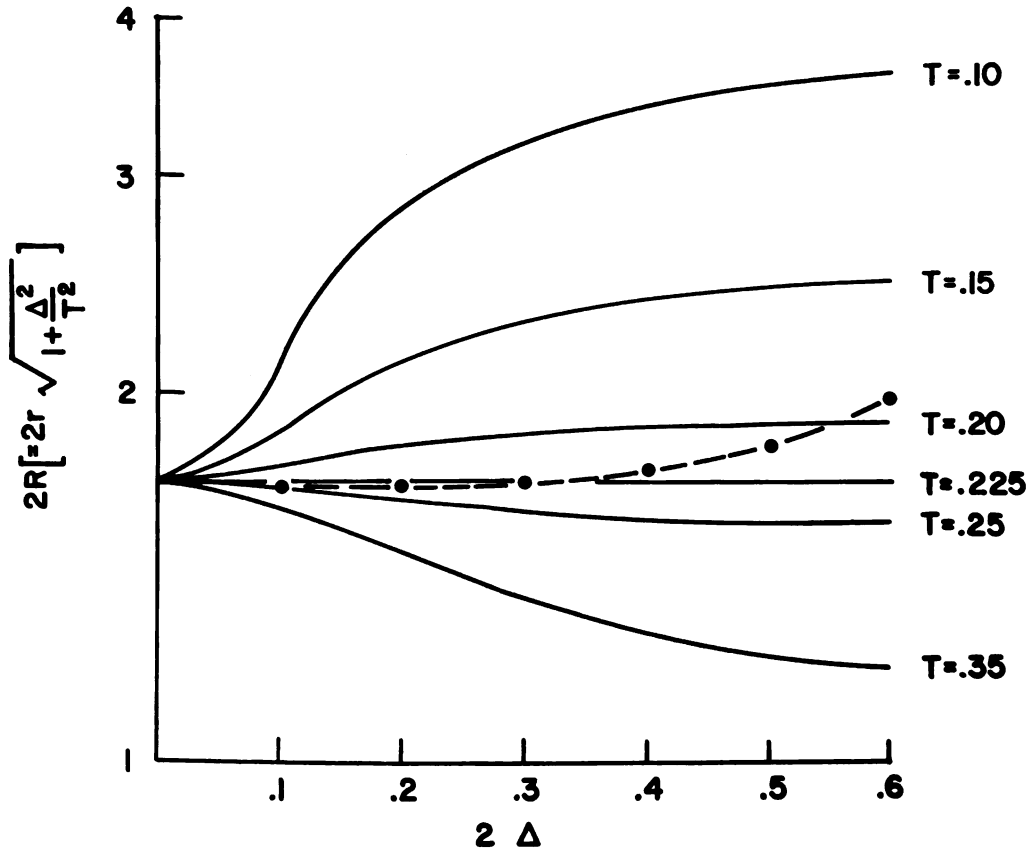


Figure 4. The error produced from an inaccurate estimate of T. The calculated sphere radius $\{t(1 + \frac{\Delta^2}{T^2})\}$ for section from a sphere of $2R=1.71$ units^2 sliced .225 units thick is plotted against the halo size (Δ) producing the solid horizontal line t=.225. A series of t values were substituted in the calculation and the error produced in R as a function of Δ shown by the remaining solid lines.

Deviation of a sample mean R as a function of Δ is represented by the dashed line. For $r \to R$ as $\Delta \to 0$, a standard deviation of 16% was found for r. Approximating this population of granules by two equal groups of granules whose radii are $\bar{R}$ + 16% and $\bar{R}$ - 16% respectively, the mean R (dashed line) for different Δ groups from this sample population was determined. The deviation of this line from the horizontal represents the maximum possible bias produced by accepting T producing $\Sigma\frac{dR}{d\Delta} = 0$ as the true slice thickness.

Variation due to errors in r and Δ measurement can be separated from variation due to the actual size distribution of zymogen granule spheres (or other errors independent of Δ). More specifically, an error of some δ in measuring Δ should produce an error (E_Δ) in R of:

$$E_\Delta = R'_\Delta - R_\Delta$$

where $R'_\Delta = r\left(1 + \frac{(\Delta+\delta)^2}{T^2}\right)^{1/2}$ and $R_\Delta = r\left(1 + \frac{\Delta^2}{T^2}\right)^{1/2}$. Likewise, an error in measuring r of δ would produce an error in R (E_r) of:

$$E_r = R'_r - R_r$$

where $R'_r = (r + \delta)\left(1 + \frac{\Delta^2}{T^2}\right)^{1/2}$ and $R_r = r\left(1 + \frac{\Delta^2}{T^2}\right)^{1/2}$ or $E_r = \delta\left(1 + \frac{\Delta^2}{T^2}\right)^{1/2}$

Since these errors, and the true variation in granule size, are random in direction, the sum of the squares of all individual deviations should produce the square of the observed deviation (S^2). Estimates for the true variation, therefore, were derived from the equation $S^2 = E_\Delta^2 + E_r^2 +$ (True Variation)^2.

To check this method for distribution analysis, an independent method for estimating the size distribution of granules was used. This involved the frequency distribution of Δ. The Δ frequency derived from spheres of a constant size should be:

$$\text{Frequency } \Delta = \cos\left(\frac{\Delta}{\Delta_{max}} \times 90°\right)$$

where Δ_{max} is the largest Δ value. This does not take into account the possibility that loss of granule sections and the probablity of overlooking a section varies as a function of Δ.

Footnotes

[1] An estimate of the variability inherent in the measurement of the derived values Δ and r (see text) was made. 25 different diameters were measured through a single granule profile from which Δ and r were calculated. $\sigma_{\frac{\Delta}{r}} / \frac{\Delta}{r}$ or the percentage standard deviation of Δ/r was used to estimate confidence in Δ and $\sigma_{\frac{d_{inner}}{d_{outer}}} / \frac{d_{inner}}{d_{outer}}$ was used for r. These estimates were used rather than absolute variation because of the eccentricity of the granules (see Footnote 2). The Δ estimate was ±8.6% and the r estimate ±1.4%. This error accounts for greater than ±1.4% of the final observed deviation (see equation 10).

[2] The diameters used in all measurements, both inner and outer, were the major axis diameter. All major axes were aligned parallel. Granules are probably round, but during the slicing process distortion occurs. The major-minor axis ratio is constant throughout a preparation, and, therefore, all major axes are directly proportional to average diameters. Use of major diameters alters the absolute calculation of D slightly but does not alter the relationship between different diameters.

[3] The units cited in this paper were not directly related to actual size because we were not sure of the exact magnification. Units relate to centimeters measured on a photograph. The average measurement of granules of about 1.71 cm at a magnification of approximately 20,000 x indicates granule size of approximately .86 μ in diameter. The sections

used were between 0.08 and 0.12 microns thick. Multiplying this by the magnification ($x \times 10^4$) yields a possible range of .16-.24 units for thickness. Our choice of 0.225 units for thickness lies within this range.

[4] If $s/p \alpha 1/C$, then:

$s/p = KV$, and V is suspending volume of supernatant and suspending volume of pellet does not, then

$$\frac{S/V}{p} = K \text{ or } \frac{[s]}{[p]} = K$$

Since at equilibrium $Flux_{out} = Flux_{in}$, then

$[s] \times K_{in} = Flux_{in}$ and $[p] \times K_{out} = Flux_{out}$

Adelson, J. and S.S. Rothman. Selective Enzyme Secretion elicited in situ by a newly purified duodenal peptide. Fed. Proc. 32; 409 Abs, 1973

Ahlroth, A. and Mutt, V. Polyacrylamide gel electrophoresis of polypeptides from the intestinal wall, with countermigration of dye. Analytic Biochem 37: 125, 1970.

Ambrus, J.L., H.B. Lassman and J.J. Morchi. Absorption of exogenous and endogenous protrolytic enzymes. Clin. Pharm. Therap. 8:362-368, 1967

Arnesjö, B. and A. Grubb. Intracellular distribution of lipase in comparison to trypsinogen, amylase and immediately measureable trypsin inhibitor(s) in rat pancreas. Acta Physiol. Scand. 75:139, 1969.

Babkin, B.P. Secretory Mechanisms of the Digestive Glands. New York: Paul B. Hoeber, Inc., 1950.

Bdolah, A., R. Ben-Zvi, and M. Schramm. The mechanism of enzyme secretion by the cell. II. Secretion of amylase and other proteins by slices of rat parotid gland. Arch. Biochem. Biophys. 104:58-66, 1964.

Bowen, R.H., Studies on the Golgi apparatus in glandcells. I. Glands associated with the alimentary tract. Quart. J. Microscopical Sci. 70:75-112, 1926.

Bowen, R.H., The cytology of glandular secretion. Quart. Rev. Biol., 4:299 484, 1929

Burwen, S.J. and S.S. Rothman. Zymogen granules: osmotic properties, interactions with ions, and some structural implications. Am. J. Physiol 222,1177, 1972a

Burwen, S.J. Zymogen granules: structure and properties. Harvard University Thesis, 1972b

Castle, J.D., J.D. Jamieson and G.E. Palade. Radioautographic analysis of the secretory process in the parotid acinar cell of the rabbit. J. Cell Biol. 53:290-311, 1972

Covell, W.P. A microscopic study of pancreatic secretion in the living animal. Anatomical Rec. 40:213-223, 1928.

Daly, M.M. and A.E. Mirsky. Formation of protein in the pancreas. J. Gen. Physiol. 36:243-254, 1952

Ekholm, R., T. Zelander and Y. Edlund. The ultrastructural organization of the rat exocrine pancreas I. Acinar cells. J. Ultrastruct. Res. 7:61-72, 1962

Elias, H.,A. Henning and D.E. Schwartz. Steriology: Applications to biomedical research. Physiol. Rev. 51: 158-199, 1971.

Fawcett, D.W. Physiologically significant specializations of the cell surface. Circulation 26; 1105, 1962

Geuze, J.J. and C. Poort- Cell membrane resorption in the rat exocrine pancreas cell after in vivo stimulation of the secretion, as studied by in vitro incubation with extracellular space markers. J Cell Biol. 57:159-174, 1973

Green, G. and R.L. Lyman. Inhibition of pancreatic enzyme secretion in the rat by trypsin and chymotrypsin in the intestine. Fed. Proc. 31:328 Abs. 1972

Greene, L.J., C.H.W. Hirs, and G.E. Palade. On the protein composition of bovine pancreatic zymogen granules. J. Biol. Chem. 238:2054-2070, 1963

Grossman, M.I., H. Greengard, and A.C. Ivy. The effect of dietary composition on pancreatic enzymes. Am. J. Physiol. 138:676-682 1942-1943

Hansson, E. The formation of pancreatic juice proteins studied with labelled amino acids. Acta Physiol. Scand. suppl 161:1-99, 1959

Harper, A.A. and H.S. Raper. Pancreozymin, a stimulant of the secretion of pancreatic enzymes in extracts of the small intestine. J. Physiol. 102:115-125, 1943

Heidenhain, R. Beiträge zur Kenntniss des Pancreas. Pflüger Archiv. Physiol. 10:557-632, 1875

Heidenhain, R. Physiologie der Absorderungsvorgange. In: Handbuch d. Physiol., Ed. L. Hermann, Leipzig F.C.W. Vogel 5:1,1 1883

Hennig, A. and H. Elias. A rapid method for the visual determination of size distribution of spheres from the size distribution of sections. J. of Microscopy 93: 101-107, 1971

Hirsch, G.C. Die wechselnde Permeabilität der Pankreaszelle als limitierender Faktor der vitalen Neutralrotfärbung. 14:517-543, 1932

Hokin, L.E. Isolation of the zymogen granules of dog pancreas and a study of their properties. Biochim. Biophys. Acta 18:379-388, 1955

Jamieson, J.D. Biology of the Secretory Process in exocrine glands. In: Salivary glands and their secretion ed. N.H. Rowe: Michigan, 1972

Jamieson, J.D. and G.E. Palade. Intracellular transport of secretory proteins in the pancreatic exocrine cell I. Role of the peripheral elements of the Golgi complex. J. Cell Biol. 34:577-596, 1967a.

Jamieson, J.D. and G.E. Palade. Intracellular transport of secretory proteins in the pancreatic exocrine cell II. Transport to condensing vacuoles and zymogen granules. J. Cell Biol. 34:597-615, 1967b.

Jamieson, J.D. and G.E. Palade. Intracellular transport of secretory proteins in the pancreatic acinar cell III. Dispociation of intracellular transport from protein synthesis. J. Cell Bio. 39:580-588,1968

Jamieson, J.D. and G.E. Palade. Intracellular transport of secretory proteins in the pancreatic acinar cell. IV. Metabolic requirements. J. Cell Biol. 39:589-603, 1968.

Jamieson, J.D. and G.E. Palade. Condensing vacuole conversion and zymongen granule discharge in pancreatic exocrine cells: metabolic studies J. Cell Biol. 48:503-522, 1971

Jones, E.W. The electrophoresis of histones in polyacrylamide gel and their quanticative determination. Biochem. J. 104:78-82,1967

Jorpes, J.E., V. Mutt, S. Magrusson and B.B. Steele. Amino acid composition and N terminal amino acid sequence of porcine secretin. Biochem. Biophys. Res. Commun. 9:275-279,1962

Keller, P.J., E. Cohen and H. Newrath. The proteins of bovine pancreatic juice. J. Biol. Chem. 233:344, 1958

Kraehenbuhl, J.P. and J.D. Jamieson. Solid-phase conjugation of ferritin to Fab-fragments of immunoglobulin G for use in antigen localization of thin sections. Proc. Nat. Acad. Sci. USA 69:1771-1775, 1972

Kühne, W. Ueber die Verdauung der Eiweisstoffe durch den Pankreassaft. Virchow's Arch. 39:130, 1867.

Kühne, W. and A.S. Lea. Beobachtungen über die Absonderung des Pankreas. Untersuchungen aus dem Physiol. Insti. Heidelberg 2:448-487,1882.

Laird, A.D. and A.D. Barton. Protein synthesis in rat pancreas. I. Intracellular distribution of amylase. Biochim. Biophys. Acta 25:56-62,1957

Laird, A.K. and A.D. Barton. Protein synthesis in rat pancreas. II. Changes in the intracellular distribution of pancreatic amylase during the secretory cycle. Biochim. Biophys. Acta 27:12-15, 1958

Langstroth, G.O., D.R. McRae, and S.A. Komarov. The synthesis and secretion of protein material by the pancreas. Canad. J. Res. D, 17:137-149,1939.

Lin, T.M. and M.I. Grossman. Dose response relationship of pancreatic enzyme stimulants: Pancreozymin and methacholine. AM. J. Physiol. 186: 52-56, 1956

Linzell, J.L. and M. Peaker. Mechanism of milk secretion. Physiol. Rev. 51:564-597, 1971

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. Protein measurements with the Folin phenol reagent. J. Biol. Chem. 193:265-275, 1951

Marchis-Mouren, G., L. Pasero, and P. Desnuelle. Further studies on amylase biosynthesis by pancreas of rats fed on a starch-rich or casein-rich diet Biochem. Biophys. Res. Commun. 13:262-266, 1963

Mathews, A. The changes in structure of the pancreas cell. J. Morph. 15 suppl: 171-222, 1899.

Meldolesi, J. and D. Cova. Synthesis and interactions of cytoplasmic membranes in the pancreatic exocrine cells. Biochem. Biophys. Res. Commun. 44:139-143, 1971

Meldolesi, J. and D. Cova. Composition of cellular membranes in the pancreas of the guinea pig. IV. Polyacrylamide gel electrophoresis and amino acid composition of membrane proteins. J. Cell Biol. 55:1-17,1972

Meldolesi, J., J.D. Jamieson, and G.E. Palade. Composition of cellular membranes in the pancreas of the guinea pig. I. Isolation of membrane fractions. J. Cell Bio. 49:109-129, 1971a

Meldolesi, J., J.D. Jamieson, and G.E. Palade. Composition of cellular membranes in the pancreas of the guinea pig. II. Lipids. J. Cell Biol. 49:130-149, 1971b

Meldolesi, J., J.D. Jamieson, and G.E. Palade. Composition of cellular membranes in the pancreas of the guinea pig. III. Enzymatic activities. J. Cell Biol. 49:150-158, 1971c

Morris, A.J., and S.R. Dickman. Biosynthesis of ribonuclease in mouse pancreas. J. Biol. Chem. 235-1404-1408, 1960.

Nossonov, D.N. Das Golgische Binnennetz und seine Beziehungen zu der Sekretion. Untersuchungen über einige Amphibiendrüsen. Arch. Mikr. Anat. 97;136-186,1923

Noll, A. Die Sekretion der Drüsenzelle. Ergebn. Physiol 4,84, 1905

Orci, L.M. Amherdt, F. Malaisse-Logae, C. Rouiller and A.E. Renold. Insulin release by emiocytosis: Demonstration with freeze-etching technique. Sci. 179:82-84, 1973

Palade, G.E. Functional changes in structure of cell components. In: Subcellular Particles, ed. T. Hayashi.New York: The Ronald Press, 1959, pp 64-83

Palade, G.E., P. Siekevitz, and L.G. Caro. Structure chemistry and function of the pancreatic exocrine cell. In: Ciba Foundation Symp. on the Exocrine Pancreas, eds. A.V.S. deReuck and M.P. Cameron. Boston: Little Brown and Co., 1962, pp 23-49

Pavlov, I.P. Beitrage zur Physiologie der Absonderung. Centralblatt f. Physiol. 2:137-138, 1888

Pavlov, I.P. Die Arbeit der Verdauungsdrusen. Weisbaden: Walther, 1898.

Pelot, D. and M.I. Grossman. Distribution and fate of pancreatic enzymes in small intestine of the rat. Am. J. Physiol. 202:285-288, 1962

Rao, G.J.S., L.A. Posner and H.L. Nadler. Deficiency of Kollikrein Activity in plasma of patients with cystic fibrosis. Sci. 177:610-611, 1972

Reboud, J.P., G. Marchis-Mouren, A. Cozzone, and P. Desnuelle. Variation in the biosynthesis rate of pancreatic amylase and chymotrypsinogen in response to a starch-rich or a protein-rich diet. Biochem. Biophys. Res. Commun. 22:94-99, 1966.

Redman, C.M. and L.E. Hokin. Phospholipid turnover in the microsomal membranes of the pancreas during enzyme secretion. J. Biophys. Biochem. Cytol. 6:207-214, 1959.

Redman, C.M., P. Siekevitz, and G.E. Palade. Synthesis and transfer of amylase in pigeon pancreatic microsomes. J. Biol. Chem. 241:1150-1158, 1966

Rinder Knecht, H., P. Wilding, and B.J. Haverback. A new method for the determination of alpha-amylase. Experientia 28:805, 1967

Rothman, S.S. Exocrine secretion from the isolated rabbit pancreas. Nature 204:84-85, 1964a

Rothman, S.S. Studies on the nature of pancreatic electrolyte secretion from the in vitro rabbit pancreas. Thesis. Univ. of Penn. 1964

Rothman, S.S. Trypsin and chymotrypsin secretion from rabbit pancreas in vitro. Am. J. Physio. 211:777-780, 1966

Rothman, S.S. "Non-parallel transport" of enzyme protein by the pancreas. Nature 213:460-462, 1967

Rothman, S.S. Transport of proteins by pancreatic acinar cells: random or selective? IN: "The Exocrine Glands". U. of Penn. Press: Philadelphia, 169-181, 1969

Rothman, S.S. Subcellular distribution of trypsinogen and chymotrypsinogen in rabbit pancreas. Am. J. Physio. 218:372-376,1970.

Rothman, S.S. Intracellular storage of exportable protein in functionally hypertrophied pancreas. 219:1652-1657, 1970b

Rothman, S.S. The behavior of isolated zymogen granules: pH-dependent release and reassociation of protein. Biochim. Biophys. Acta 241:567-577, 1971.

Rothman, S.S. The association of bovine α-chymotrypsinogen and trypsinogen with rat zymogen granules. Am. J. Physio. 222:1299-1302, 1972.

Rothman, S.S. The molecular regulation of digestion: Short-term and bond specific . Am. J. Physiol. In press

Rothman, S.S., and F.B. Brooks. Electrolyte secretion from rabbit pancreas in vitro. Am. J. Physiol. 208:1171-1176, 1965

Rothman, S.S., and L.D. Isenman, Secretion of digestive enzyme derived from two parallel intracellular pools. Am. J. Physiol. In Press

Rothman, S.S. and L.D. Isenman, The "rapid" secretion of newly synthesized digestive enzymes. In preparation.

Rothman, S.S. and S. ITO, unpublished data

Rothman, S.S. and H. Wells. Selective effects of dietary egg white trypsin inhibitor on pancreatic enzyme secretion, Am. J. Physio., 216;504-507, 1969.

Sabatini, D.D. and G. Bloebel. Controlled proteolysis of mascent polypeptides in rat liver cells. II. Location of the polypepeptides in rough microsomes. J. Cell. Biol. 45;146, 1970.

Schramm, M., Z. Selinger, Y. Solomon, E. Eytan and S. Batzri; Pseudopodia formation by secretory granules. Nature N.B. 240: 203-305, 1972.

Siekevitz, P. and G.E. Palade. A cytochemical study on the pancreas of the guinea pig. I. Isolation and enzymatic activities of cell fractions. J. Biophys. Biochem. Cytol. 4:203-218, 1958a

Siekevitz, P. and G.E. Palade. A cytochemical study on the pancreas of the guinea pig. II. Functional variation in the enzymatic activity of microsomes. J. Biophys. Biochem. Cytol. 4:309-318, 1958b

Siekevitz, P. and G.E. Palade. A cytochemical study on the pancreas of the guinea pig. V. In vivo incorporation of leucine-1-C^{14} into the chymotrypsinogen of various cell functions. J. Biophys. Biochem. Cytol. 7:619-630, 1960.

Sjöstrand, F.S. The fine structure of the exocrine pancreas cells. In: Ciba Foundation Symp. on the Exocrine Pancreas, eds. A.V.S. deReuck and M.P. Cameron. Boston: Little Brown and Co., 1961, pp 1-19

Sjöstrand, F.S. and V. Hanzon. Ultrastructure of Golgi apparatus of exocrine cells of mouse pancreas. Exp. Cell. Res. 7:415-429, 1954

Sluiter, J.W. Zeitschrift Zellforsch. Mikr. Anat. 33:187, 1944. Cited in: Cell secretion: a study of pancreas and salivary glands by L.C.U. Junqueira and G.C. Hirsch. Internat. Rev. Cytol. 5:323-364, 1956.

Smith, U., D.S. Smith, H. Winkler and J.W. Ryan. Exocytosis in the adrenal medulla demonstrated by freeze-etching. Sci 179; 79-81, 1973.

Van Lancker, J.L. and R.L. Holtzer. Tissue fractionation studies of mouse pancreas. Intracellular distribution of nitrogen, deoxyribonucleic acid, ribonucleic acid, amylase, acid phosphatase, deoxyribonuclease, and cytochrome oxidase. J. Biol. Chem. 234:2359-2363, 1959.

Vandermeers-Piret, M.C., J. Camus, J. Rathe, A. Vandermeers, and J. Christophe. Distribution of hydrolases in rat pancreas; some properties of the zymogen granules. Am. J. Physiol. 220: 1037-1045, 1971.

Venable, T.H. and R.A. Coggeshall. A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. 25:407-408, 1965

Webster, P.D. Induction of pancreatic amylase synthesis following methacholine to pigeons. Federation Proc. 27:582, 1968.

Wicksell, S.D. The corpuscle problem. A mathematical study of a biometric problem. Biometrika 17:84-99,1925

Yasuda, K. and A.H. Coons. Localization by immunofluorescence of amylase trypsinogen and chymotrypsinogen in the acinar cells of the pig pancreas. J. Histochem. Cytochem. 14:303-313, 1966.

UC San Francisco LIBRARY
UNIVERSITY OF CALIFORNIA

FOR REFERENCE

NOT TO BE TAKEN FROM THE ROOM

CAT. NO. 23 012

PRINTED IN U.S.A.

COPY 1