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DIGESTIVE ENZYME TRANSPORT AND SECRETION IN THE PANCREAS

by

Lois Donna Isenman

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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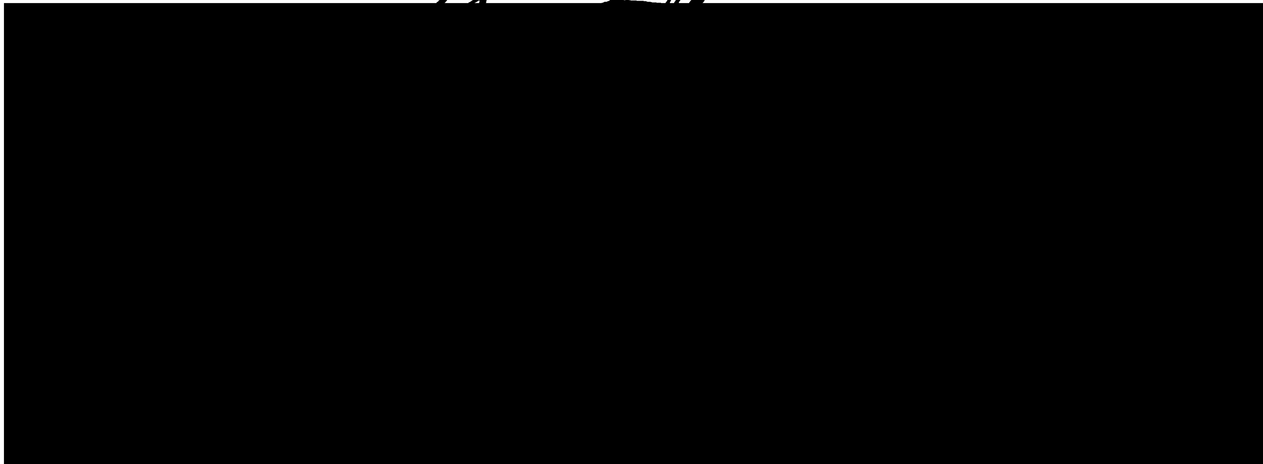

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DIGESTIVE ENZYME TRANSPORT AND SECRETION IN THE PANCREAS

Lois Donna Isenman

Abstract

Evidence is presented in this study supporting a new model for digestive enzyme transport and secretion in the pancreatic acinar cell called the equilibrium model (Rothman, 1975). The model proposes that digestive enzyme is present in the cytoplasm of the cell and that this enzyme equilibrates with enzyme in the ductal, the basolateral, and the storage compartment via bidirectional concentration-dependent fluxes.

The whole pancreas in short-term organ culture was first preincubated with ^{14}C-leucine and then ductal secretion was stimulated with acetyl-β-methylcholine chloride (MCh) or cholecytokinin-pancreozymin (CCK-PZ) in the continued presence of label. The specific radioactivity (S.R.A.) of secretion decreased dramatically, indicating the existence of at least two pools of enzyme with one turning over at a faster rate than the other. With MCh, a dramatic decrease also occurred in the absolute amount of labeled enzyme in secretion and this decrease was maximal during the 5 minute delay prior to the output response. The equilibrium model suggests that cold enzyme is released from storage during the delay, mixes with the hotter enzyme pool and competes with it for sites on the existing transport capacity; later in time the transport capacity itself is greatly augmented.

The appearance of amylase in the medium bathing the whole rabbit pancreas _in vitro_ reached an apparent steady-state value with time, suggesting the equilibration of a cell to bath and bath to cell flux.

In contrast, ductal secretion was linear with time. Amylase secretion into the bath at the initial rate was at least 3-fold the concomitant apical flux. Bath amylase was stimulated by MCh but not by CCK-PZ.

When a large concentration of amylase was added to the bathing medium of the *in vitro* pancreas, ductal amylase secretion increased greatly and, similarly, adding chymotrypsinogen to the bath augmented ductal chymotrypsinogen secretion. Augmented amylase output was accompanied by a striking inhibition in the S.R.A. of amylase, as well as in the amount of endogenous amylase in secretion. Both were sustained in the steady-state, though the amylase output returned towards its basal level. The data suggests that the exogenous enzyme enters the cell, mixes with the endogenous enzyme and competes with it for transport sites. Amylase in the bath also stimulated a roughly parallel chymotrypsinogen secretion and vice versa. However, since the individual data points show that the ratio of the two enzymes in secretion becomes chymotrypsinogen-dominant relative to controls when the exogenous amylase is added to the bath, the secretion of the two enzymes cannot be strictly linked.

The traditional model predicts that enzyme secretion should continue unabated when fluid secretion from the pancreas is either totally blocked or reduced by a graded backpressure. Instead, amylase secretion into the duct decreased in parallel with the inhibition in fluid flow, leaving the concentration of enzyme in secretion unchanged. Such behavior is explained most simply by the linkage of fluid and amylase secretion via concentration-dependent bidirectional fluxes of enzyme across the apical membrane. The constancy of enzyme concentration suggests that the concentration of enzyme in the duct system is maintained close to its equilibrium value.

The temperature dependence of apical and basolateral amylase fluxes was measured in the *in vitro* pancreas from 5° to 38°. Both were V-shaped, with high secretion rates at low and high temperatures and an inflection point around 20°. Since release of amylase from zymogen granules only increased with increasing temperature, the high secretion rates observed at low temperature apparently result from a temperature dependent characteristic of plasma membrane permeability. In contrast, fluid flow only increased with temperature. From 20° to 38°, fluid secretion and amylase secretion related to temperature in an identical manner, leaving concentration constant. This again suggests that fluid and enzyme secretion are linked by bidirectional, concentration-dependent fluxes across the apical membrane. Taken together these studies provide provocative evidence in support of the equilibrium model.

DEDICATION

This study is dedicated to Bruce Isenman. He wanted to be a scientist and I believe that nothing could have stopped him had he lived.

ACKNOWLEDGEMENTS

My deepest gratitude to Stephen Rothman, my advisor, for his support and encouragement over the years. I would also like to express my appreciation to all the members of the Rothman group. Thanks also to Patti Tabor and Cordelle Yoder for typing this study and to Annette Loewe for her work on the figures.

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INTRODUCTION

Early Work

The first extensive investigation of pancreatic morphology and function was performed by Rudolph Heidenhain in the 1870's. Only rudimentary information about the gland was available prior to the publication of Heidenhain's remarkably thorough experimental studies (Heidenhain, 1875). Pancreatic juice had first been collected in 1663 by Regner de Graff (Fulton, 1930) who used a quill from wild duck to cannulate the major collecting vessel of dog pancreas. In the 1850's Claude Bernard experimented on the composition and function of pancreatic juice (Bernard, 1856). He collected secretion from the pancreatic duct thereby preventing the flow of pancreatic juice into the intestine and was able to demonstrate that both the absorption and the digestion of fatty substances from the intestine were impaired in its absence. Bernard confirmed his observation that pancreatic juice was essential to the process of digestion with a number of other experimental findings. On the molecular level, Kühne in 1867 published observations showing that the ability to hydrolyze protein developed with time when either newly secreted pancreatic juice or extract of fresh pancreas were left standing at room temperature; he named the agent responsible for the development of this protease activity trypsin.

Heidenhain examined fixed tissue from dog pancreas and noted the presence of 3 distinct regions in the acinar cells, 1) an intensely basophilic zone in the basal portion of the cell 2) a large, basally oriented nucleus 3) a supranuclear zone densely packed with spherical granules. He compared the morphology of acinar cells from fasted and fed animals

and observed that with feeding the cells appeared smaller, the basophilic portion seemed enlarged relative to the granular zone and a large number of granules had disappeared. He noted, furthermore, that those granules that did remain were reduced in size and tended to be tightly clustered in the apical region of the cell.

In another series of experiments Heidenhain correlated the proteolytic activity of pancreas extract from fasted dogs and from dogs killed at various times after feeding with histological observations of the tissue. He used the solublization of a particulate fraction from blood as his assay of proteolytic activity. He found that both protease activity and the number of granules reached a minimum about 6 to 10 hours after feeding, whereas complete recovery of both parameters occurred about 14 to 16 hours after a meal. He called this depletion and recovery of granules and enzymatic activity the "secretory cycle". From these observations he postulated that in dog pancreas: 1) digestive enzymes are stored in zymogen granules; 2) digestive enzymes are released into the duct with feeding; 3) granules and their enzyme stores are replenished during the quiescent period between feedings.

Furthermore, since proteolytic activity developed in pancreatic juice and tissue extract only when they were left standing for a time, Heidenhain postulated that the proteolytic enzymes were actually stored in precursor form. He called the precursors "zymogens", meaning ferments, and so the enzyme-containing storage granules have come to be called zymogen granules.

An early confirmation of Heidenhain's observations came from Kühne and Lea (1882) who were able to follow the morphological changes that occur with feeding in rabbit pancreas in the light microscope. They

noted that zymogen granules had different refractive qualities than the surrounding medium and were able to watch the decrease in the number of granules, as well as the clustering of the remaining ones in the apex of the cells, that occur with feeding. Covell in 1928 published a claim that he had seen the release of intact granules into the acinar lumen in the microscope. He also reported that he had followed the movement of secretion granules into vacuoles formed from apical membrane, as well as the subsequent release of such vacuoles into the lumen. These observations by Covell are the first reference in the literature to an exocytosis-like release process. Another influential early study was by Hirsch (1932) who followed the regranulation process in live material and claimed to have observed the transformation of vacuoles first appearing in the Golgi region of the cell into mature zymogen granules.

Heidenhain's extraordinary studies on pancreatic function have stood the test of time very well indeed, and in some important ways we have not really progressed much beyond his pioneering observations. Zymogen granules apparently are a storage depot for digestive enzymes. Consensus, however, has not been reached on the converse question -- i.e., is all the digestive enzyme in the cell sequestered in the zymogen granules? The currently popular paradigm assumes that this is so and the cisternal packaging-exocytosis model of enzyme transport and secretion logically follows from this assumption. The purpose of the present study, in contrast, is to offer evidence in support of an alternative hypothesis which proposes that digestive enzyme exists free in the cytoplasm of the cell, that it exists in equilibrium with the enzyme in zymogen granule pool, and that the cytoplasmic pool of digestive enzyme is the immediate precursor pool to secretion.

Cell Fractionation Studies

Chemical proof for the secretory cycle proposed by Heidenhain as well as detailed study of the intracellular events involved in protein synthesis and transport waited upon the development of adequate procedures to isolate the various subcellular structures. An early attempt at cell fractionalization is reported by Warburg in 1913; by the 1950's protocols had been developed to isolate and test a nuclear, a mitochondrial, a microsomal, and a post-microsomal fraction. The general procedure involves first mechanical rupture of the cells and then differential centrifugation to isolate the different ultrastructural components.

Zymogen Granules

Claude (1943) was the first to attempt the isolation of zymogen granules from the pancreas, however his secretory granule fraction (2,000 g) was highly contaminated with mitochondria. In 1955, Hokin reported the isolation of a reasonably homogeneous zymogen granule fraction from dog pancreas. He noted the quality and composition layer by layer of material sedimenting from a homogenate of pigeon pancreas at different gravitational forces and isolated a white colored material sedementing between 600 and 1,000 g with similar staining characteristics to granules *in situ*. In the light microscope this fraction appeared composed of vesicles from 0.5 to 1.5 μ, or about the same size as zymogen granules *in situ*. Chemical analysis of the zymogen granule fraction indicated that it was enriched in amylase activity per mg nitrogen 2-fold relative to the homogenate and 1,000-fold in protease activity per mg nitrogen [probably due to the presence of an inhibitor in another cell fraction lowering homogenate activity (Northrop, Kunitz

and Herriott)]. Relatively low phospholipid content (7 μg phospholipid-P per mg nitrogen vs 66 for the microsomal fraction) and low RNA content (3 μg RNA-P per mg nitrogen vs 152 for the microsomal fraction) suggested that the zymogen granule fraction was relatively free of microsomal contamination. Amylase was found in all other cell fractions except the microsomes, however, was only enriched relative to the homogenate in the zymogen granule fraction. This relative enrichment of digestive enzyme in the granules confirmed Heidenhain's observation that zymogen granules contain digestive enzyme, even if the enzyme is not exclusively stored in the granules.

Additional support for this view came from the work of Laird and Barton (1958), who demonstrated that the mass of the isolated secretory granule fractions decreased and increased with the secretory cycle. Evidence supporting Heidenhain's hypothesis also came from the work of Greene, Hirs and Palade (1963), and Keller and Cohen (1961) who used chromatography to compare the protein content of bovine pancreatic juice and zymogen granules and found that the two were reasonably similar. In addition Greene et al. found that 90% of the protein in zymogen granules could be identified as digestive enzyme. The properties of zymogen granules will be discussed further in following sections.

Microsomes

A submicroscopic particulate fraction referred to as microsomes was first isolated by Claude in 1943. He showed that microsomes had the same staining characteristics as the so-called ground substance of the cytoplasm in the light microscope. Brachet (1944) showed that the staining characteristics of ground substance resulted from the presence of large amounts of RNA, and Claude (1948) showed that 50% of total mouse liver

RNA was contained in the microsome fraction. Porter (1953) demonstrated with the electron microscope the existence of a reticular membranous substructure below the resolution of the light microscopic which had the same staining characteristics as the ground substance. He called this structure the endoplasmic reticulum since it was contained predominantly in the central or endoplasmic region of the cell.

Microsomes in pancreas were first isolated by Siebert (1955), and Peterman (1954) mentions nucleoprotein particles from pancreas similar to those found in liver. In a fascinating study, Allfrey et al. (1953) demonstrated that isolated pancreatic microsomes incorporate amino acids and that this incorporation increases when protein synthesis is stimulated. They also showed that peptides containing ^{15}N appear first in association with the microsomal pellet and only later in the suspending medium. Moreover they demonstrated that only part of the pellet protein is involved in amino acid uptake, that part solublized by RNAase.

Palade and Siekevitz (1956b) studied the microsomal fraction of guinea pig pancreas in the electron microscope. They noted that microsomes were composed of spherical vesicles from 80 to 300 mμ, banded by a continuous membrane studded with RNA-rich particles about 150 Å in diameter. They identified microsomes as fragments of endoplasmic reticulum on the basis of the presence of these small dense particles in micrographs of both. They postulated that fragmentation and vesicle formation occur either as a result of mechanical shear with subsequent membrane healing to form vesicles or as a response to the chemical composition of the suspending medium.

In contrast to Hokin, Laird and Barton (1957) found a significant amount of amylase associated with the microsomal fraction. They tested

the intracellular distribution of amylase in both starved and pilocarpine treated rat pancreas. They found the microsomal and supernatant fraction together accounted for over 50% of total amylase; the enzyme was equally distributed between the two fractions in starved glands while in stimulated glands it was higher in the microsomal fraction. They subfractionated the microsomal material and found that within a given physiological state, independent of the gravitational force used, a constant ratio of amylase, RNA and phospholipid precipitated in 0.88 M sucrose. They proposed that endoplasmic reticulum fragments of different sizes were being sedimented at the various speeds. Since the ratio of amylase to the other components did vary in a consistent manner with the physiological state of the gland, absorption alone would not likely account for the results. They suggested that the amylase might be manufactured in association with the ribonucleoprotein particles of microsomes which had been demonstrated to be capable of protein synthesis in liver.

Intracellular Distribution of Enzyme

The process of cell fractionation has serious limitations as an analytic procedure. Contamination or inhomogeneity of a given fraction is often a severe problem and can lead to ambiguous results (note the question about Palade's work on intracisternal granules presented below). Moreover, some organelles are ruptured during the homogenization procedure and a number of investigators feel that leakage of enzyme into the soluble fraction is a serious problem. A related problem is the absorption of soluble enzyme molecules to membranes which may lead to a redistribution of digestive enzyme among the various fractions. In spite of these problems, cell fractionation has been historically and remains an important tool for qualitative investigation of the function of subcellular organelles.

The results of several early cell fractionation studies are presented below. There was general agreement that secretory granules were concentrates of digestive enzyme. In addition, it appeared likely that microsomes were involved in some way in the replenishment of enzyme stores. However, there was, and still is, considerable controversy about the soluble fraction of the cell represented by the post-microsomal supernatant. The question is--does enzyme exist in the cytoplasm of the acinar cell in situ or is its presence in the post-microsomal supernatant fraction entirely an artifact produced by the fractionation procedure?[1]

All data presented below is for fasted animals. Siekevitz and Palade (1958a), working with guinea pig, found that 30 to 40% of the total trypsin activable proteolytic enzyme (TAPase) could be recovered in a broad zymogen granule fraction. In a fraction isolated for purity, the specific activity of the granules was 8 to 40x the specific activity of the whole homogenate. They found microsomes from guinea pigs to contain as little as 5% or as much as 30% of the total digestive enzyme activity of the gland. One third of the total RNA activity of the gland was found in the post microsomal supernatant, while 10 to 20% of the TAPase activity was found in this fraction. Hansson (1959), also working with guinea pigs, found about 40% of both amylase and the proteases in his zymogen granule fraction. He found 20% of the amylase as well as most of the lipase in the soluble fraction of the cell. Redman and Hokin (1956), working with pigeon pancreas, found greater than 85% of the amylase in the soluble portion of the cell.

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[1] Recently, Scheele, Palade, and Tartakoff (1978) have used a double label technique in an attempt to assess quantitatively the extent of digestive enzyme relocation occurring during homogenation and centrifugation of pancreatic tissue. It should be stressed that they assume that all enzyme found in the cytoplasmic compartment represents leakage from other cell compartments.

Laird and Barton (1958) followed the depletion and recovery of amylase in the various subcellular fractions from pancreas following stimulation. They found that loss of activity occurred first from the zymogen granule fraction. Minimum activity occurred roughly synchronously in microsomes and secretory granules, while minimum activity occurred somewhat later in the soluble fraction. Recovery of activity occurred at about the same time and rate in microsomes and the soluble fraction, with zymogen granules following considerably behind.

Cisternal Packaging - Exocytosis Model

In the late 1950's, George Palade and Philip Siekevitz proposed a complex hypothesis for the intracellular events involved in the synthesis and transport of digestive enzyme by the pancreas. The schema has become the accepted paradigm for protein synthesis and transport in the pancreatic acinar cell and most other protein secreting cells as well. Central to the model is the assumption that digestive enzymes are segregated in the cisternae of the endoplasmic reticulum immediately after they are synthesized and that this isolation from the cycosol is maintained through extrusion from the cell. The sequestered enzyme molecules are thought to move to the duct lumen via a sequence of membrane-bound compartments.

Palade himself in 1958 wrote, "We still need confirmatory evidence for the basic assumption that the synthesis of exportable proteins and their intracellular transport are carried through by the cell in two distinct and well separated phases, the first the cytoplasmic matrix and the second the intracisternal medium." In this statement Palade is acknowledging that sufficient evidence did not exist to confirm the basic assumption of the theory, that once a protein molecule is synthesized on

the ribosome, it is necessarily removed from communication with the cytosol by sequestration in the cisternae of the endoplasmic reticulum. In spite of the fact that such confirmatory evidence has not been forthcoming in the intervening years, the uncertainty surrounding this assumption has been all but forgotten.[2] This illustrates quite well the major flaw in the whole body of evidence supporting the Palade-Siekevitz hypothesis. Assumptions and tentative conclusions are generally presented in a cautious and circumspect manner at first, but then are later stated dogmatically or used as proven fact without additional confirming evidence. The cisternal packaging-exocytosis model remains only a hypothesis, though it is not generally viewed in this limited light.

According to the Palade and Siekevitz model, all digestive enzyme is synthesized on the attached ribosomes and immediately transferred into the cisternae of the endoplasmic reticulum. Small vesicles then bud off from the endoplasmic reticulum and transfer the enzyme to the Golgi region. At the Golgi the enzyme is packaged into condensing vacuoles which mature into zymogen granules as they move to the apex of the cell. The final step in the sequence is the exocytosis of granule contents, a process in which a specialized fusion of the membrane of the secretion granule and the cell membrane produces a direct connection between the enzyme in the granule and the extracellular environment.

Siekevitz and Palade used a combination of evidence from cell fractionation studies and high resolution electron microscopy to support their hypothesis. They found that microsomes could contain as little as

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[2] Blobel's work on signal peptides is taken by some to be confirmation of cisternal packaging. See discussion on page 19-20.

5% or as much as 30% of the total digestive enzyme activity of a guinea pig pancreas and hypothesized that the difference had to do with the state of the gland (1958a). They compared micrographs of the microsomal fraction from starved and fed animals and observed the presence of large aggregations of electron dense material in the cisternae of the endoplasmic reticulum of fasted animals sacrificed 1 hour after feeding. The material had an opacity and density similar to that of zymogen granules; in addition, a presumed intracisternal granule fraction isolated by cell fractionation was found to have RNAase and TAPase activity as high as the zymogen granules. From this they concluded that the intracisternal granules were composed of aggregated digestive enzyme and that digestive enzyme was transported into the cisternae of the endoplasmic reticulum at some point after synthesis. Though intracisternal granules are not found in most species, and though one of the authors at a later time states that the results were possibly due to contamination by elements from the Golgi complex (Caro and Palade, 1964), this study served as important and dramatic evidence in support of cisternal packaging.

Additional evidence for the schema was found in a study in which guinea pigs were sacrificed at various time intervals after an injection of labeled amino acid and each pancreas fractionated and analyzed for label incorporation into protein (Siekevitz and Palade, 1958c). They found, as had Allfrey et al. (1953), that the relative specific activity (defined as counts per minute, per milligram protein per gram pancreatic pulp, divided by the specific activity of acid-soluble amino acids in the final supernatant) of protein was highest first on the attached ribosomes; by 10 minutes, however, the relative specific activity of the

deoxycholate (DOC) soluble fraction[3] was higher than that of the attached particles. The relative specific activity of the zymogen granule fraction was low at 3 minutes; by 45 minutes it was the fraction with the highest S.R.A. The curve of the microsomal S.R.A. vs time crossed the corresponding function for the zymogen granules and this was interpreted to suggest a transfer of enzyme from one compartment to the other.

A specific digestive enzyme, chymotrypsinogen, was isolated in a similar series of experiments (Siekevitz and Palade, 1960b). Its specific radioactivity (cpm/mg enzyme) was highest first on the attached ribosomes. At 15 minutes the S.R.A. of the enzyme was roughly equal in all fractions, and at 45 minutes its S.R.A. in the zymogen granule fraction was 2x as high as in the microsomes. The authors concluded that their data was consistent with the view that digestive enzymes are synthesized on attached particles and transported with time to other intracellular compartments. They also mention, however, that the kinetics are consistent with another or other sites of synthesis as well.

Two fractions of unattached ribosomes were isolated (105,000 g for 3 hours, or 105,000 g for 15 hours) and were found to have considerable digestive enzyme activity (Table 1). The data shows that at 1 minute post pulse the S.R.A. of chymotrypsinogen associated with both free ribosome fractions was much lower than the S.R.A. of enzyme associated with the attached particles, whereas at 2.5 minutes the chymotrypsinogen S.R.A. of both free ribosome fractions was 70% that of the attached particles. Data from a gland fractionated at 3 minutes post pulse shows

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[3] DOC solublizes membranes and the DOC-soluble fraction is generally considered to represent enzyme within the endoplasmic reticulum. The actual content of the DOC-soluble fraction, however, is subject to some ambiguity as will be discussed shortly.

Table I

From Siekevitz and Palade, a Cytochemical Study on the Pancreas of the Guinea Pig V. In vitro Incorporation of Leucine-1-^{14}C Into the Chymotrypsinogen of the Various Cell Fractions, Journal of Biophysical and Biochemical Cytology 7:627, 1960. The data relevant to the current discussion has been underlined.

Specific Radioactivity of α-Chymotrypsinogen Isolated from Various Pancreatic Cell Fractions after in vivo Labeling with DL-Leucine-1-^{14}C

The details of the experiments are given in the text. The figures are C.P.M./mg enzyme.

Cell Fraction	Time after injection 1 min.	2.5 min.	3* min.	3 min.	15 min.	45 min.
Zymogen granule fraction	---	---	215	1,770	10,300	58,500
Mitochondrial fraction	---	---	2,240	4,100	13,950	51,250
Microsomal fraction (whole microsomes)	---	---	3,140	---	---	27,700
MS_{120} (attached particles)	22,100	13,780	---	10,000	15,480	---
MS_{30} (intracisternal granules)	7,970	2,920	---	2,770	18,300	---
SS_{120} (microsomal contents)	---	8,160	---	5,740	14,950	---
First postmicrosomal fraction (PM_1) (free particles)	2,840	9,280	---	2,970	---	---
Second postmicrosomal fraction (PM_2) (free particles)	3,900	9,150	---	8,370	---	---

continued

Table I (continued)

	Time after injection					
	1 min.	2.5 min.	3* min.	3 min.	15 min.	45 min.
PM_1 and PM_2 in a common pellet[2]	---	---	---	---	---	27,500
Final supernatant	---	6,220	---	3,930	---	24,450

* In this experiment only 1 mg. of 4.66 μc/μm DL-Leucine-1-^{14}C was injected to each animal. In all the other experiments the amount injected was 2 mg.

one free ribosome fraction with an S.R.A. 30% and the other 80% of the attached particles. Finally, for 45 minutes the data shows that the S.R.A. of the combined free ribosome fraction is similar to that of the whole microsome fraction (Table 1). The authors conclude that the free particles are probably particles that were attached to the microsomal membrane and then for some reason have become detached.[4] To explain why then the S.R.A. of chymotrypsinogen associated with the free ribosomes was lower than that associated with the attached particles, they suggest that the chymotrypsinogen attached to the free particles could reflect a mixture of hot and cold chymotrypsinogen artifactually relocated from other cell components. They also suggest that the lower S.R.A. of the free ribosomes could result from the inability of ribosomes to release synthesized enzyme unless they are attached to the membrane. No evidence was presented to support either hypothesis.

Palade (1975) does acknowledge in his Nobel Prize speech that continued uncertainty exists around the role of the free ribosome in protein synthesis. His presentation of the issue, however, is extremely confusing and even misleading. He selects data from the 1960b publication showing that at both 1 minute and 3 minutes post pulse, the S.R.A. of chymotrypsinogen associated with one fraction of free particles is much lower than that of the S.R.A. of chymotrypsinogen associated with the attached particles (Table 2). He ignores the data from 2.5 minutes which shows that both free ribosome fractions have an S.R.A. 70% that of the attached

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[4] This conclusion is reached in spite of previous work by the two investigators that had demonstrated that incorporation of ^{14}C adenine into RNA occurred at a considerably slower rate in free particles than in attached particles and thereby argued that free and attached particles represent distinct ribosomal populations (Siekevitz and Palade, 1958b).

Table II

From Palade, Intracellular Aspects of the Process of Protein Synthesis, Science, 189:347, 1975.

Specific radioactivity of chymotrypsinogen isolated from attached and free polysomes in guinea pig pancreas after *in vivo* labeling with [^{14}C] leucine. Data are counts per minute per milligram of chymotrypsinogen (estimated from enzyme activity). [From (18)].

	Time after [^{14}C] leucine	
Fraction	1 minute	3 minutes
Attached polysomes	22,100	10,000
Free polysomes	2,800	3,000

ribosomes as well as the data from 3 minutes which shows that the S.R.A. of the other free ribosome fraction is 80% that of the attached particles. Therefore of the 4 original values for the interval from 2.5 to 3.0 minutes, 3 are around 75% while only one is much lower at 30%, yet it is this one lower value that was chosen to represent the group. Furthermore, Palade omits any mention of the 45 minutes time point which shows the chymotrypsinogen S.R.A. of the combined free ribosome fraction equal to the chymotrypsinogen S.R.A. of the whole microsomal fraction. Large differences, therefore, between the S.R.A. of chymotrypsinogen associated with the free and the attached particles are not clear from the original data. Palade, having apparently selected for display the data points consistent with his current view, however, is now able to conclude that "radioactive chymotrypsinogen appeared preferentially associated with attached polysomes isolated from the guinea pig pancreas", and that free ribosomes in the pancreas as in the liver synthesize enzyme primarily for intracellular use.

The role of the free ribosome in protein synthesis in the pancreas still remains to be clarified, even after all these years of investigation. It is an important issue since synthesis of digestive enzyme on free ribosomes is not readily consistent with the cisternal packaging-exocytosis model. Certainly it is possible that artifactual adsorption accounts for at least some of the digestive enzyme associated with the free ribosomes. In this context, in a recent publication, Scheele, Tartikoff and Palade (1978) attempt to assess the relocation artifacts that occur during cell fractionation of pancreatic tissue. In their abstract they state that the free ribosome fraction is the fraction most contaminated by adsorption, however no direct data is shown to support

this contention in the body of the paper. They found that when exogenous labeled digestive enzyme was added to the homogenization medium the individual particulate fractions were enriched and the post-microsomal supernatant fraction impoverished in specific exogenous enzymes as a function of the isoelectric point of the enzyme. They hypothesize therefore, that selective adsorption of native cationic proteins occurs on the various particulate fractions during fractionation, leaving the supernatant enriched in native anionic proteins. Free ribosomes were found to be especially enriched in high isoelectric point enzyme and the authors apparently therefore conclude that <u>total</u> adsorption is greatest on this fraction. This conclusion is not justified by the data. Rather, those data simply indicate that free ribosomes have a higher concentration of negative surface charge than other fractions. Total adsorption or contamination of a fraction would include proteins adsorbed to positive surface charges as well, and it is possible that another fraction could have a larger amount of total adsorbed protein per relevant unit when both anionic and cationic proteins are taken into account. To make the point they claim to, the authors must show either the percentage of total label associated with the free ribosomes or the amount of label per total protein associated with free ribosomes is higher than for other fractions. The relevant data is presented for other fractions but does not appear for free ribosomes. Scheele has acknowledged that only a miniscule amount of the exogenous enzyme was associated with the free ribosomes (personal communication), though this was not clear from the paper. Moreover, the experimental data indicates that almost no enrichment of chymotrypsinogen occurs on free ribosomes even though the isoelectric point of the enzyme is high; in any case therefore, it cannot

be concluded from this study that artifactual adsorption of chymotrypsinogen to free ribosomes can explain the original Siekevitz and Palade data.

Cell free systems were used to provide additional support for the hypothesis that vectorial transport of newly synthesized enzyme occurs from attached ribosome to the cisternae of the endoplasmic reticulum. Redman, Siekevitz and Palade (1966) found that 52% of the newly synthesized amylase was found in the deoxycholate-soluble subfraction, while 30% of the newly synthesized amylase was found in the incubation medium. In addition, Redman and Sabatini (1966) showed that puromycin, which releases nacent polypeptide chains from ribosomes, caused a decrease in the radioactivity of ribosome-associated protein and polypeptides while simultaneously causing an increase in the radioactivity in the DOC-soluble fraction, when added to a microsomal synthesizing system. More recently Blobel and Dobberstein (1975) have shown that many exportable proteins may be synthesized with an N-terminal amino acid sequence that directs the nacent protein chains through the membrane of the endoplasmic reticulum and into the cisternal space. In the cisternae, the "signal" peptide is thought to be clipped off enzymatically. They have shown that approximately 60% of the enzyme synthesized by pancreatic microsomes is protected from trypsin proteolysis and therefore could be sequestered. The other 40% they assume has escaped into the incubation medium from microsomal vesicles broken during incubation. The above data is consistent with sequestration of some of the newly synthesized enzyme, however, most often it is interpreted to suggest that obligatory sequestration of all digestive enzyme occurs in the pancreas. The existence of a mechanism whereby digestive enzyme can be transported vectorially from ribosome

to the cisternae of the endoplasmic reticulum does not indicate that all enzyme follows this route. To prove obligatory sequestration, it must be shown that indeed all enzyme is sequestered.

Autoradiography was employed in an attempt to demonstrate the role of the Golgi in the transport sequence. Caro and Palade (1964) showed that 5 minutes after injection of ^{3}H leucine into guinea pigs, autoradiographic grains in slices of pancreas are concentrated over the basal cytoplasm. In addition, a few grains are found in the region of the smooth surfaced vesicles in the periphery of the Golgi. By 20 minutes the majority of the autoradiographic grains were associated with the partially filled Golgi vacuoles or condensing vacuoles, with only a few in the region of the peripheral Golgi elements. At 45 minutes grains were equally divided between condensing vacuoles and zymogen granules and at 4 hours they appeared only over the zymogen granules and the luminal space. No grains were found over the intracisternal granules and the authors suggest that the intracisternal granule fraction described in a previous publication may in fact have been heavily contaminated with Golgi vacuoles.

Assuming cisternal packaging, Caro and Palade find their data consistent with the hypothesis that digestive enzyme is transferred from the cisternae of the endoplasmic reticulum to the smooth surfaced vesicles and then to condensing vacuoles. The authors are aware that a definite designation of grains to the cisternae cannot be made. Furthermore, it is my view that the grains do not appear to be especially associated with the smooth surfaced vesicles. In any case, the resolution of the technique, 0.1 to 0.2 μ, is not good enough to make the apparent association of a few grains evidence for a role of the small vesicles in transport since vesicle size varies from 0.04 to 0.06 μ. A more cautious

reading of the evidence suggests then that enzymes synthesized in the basal portion of the cell may pass through the region of the cell occupied by the peripheral elements of the Golgi en route to the condensing vacuoles or the cell lumen.

In a similar pulse-chase autoradiographic study, Jamieson (1968a) found that greater than 20% of the autoradiographic grains were associated with the basal cytoplasm or endoplasmic reticulum at two hours post pulse. Such lingering of enzyme in the basal portion of the cell cannot be explained in any simple manner by the Palade model in which enzyme is viewed as moving en masse from compartment to compartment. It is, however, consistent with the model presented in this study, the equilibrium model.

In another attempt to demonstrate movement of enzyme from the cisternae of the endoplasmic reticulum to the smooth surfaced vesicles seen at the periphery of the Golgi, Jamieson (1967a) isolated a rough and a smooth vesicle fraction by gradient centrifugation. Using guinea pig slices, he found that at 3 minutes post pulse the S.R.A. of protein associated with the rough surfaced vesicles was higher than that of the smooth vesicles, whereas at 7 minutes the S.R.A. of the smooth vesicles was double that of the rough. He also incubated rough and smooth microsomes isolated at 17 minutes post pulse in 0.15 m $NaCl-HCO_3$ pH 8.4 and found that the S.R.A. of the protein in the soluble fraction was much higher than the S.R.A. of the protein in the sedimentable fraction. $NaCl - HCO_3$ had been shown to release the contents of zymogen granules. Jamieson, apparently on this basis alone, assumed it would do the same for microsomal contents and concluded that his data demonstrated that newly synthesized digestive enzyme was contained within the vesicles rather than associated with the membrane.

Experiments by Rothman (personal communication) urge caution in the interpretation of all the above work with isolated microsomes. His data challenge the assumption that either the NaCl - HCO_3 or the DOC-soluble fraction necessarily represents enzyme contained within vesicles. He shows that microsomes collected from the in vitro pancreas after a 3 minutes pulse and a 5 minutes chase release 70% of their TCA precipitable protein with mere dilution and resuspension in 3 ml of medium. This enzyme therefore is apparently absorbed to the membrane or contained within the intervesicular spaces.[5]

After dilution only 1.5% of total tissue protein-associated DPM (or 15% of the labeled protein in the microsomal fraction) was recovered in the DOC-soluble fraction. Yet, the cisternal packaging model predicts that most of the newly synthesized enzyme should be in microsomal vesicles (DOC-soluble fraction) at this time. Moreover, when inulin was added to the homogenization medium with the tissue, it appeared in the DOC-soluble fraction in amounts proportional to that of labeled protein. Since it is clear that the inulin must have entered the vesicles during the homogenization procedure, it is also possible that the labeled protein was external to the E.R. prior to homogenization as well. If, on the other hand, we assume instead that all protein is sequestered in the endoplasmic reticulum prior to homogenation, then the large amount of protein lost during the procedure would suggest an equilibration or approach to equilibration between the digestive enzyme contents of the endoplasmic reticulum vesicles and the medium. If this were so, homogenization in smaller volumes should increase both the amount and the

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[5] A transmembrane equilibrium of digestive enzyme is unlikely here since a 10x dilution lead to only a 10% increase in label in the medium.

proportion of protein remaining in the vesicles or the DOC-soluble fraction. This was not observed and the data suggests either that the protein enters vesicles only with homogenation or alternatively that the DOC-soluble fraction does not coincide with the contents of the endoplasmic reticulum. In either case these data indicate that firmer evidence than is presently available is needed to prove that cisternal packaging of some or all digestive enzyme does occur.

According to the Palade model, enzyme moves from the cisternae of the endoplasmic reticulum via the smooth surfaced vesicles of the Golgi to the condensing vacuoles, which mature into zymogen granules. Jamieson (1967b) isolated a condensing vacuole fraction and found that half maximal labeling of these vacuoles occur at approximately 37 minutes post pulse. At 57 minutes he found the label equally divided between the zymogen granules and the condensing vacuoles.

Jamieson (1968b) studied the energy dependence of enzyme release into the medium. Stimulated secretion was inhibited by Antimycin A and other inhibitors of respiration-dependent ATP formation. Basal output was not affected by such inhibitors, but since he viewed such basal output as leakage, he concluded that the extrusion process, exocytosis, is an energy dependent event.

The final step in the Palade model is the fusion of the zymogen granule membrane with the cell membrane and the extrusion of the granule's enzyme content into the lumen, in a process called exocytosis. The evidence for the process is morphographic, yet fusion profiles, or omega figures as they are called, are in fact rare occurrences in pancreas. In addition, one would expect that with stimulation, the frequency of such figures would increase, and roughly in proportion to the increase

in enzyme output. An increase in fusion figures with stimulation in the pancreas has been demonstrated by only one investigator (Ichikawa, 1965). His evidence suggests that sequential fusion of vesicles occurs, that is, once a vesicle has fused with the membrane, other vesicles may fuse to it then and so on. Of course it is possible that exocytosis may occur too rapidly to be seen easily using traditional electron microscopic techniques.

Initially it was thought that the membrane of the fusing granule remained and became incorporated into the apical plasma membrane. This concept of membrane flow proposed that digestive enzyme moved with its membrane from compartment to compartment, and that new membrane was added at the transitional elements of the E.R., while old membrane was removed from the plasma membrane. Work by Meldolesi and Palade (1971a-d) argues, however, that the membranes of the various compartments are too dissimilar with respect to both protein and phospholipid content for such a process to occur. The current view is that condensing vacuole membrane is produced by the Golgi and that the zymogen granule membrane is removed from the plasma membrane immediately after fusion.

In summary, the evidence in support of the cisternal packaging-exocytosis model is equivocal on many points. It does suggest that a portion of the newly made enzyme moves as a front from the basal cytoplasm to the Golgi and then to the apex of the cell. Whether or not the enzyme is necessarily contained within vesicles part or all the way remains a question. The model disregards all data suggesting that digestive enzyme can exist free in the cytoplasm of the cell as well as that suggesting digestive enzyme may be synthesized by free as well as attached ribosomes. These data are considered artifactual, mostly it appears because cisternal packaging is assumed. Finally, the evidence for exocytosis is meager,

however, if obligatory sequestration in impermeable vesicles is assumed to occur, an exocytosis-like release process must also be assumed to occur.

Equilibrium Model

In the past decade, a large body of evidence has accumulated, mostly from the laboratory of Rothman, but from other laboratories as well, which cannot be easily explained by the cisternal packaging-exocytosis model. These data have suggested an alternative model for digestive enzyme transport and secretion in the pancreas which has been termed the equilibrium model. In contrast to the basic assumption of the traditional view, that all enzyme is sequestered immediately after synthesis in membrane enclosed vesicles, a central tenel of the equilibrium hypothesis is that a portion of the digestive enzyme content of the cell is free in the cytoplasm of the cell. The model proposes that digestive enzyme stored in zymogen granules equilibrates with the enzyme content of the cytoplasm and that secretion itself occurs from the cytoplasmic pool across both the apical membrane and the basolateral. The equilibrium model views digestive enzyme transport and secretion as analogous to the simple diffusion of small molecules. It proposes that enzyme molecules move individually rather than en masse and that concentration-dependent, bidirectional fluxes of enzyme occur across membranes specialized for their transport.

Non-Parallel Secretion

The initial observation by Rothman (1967) at variance with the cisternal packaging-exocytosis model was that the gastrointentinal hormone cholecystokinin-pancreaozymin (CCK-PZ) led to a proportionally larger

increase in the secretion of trypsinogen from the *in vitro* pancreas than chymotrypsinogen. The observation reopened an old controversy; Pavlov in the early part of the century had championed the view that digestion, like metabolism, was a finely evolved and carefully regulated process in which specific enzymes would be secreted only in response to a need for that enzyme in the intestine (Pavlov, 1910). Babkin (1906, 1950), a student of Pavlov, championed the view that the proportions of different enzymes in secretion are invariant and argued that Pavlov's data was invalid because the proteolytic enzymes in his samples had not been properly activated prior to assay. Babkin's viewpoint is the generally accepted one today, though much evidence exists to the contrary. If secretion occurs as is envisioned by the cisternal packaging-exocytosis model, such constancy in the proportions of the different enzymes in secretion, or "parallel secretion" as it is often referred to, is required. This is so because each zymogen granule contains all the enzymes and there is no evidence for a significant variance in the amount of any given enzyme from granule to granule or from region to region of the pancreas (Kraehenbuhl, 1977).

According to the currently popular schema, then, the only place that the proportions of the different digestive enzymes in secretion can be modified is on the ribosome, before packaging occurs. Changes in the synthetic rate of one enzyme relative to another have been shown to occur in response to diet; however such changes take a number of days to develop at the minimum (Marchis-Mouren et al., 1963; Desnuelle, 1962). Alterations in synthetic rate cannot account for the numerous examples of short-term non-parallism reported in the literature (for review, see Rothman, 1977).

Recently, Rothman (1978b) repeated his initial observation showing short-term selective enhancement of trypsinogen with a preparation of pure CCK-PZ. He found that CCK-PZ increased trypsinogen secretion 4x that of chymotrypsinogen secretion, though parallel responses of the two enzymes were elicited by both acetyl-β-methylcholine chloride and a synthetic preparation of the C-terminal fragment of the hormone itself. With CCK-PZ the chymotrypsinogen response was 4x basal, while with the C-terminal peptide it was 15x the basal rate. The remainder of the molecule, therefore, must act in some manner to inhibit chymotrypsinogen secretion. This fascinating observation provides powerful support for the view that mechanisms exist whereby the secretion of different enzymes can be independently regulated.

Adelson and Rothman (1974) have described an enzyme-specific hormone called chymodenin which substantially enhances the short term secretion of chymotrypsinogen without stimulating the output of other enzymes to any large extent. In addition, they demonstrate that in the presence of chymodenin, the amount of chymotrypsinogen secreted by individual glands closely correlates to the amount of lipase secreted, while in the absence of the hormone, the amount of the two enzymes secreted correlates only poorly. Chymodenin is found in the intestinal mucosa and the authors are of the opinion that other enzyme-specific hormones might also be found there.

Another observation by Rothman (1974) suggests that the regulation of digestion indeed might be as subtle a process as Pavlov envisioned. He tested the effect of lysine, the end product of trypsin hydrolysis, on trypsinogen secretion. He found that the amino acid stimulated trypsinogen secretion when perfused into the duodenum at a concentration of 10^{-3} mM without increasing the output of chymotrypsinogen or other

digestive enzymes. A similar concentration of lysine added to the bath of the *in vitro* pancreas, however, decreased the ratio of trypsinogen to chymotrypsinogen in secretion, suggesting that a negative feedback loop may exist at the cellular level.

Rothman (1976a) also found that although glucose, an end-product of digestion, does not stimulate overall enzyme output when perfused in the duodenum, it does change the relative proportions of 3 enzymes found in secretion. These differences are masked when data from individual preparations are meaned, apparently because of a large variability in the general secretory activity of different glands even in the same physiological state. The changes, however, become evident when individual data points for each enzyme pair are plotted against each other; for example, there was a larger than 3-fold change in slope in the function relating trypsinogen to chymotrypsinogen secretion for the control and the + glucose condition. Furthermore, two of the functions have positive intercepts, which indicates either that the secretion of one enzyme can occur in the absence of the other or, alternatively, that the relationship of the two variables is non-linear. Either possibility is inconsistent with a parallel transport of the two enzymes.

Cytoplasmic Pool

Central to the equilibrium model is the view that digestive enzyme does exist free in the soluble portion of the cell. In an attempt to determine if the enzyme found in the cell fraction containing the contents of the soluble portion of the cell could indeed be entirely attributed to enzyme lysed from zymogen granules or microsomes during homogenization and centrifugation, as proponents of the traditional model assume, Rothman (1970a) examined the relative amount of trypsinogen and

chymotrypsinogen in the various cell fractions and in secretion from rabbit pancreas. Since the two molecules are closely related in both their structure and physical characteristics, it would be expected that they would respond to the stresses of the fractionation procedure in roughly similar ways. Rothman found, however, that while the soluble fraction contained 32% of the total trypsinogen in the homogenate, it only contained 18% of the total chymotrypsinogen. Furthermore, he found that the ratio of trypsinogen to chymotrypsinogen in both the zymogen granule fraction and the microsomal fraction was about 3.3, while this ratio in the soluble fraction was 9.1. Moreover, in secretion the ratio of the two enzymes was 6, which is midway between that of the zymogen granules and microsomes on the one hand and of the soluble fraction on the other. These observations suggest that enzyme does occur naturally in the cytoplasm, and in addition support the view that secretion is in part derived from the cytoplasmic pool.

Egg white trypsin inhibitor fed to rats for several days causes hypertrophy of the pancreas and an increase in the tissue content of digestive enzyme in the gland. When Rothman (1970b) examined the effect of trypsin inhibitor on the digestive enzymes contained in the various subcellular fractions, he found that most of the increase was not in the zymogen granules, as would be expected, but rather that 80 to 100% of the increase occurred in the soluble fraction of the cell. Since secretin did not "wash away" the extra enzyme in the PMS, the effect was not likely to result from an increase in the enzyme content of the ducts which would be released into the soluble fraction during homogenization. In another series of experiments (1969) he found that the tissue content of amylase and chymotrypsinogen in trypsin inhibitor fed rats was increased significantly relative to controls, while trypsinogen content was only slightly

increased. In contrast, trypsinogen secretion from trypsin inhibitor fed rats was augmented relative to controls whereas chymotrypsinogen and amylase secretion were not. Furthermore, MCH, which generally produces parallel increases in the secretion of these enzymes, produced a trypsinogen response 5x normal, a chymotrypsinogen response 2x normal, and had no effect on amylase secretion in trypsin inhibitor fed animals. These intriguing results are entirely inconsistent with exocytosis and in addition indicate a special adaptation of the transport mechanism to diet.

Rothman (1970a) also observed that the increase in the ratio of trypsinogen to chymotrypsinogen in secretion from rabbit pancreas that occurs with CCK-PZ stimulation (see last section), is accompanied by a reciprocal decrease in the ratio of these two enzymes in the soluble fraction. Since the ratio of the two enzymes remains constant in the other cell fractions, this change cannot reflect enzyme artifactually derived from other cell compartments. The simplest explanation of these results, therefore, is that a direct transport of enzyme occurs from cytoplasm to duct lumen.

Evidence from Dandrifosse (1970), a Belgian researcher, also suggests that molecular transport of enzyme occurs from cytoplasm to lumen across the cell membrane. He labeled amylase in pigeon pancreas slices and found two distinct amylase fractions, one that precipitates and one that is soluble at pH 4. Whereas a large portion of the amylase in the incubation medium (secreted amylase) was pH 4 precipitable, only very small amounts of this material were found in the various subcellular fractions. Dandrifosse concludes, therefore, that the pH 4 precipitable material is the amylase that is secreted. Furthermore, the S.R.A. of the pH 4 soluble material was high in the microsomes and zymogen granules and much lower

in the supernatant fraction. Since the S.R.A. of the secreted amylase (pH 4 precipitable material) was very close to that of the pH 4 soluble enzyme in the supernatant, Dandrifosse suggests that the pH 4 soluble enzyme is secreted from the cytoplasm to the lumen and in the process is modified so that it precipitates at pH 4.

Early evidence by Lin and Grossman (1956) and more recent evidence by Rothman (1975a) suggests that enzyme secretion can occur in the absence of zymogen granules. Rothman notes that if the exocytosis of zymogen granule contents were the only method of exit of enzyme from the cell, enzyme output would have to relate in a linear manner to the number of granules lost with repeated injections of secretagogue acetyl-β-methylcholine chloride (as long as the rate of granule loss is substantially greater than the rate of granule formation during administration of the secretagogue). The function relating the number of granules lost to enzyme output was in fact highly non-linear. By extrapolating this function to 100% granule loss, Rothman determined that indeed only about 60% of the actual enzyme output could be accounted for by the loss of zymogen granule content. Enzyme in secretion, therefore, must be derived from another intracellular pool in addition to zymogen granules and this pool is likely cytoplasmic. Since the rate of appearance of newly labeled protein in secretion was somewhat depressed rather than increased by serial MCh injections, it is not likely that the augmented protein output in response to repeated MCh injections can have come from the exocytosis of a population of rapidly turning over vesicles containing newly synthesized enzyme. The data is consistent either with a final common pathway via a non-exocytotic mechanism, as is suggested in Part I of this study, or with an independent non-granular pathway.

Finally, according to the cisternal packaging-exocytosis construct, movement of newly synthesized enzyme from ribosomes to lumen takes 35 to 40 minutes at a minimum. Rothman (1976), however, found that a small amount of newly labeled enzyme could be collected in secretion in the first 5 minutes after isotope was added to the *in vitro* pancreas. The appearance of this newly synthesized enzyme in secretion tended to reach a constant level by 15 minutes. Rothman estimated the minimum transit time of this newly synthesized enzyme to be 2.5 minutes. That this enzyme is not an artifact is suggested by the fact that an inverse relationship exists between the "steady-state" level of the new protein in secretion and the total protein output for individual glands, as well as the fact that MCh inhibited this early release of newly synthesized enzyme by about 1/2 and this inhibition would not have occurred if the newly synthesized enzyme were leaked from damaged cells (Figure 7). Rothman suggests that the early secreted enzyme is released directly from the ribosomes into the cytosol. He estimates, from the S.R.A. of early samples relative to a theoretical steady-state S.R.A., that it represents about 2.5% of secreted enzyme.

Evidence for Equilibration of Digestive Enzyme Across Zymogen Granule Membranes

Liebow and Rothman (1972) demonstrated that zymogen granule membranes have an enzyme specific, bidirectional permeability to digestive enzymes. They found that increasing the volume of the medium in which zymogen granules were suspended increased the amount of enzyme released into the medium. A log-log plot of supernatant/pellet vs dilution resulted in a slope of 1. This indicates a first-order equilibrium with release dependent on the amount of enzyme in the pellet. Therefore, enzyme binding within the granule must occur in such a manner that all molecules are in equilibrium with the medium.

Release was enzyme specific and equilibrium occurred slowly over time. In response to an eight-fold dilution, amylase release was 80% at 240 minutes with a half time of 30 minutes. Chymotrypsinogen release, in contrast, was 50% at 240 minutes with a $t_{1/2}$ of 60 minutes. Since chymotrypsinogen from reaggregated material equilibrated within the 10 minutes centrifugation interval, the slow approach to equilibrium in the intact granules can be attributed to the presence of the intact membrane. (Zymogen granule contents which have been solubilized by high pH become reaggregated when the pH is lowered back to 5.5.)

Bidirectionality of the enzyme flux was demonstrated by showing that concentrating a diluted suspension of granules led to a redistribution of enzyme back into the granules from the supernatant. In addition, Liebow and Rothman demonstrated that uptake of H^3 α-chymotrypsinogen into granules increased with time relative to the uptake of I^{131} albumen. They also showed that with continuous removal of the suspending medium by filtration, enzyme release from granules, rather than approaching zero as it does in the absence of filtration, remained constant over time. This indicates that filtration, by removing the effluent, reduced the back flux of enzyme from suspending medium to granule.

The Q_{10} for the release of chymotrypsinogen from granules was over 5, suggesting that the release process does not occur by simple diffusion but is reaction mediated. The Q_{10} for amylase release was considerably less. Liebow and Rothman suggest that since amylase is a molecule 2x the size of chymotrypsinogen, the transport process must depend on conformational rather than on physical characteristics of the molecules. This again suggests that enzyme release from granules is reaction mediated rather than a diffusional process.

Liebow notes that isolated zymogen granules are unstable and tend to release enzyme in both isotonic KCL and at pH 7 (Burwin and Rothman), conditions which mimic the intracellular environment. He suggests, however, that these forces favoring enzyme release would be counteracted by the very small volume of the <u>in situ</u> suspending medium, the cytoplasm, and, therefore, that equilibration within the cell would leave most of the enzyme in the granules.

In the electron microscope, filled zymogen granules appear as relatively uniform, electron opaque structures. In an attempt to gain insight into granule ultrastructure, Ermak and Rothman (1978) studied the morphology of a range of partially empty zymogen granules produced by dilution. They observed a reticular meshwork attached at regular intervals to the granule membranes, composed of electron dense strands 50 to 100 μm thick, which define polygonal and spherical spaces ranging from 20 to 100 μm in diameter. Loss of enzyme appeared to occur in a relatively even way throughout the granule with the strands becoming thinner and the spaces enlarging as the granules lost their contents. This even loss of material throughout the granule is consistent with the kinetic data suggesting that all the enzyme in the granule is in equilibrium with the suspending medium. Granules which had been treated with detergent to remove their membranes also displayed a reticular structure. In addition, when released digestive enzyme was reaggregated with granule membrane, a similar but more open and loosely packed morphology was observed.

A straight or branching filamentous material connecting the thicker strands was sometimes seen in partially empty or "reticulated" granules, and might represent the basic structural molecules to which the enzyme binds. Filamentous strands were often seen in granules which were virtually empty of all their enzyme content.

Reticulated granules occur frequently in dissociated acinar cells and their number tends to increase with increasing incubation time. They have been called "loose condensing vacuoles" in autoradiographic studies because they become labeled before the zymogen granules. Ermak proposes an alternative explanation for the early labeling of these structures; he suggests that uptake of enzyme from the cytoplasm occurs in these partially empty granules before it does in filled granules. In support of the notion that reticular granules are not condensing vacuoles is the fact that condensing vacuoles are osmotically active whereas reticulated granules are not (Jamieson, 1971).

Zymogen Granule - Chemical Evidence for Zymogen Granule Structure

Hokin (1955) had found that zymogen granules were most stable around pH 4.5. He observed that release of digestive enzymes from the granules, or "lysis", occurred when the pH of the suspending medium was raised to 7 or 8, and that this release was accompanied by a decrease in the turbidity of the medium. Rothman (1971) measured the percent release of amylase, chymotrypsinogen and trypsinogen as a function of pH and found that the curves were different for the 3 enzymes. In addition, he observed that reaggregation or reassociation of a large part of the released enzyme occurred with the empty membranes and the solution again became turbid when the pH was returned to 5.5. This reaggregation of protein did not occur without the membranes. Furthermore, he found that the maximum reassociation of exogenous chymotrypsinogen and trypsinogen with zymogen membranes occurred in proportions similar to those found in the native granules (Rothman 1972). In contrast, bovine serum albumen did not associate with the empty membranes to any significant extent. The data suggested that the zymogen granule was an ordered

structure in which both site specific digestive enzyme - membrane and digestive enzyme - digestive enzyme interactions occur.

Burwen and Rothman (1972) found rat granules to be quite stable in H_2O, 0.3 molar sucrose and 0.3 molar urea. This suggested that digestive enzymes were stored inside the granules in osmotically inactive form, and confirmed observations by Hokin (1955) and Jamieson (1971b) on other species. In contrast, they found that zymogen granules were unstable in electrolyte solutions. In addition, they noticed that general differences exist in the capacity of monovalent and divalent cations to release enzyme and also that the different enzymes have different release tendencies or sensitivities to the same cation.

Burwen and Rothman observed that the turbidity of a suspension of zymogen granules, which is determined primarily by the number of granules present in solution, was unchanged by the addition of 0.1 M $CaCl_2$. However, under these circumstances, almost complete release of amylase and chymotrypsinogen had occurred from the granules with 50% release of total protein and lipase. Since complete release of at least two enzyme species occurred, the number of granules and therefore the turbidity of the solution should have also decreased if each granule contained only one enzyme species. Since turbidity was unchanged, the authors concluded that each granule must contain a mixed array of enzymes. This conclusion was subsequently confirmed by Kraehenbuhl (1977) with antibody studies. In summary then, enzyme specific release occurs from granules containing a mixed array of enzymes, in response to the pH, the electrolyte content and the volume of the suspending medium.

Tissue Uptake Studies

Studies by Liebow and Rothman (1976) provide direct evidence for species - specific transport of enzyme across the plasma membrane. They reasoned that such a process would likely be bidirectional and equilibrated slices of rabbit pancreas with ^{3}H chymotrypsinogen and ^{131}I albumin in order to measure label uptake into the various subcellular fractions. Cellular uptake of digestive enzyme demonstrated by the fact that the S.R.A. (^{3}H cpm/mg protein) of the zymogen granule fraction was 14x that of the homogenate after 5 minutes of incubation. Selective enzyme uptake is indicated by the fact that chymotrypsinogen uptake was 17x the ^{131}I albumin uptake into the homogenate and 150x the albumin uptake into the zymogen granule fraction. After 5 minutes of incubation, the S.R.A. of chymotrypsinogen (^{3}H/μg chymotrypsinogen in fraction) was highest in the PMS and next highest in the zymogen granules. By 60 minutes, both the S.R.A. of the PMS and the zymogen granules were close to that of the bathing medium, suggesting isotopic equilibration with the exogenous chymotrypsinogen. The S.R.A. of the other fractions remained low. This evidence suggests that enzyme uptake occurs first from bath to cytoplasm and then from cytoplasm to zymogen granule.

Enteropancreatic Circulation

Slices of pancreatic tissue expose both the apical and the basolateral membranes of surfaces of the cell to the medium. In a control experiment, Liebow and Rothman (1975) measured ^{3}H chymotrypsinogen uptake in unsliced strips of pancreas tissue, which expose mostly the basolateral surfaces of the cells to the medium. Since uptake was still substantial, it was evident that the basolateral as well as the apical membrane of the cell

was permeable to digestive enzyme in the bath to cell direction. Part II of the current study demonstrates the cell to bath basolateral flux predicted by this observation.

Since exogenous and endogenous chymotrypsinogen had been shown to equilibrate inside the cells, it seemed likely that exogenously added enzyme could also be secreted by the cells. Liebow and Rothman (1975) demonstrated that exogenous chymotrypsinogen added to the bathing medium of the *in vitro* pancreas appeared in secretion. A cellular route through the pancreas was suggested by the observation that adding a large concentration of cold chymotrypsinogen to the bath transiently decreased the amount of labeled enzyme in secretion. If chymotrypsinogen crossed the gland via a diffusive route, either no change or an increase should have occurred; the decrease that was observed suggests saturation kinetics and inhibition of some sort and therefore a cellular route. A cellular transpancreatic route was also suggested by the observation that CCK-PZ, which increases amylase secretion from the acinar cell, increased the rate of secretion of labeled amylase added to the bath of the whole rabbit pancreas in short term organ culture. Further evidence for a cellular route from bath to duct is presented in Part IV of the current study.

Liebow and Rothman reasoned that if enzyme can be absorbed from blood and resecreted, a recycling mechanism could exist for digestive enzymes after they are secreted into the gut by the pancreas. Indeed the intestinal epithelium was known to be permeable to some digestive enzymes. They instilled labeled chymotrypsinogen into rabbit intestine just below the entrance of the pancreatic duct and found that labeled molecules did appear in pancreatic secretion. The peak response occurred in 15 minutes. Since 70% of the secreted label comigrated with bovine

chymotrypsinogen, the circulated molecules were for the most part intact digestive enzymes. They proposed an enteropancreatic circulation of digestive enzyme, analogous to the well established enterohepatic circulation of bile, in which enzyme is absorbed intact into the blood from the gut and then transported through the pancreas back into secretion.

Göetze and Rothman (1975) show that the circulation process could be a bulk phenomenon. They compared the fall-off in MCh stimulated secretion over a 3 hour period when secretion was reinstilled into either blood or intestine or simply diverted from the system. When compared to diverted controls, mean protein output was augmented by about 60% of the amount that had been reinstilled. Input and augmented output in individual experiments were highly correlated with a slope of almost 1, indicating a high efficiency process. The function had a relatively large positive intercept, suggesting that the system has a substantial capacitance.

Enteropancreatic circulation requires the capacity for large scale absorption of digestive enzyme by the intestine. Göetze and Rothman (1978) measured the transport of amylase across rabbit ileum in a modified Ussing chamber. They found that amylase was transported preferentially in the mucosal to serosal direction with a rate 2-3 orders of magnitude greater than for inulin. They estimated that the amylase absorption capacity of the intestine is of sufficient magnitude to account for the circulating fraction of the enzyme.

A Model for Membrane Transport

The kinetic evidence suggests that the plasma membrane and the zymogen granule membranes of the acinar cell, as well as the intestinal epithelium are permeable to digestive enzyme. The mechanism by which digestive enzymes pass through the various membranes is not yet known.

However, views on membrane structure have changed radically in the last decade. When the cisternal packaging model was first elaborated, the membrane was viewed as a static hydrocarbon bilayer; we see it now as a mosaic of protein and lipid in constant flux. We know proteins both penetrate the bilayer and span its width. Indeed, there is evidence on the degradation of membrane proteins indicating that they are in equilibrium with their own species in the cytoplasm of the cell (Schimke, 1974). It is not a large jump to envision the movement of globular protein molecules through the membrane. Protein movement through the membrane could occur by interactions with other proteins in the membrane or, alternatively, by interactions with either cellular or membrane lipids.

Rothman (1978b) demonstrates, for example, that digestive enzymes can easily be made hydrophobic by interactions with a few polar phospholipids. He shows that phosphatidyl inositol has a high affinity for chymotrypsinogen and coprecipitates with the enzyme to form a water-insoluble complex. A similar reaction occurs with trypsinogen, but not with α-chymotrypsin, albumin, carbonic anhydrase or lactic dehydrogenase. The reaction is facilitated by 10^{-4} mM Ca^{++} and the stoichiometry of the complex is ~3 · 3 · 1, phospholipid · Ca^{++} · chymotrypsinogen. Since the complex is unstable in ionic solutions, the association is primarily electrostatic. Rothman suggests that the mechanism for membrane transport of digestive enzymes might involve protein-lipid interactions of this type, and points out that the surface of the enzymes to be transported might be organized so that only a few lipid molecules are required to make them hydrophobic.

METHODS

Biological Preparations

The pancreas of the rabbit is a diffuse anatomical structure suspended within the mesomentum of the first duodenal loop. The pancreatic tissue lies in discrete clumps which tend to run in bands within the mesentary. Since the thickness of the tissue is only on the order of 0.2 mm, adequate oxygenation can occur from an external source and the gland, therefore is, well suited for _in vitro_ study.

The preparation used for most of the experiments in this study was the whole rabbit pancreas in short-term organ culture, developed by Rothman (1964). In contrast to most other _in vitro_ pancreas preparations, this preparation maintains the natural polarity of the gland; the bathing medium is in contact only with the basolateral surface of the cells while apical secretion is gathered separately via a cannula inserted into the major collecting duct.

Glands were obtained from male, white New Zealand rabbits (2-3 kgs body weight). Following an overnight fast (14-18 h), the animals were anesthetized with a mixture of allobarbitol (0.34 mmole/kg body weight), urethan (3.1 mmole/kg) and monoethylurea (3.2 mmole/kg) administered intraperitoneally. After the abdominal cavity of the animal was opened by a midline incision, the duodenal loop containing the pancreas was located and gently eased out of the cavity. An incision was made in the intestinal wall adjacent to the papilla of the pancreatic collecting duct, and polyethylene tubing (P.E. 10) was inserted about an inch up into the duct through the papilla and tied in place with 000 silk thread. Once good flow had been obtained, the caecum, which is attached to a small internal loop of intestine within the larger loop, was peeled away and

the whole duodenal loop removed as a unit. Since pancreatic tissue can extend right to the pylorus of the stomach and sometimes as far dorsally as the liver, care was taken at these borders to include all the tissue. (This is necessary in order to maintain the integrity of the duct system and thus the polarity of the gland). After excision, the gland was rinsed carefully in 3 volumes of saline and then attached with thread to a plexiglas frame with the orientation displayed in Figure 1. After the cannula was threaded through a conduit leading from the inside to the outside of the vertical bathing chamber, the frame with mounted tissue was lowered into the chamber. 400 ml of a HCO_3^- buffered Krebs-Henseleit solution (Krebs and Henseleit, 1932) containing a nutrient mixture of amino acids recommended by Eagle (1959) and 5.5 mm glucose was pre-equilibrated with a mixture of 95% CO_2 and 5% O_2 and added to the chamber. During incubation, gas was continuously bubbled into the medium through a scintered glass filter built into the bottom of the chamber. In general, about 15 minutes elapsed between the excision of the gland from the animal and the addition of the bathing medium to the gland in the chamber. The temperature of the bathing medium was carefully maintained at 30° by an infrared light regulated by a thermistor probe (except in the temperature-dependence studies where temperature was varied as stated). Flow ranged from 240 to 636 λ/h. The mean flow rate was 360 λ/h ± 162 S.E.M.

A strip preparation was needed for one of the studies. After an overnight fast, animals were sacrificed with 50 cc of air injected into an ear vein. The abdominal cavity was opened quickly, and then the pancreas with its intestinal border was removed from the animal and draped over a saline filled petri dish. After the mesentary was gently peeled away

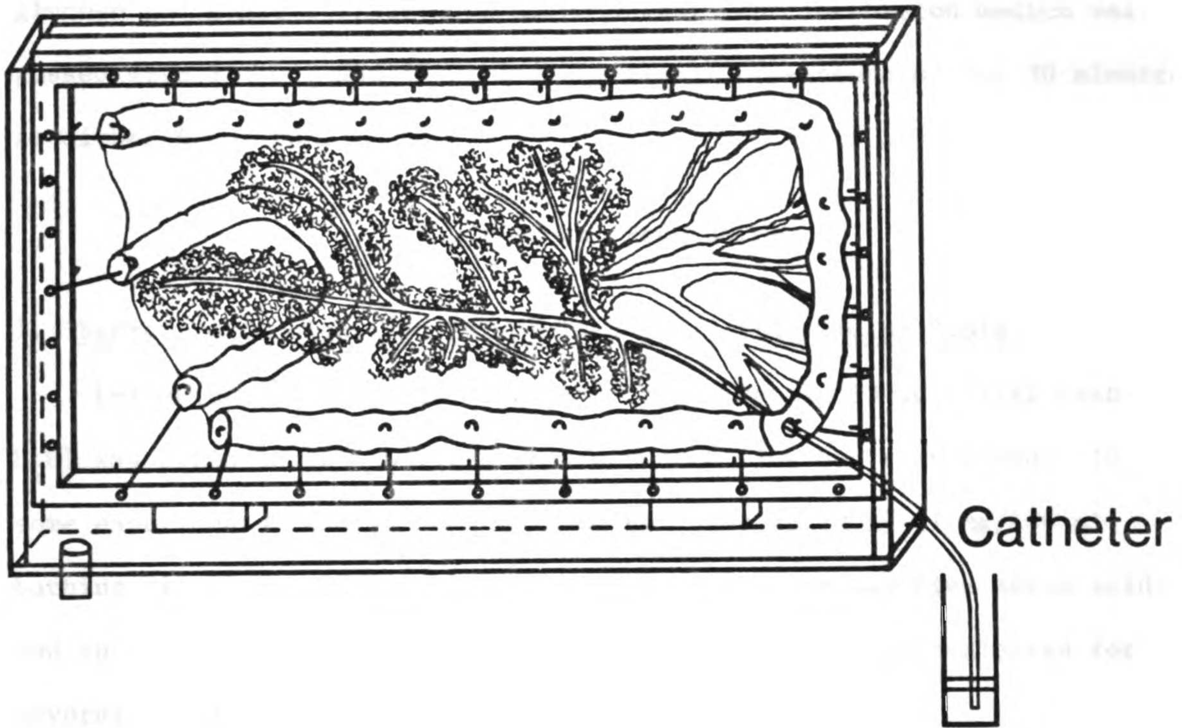

Figure 1. Rabbit pancreas in short term organ culture. The duodenal loop containing pancreatic tissue is shown mounted on its plexiglas frame. Ductal secretion is collected via the indwelling catheter.

with forceps, strips of pancreatic tissue were removed and placed immediately in HCO_3^- buffered Krebs-Henseleit fluid enriched with 5.5 mM glucose and Eagle's mixture of amino acids. The incubation medium was gassed with 95% CO_2 and 5% O_2 and the tissue pre-incubated for 30 minutes at either 4° or 20°.

Experimental Procedures

I. Secretion of Digestive Enzyme from Two Intracellular Pools

L-leucine-^{14}C (180 mCi/mmole and approximately 63 μCi/liter bath H_2O) was added to the *in vitro* pancreas once flow had stabilized. In some experiments, acetyl-β-methylcholine chloride (MCh) (1 mg/100 ml bathing fluid) was added at the same time as the radioactive amino acid and in others it was added after 2 hours. Secretion was collected for several hours.

Secretion samples were diluted in 3 ml H_2O, and protein content was estimated by ultraviolet (UV) absorption at 280 nm. Absorption measurements were compared to a chemical estimate of protein content using the method of Lowry (1951). The two measurements were directly proportional in the range used, with 280 Å giving approximately one half the value of the Lowry estimate. 3 mls of 20% TCA were added to the sample to precipitate the digestive enzyme which was then trapped (and separated from the free ^{14}C-leucine) on 0.22 μm cellulose ester filters (Millipore). The filters were washed with 30 ml of 10 mM cold leucine in 10% TCA, dried and their radioactivity counted.

II. Basolateral Flux of Amylase

The *in vitro* pancreas was incubated for 1 hour after surgery, the bathing medium was then changed and measurements initiated. In the

equilibration study, ductal secretion was collected and bathing medium sampled at 15 or 30 minute intervals for 3.5 hours. Two milliliters of the bathing solution were removed for each sampling period; since the total removed during the experiment accounted for less than 10% of the total chamber volume, no replacement was made. In some experiments, pancreozymin (0.1 mg of hormone per mg of peptides) (6.0 Ivy dog units/ 100 ml bath) was added to the medium at 190 minutes, and acetyl-β-methylocholine chloride (1 mg/100 ml bath) was added at 250 minutes. After these agents were added, bath and duct samples were collected at 10 minute intervals.

In one experiment, after the hour of pre-incubation, the bath was changed and 2 ml bath samples were collected at 10 intervals for 30 minutes. The bath was then changed and rinsed again and the procedure repeated 6 times.

Bath and secretion samples were assayed for amylase activity. In some experiments, bath samples were assayed for lactate dehydrogenase activity and RNA content in addition.

III. Temperature Dependence of Apical and Basolateral Secretion

The temperature dependence of apical secretion and the initial rate of amylase release into the bathing medium were measured in the whole rabbit pancreas in short-term organ culture between 5° and 38°. After an hour of equilibration at 30°, glands were equilibrated at a given temperature for 30 minutes (5°, 10°, 20°, 30°, 35°, and 38°C successively), then the bath was changed and rinsed and 2 ml bath samples were removed at 0, 10, 20, and 30 minutes. Ductal secretion was collected at each temperature for 30 minutes. Samples were assayed for amylase activity.

To determine the temperature dependence of enxyme release from zymogen granules, strips of pancreas from the same gland were incubated at either 4° or 20° for at least 30 minutes. Approximately 100 mg of tissue were removed from the incubation medium, blotted on filter paper and weighed. Two batches of tissue at each temperature were homogenized in quick succession in water-jacketed, constant-temperature homogenization vessels which had been pre-equilibrated to either 4° or 20°. 1 ml of 0.3 M sucrose (10x tissue weight) at the appropriate temperature was added with the tissue to the vessel, and homogenization performed with 3 long and 3 short shearing strokes of a teflon pestle (clearance 0.13 to 0.15 mm).

Cell fractions were separated by differential centrifugation (Hokin, 1955; Rothman, 1970; Burwen, 1972b). All operations were performed at 4° on the tissue samples incubated and homogenized at 4°, and at 20° on the tissue incubated and homogenized at 20°. The homogenate was centrifuged at 750 g for 10 minutes using a Sorval SS-34 fixed angle rotor, and the pellet containing the whole cells and nuclei discarded. The supernatant was spun at 1,000 g for 10 minutes, and the second pellet consisted of a central, white zymogen granule zone surrounded by a buff-colored ring of mitochondria. The 1,000 g supernatant was decanted and saved and then as much of the mitochondrial ring as possible was removed from the pellet with a cotton-tipped stick. The remaining material was resuspended in 1/5 the original volume of sucrose and centrifuged again at 750 g for 10 minutes. The supernatant was then spun at 1,000xg for 10 minutes, and the 1,000 g sediment, the purified zymogen granule fraction, was resuspended in 1 ml of 0.1 M Na phosphate buffer (pH 7.4). The supernatant saved from the first 1,000 g spin was placed in a Type 50

Beckman titanium rotor and centrifuged at 133,573 g for 1 hour. The supernatant from this spin, the post-microsomal supernatant fraction, and the zymogen granule fraction were assayed for amylase activity.

IV. Transpancreatic Transport of Amylase

After an hour of incubation, the bathing medium of the whole rabbit pancreas in short-term organ culture was changed, and control secretion samples collected at either 10 or 20 minute intervals (as specified) for an additional hour. At this time, either porcine alpha amylase (Sigma, Type VI-A), the same material repurified (as described below), or 5x crystallized bovine chymotrypsinogen A (Worthington Chemical Co.) was added to the bathing medium at specified concentrations. Secretion was then collected at 10 minute intervals for 1 hour and at 15 minute intervals for an additional hour. Samples were assayed for either amylase activity alone or both chymotrypsinogen and amylase activity.

In a number of preparations, 4 mC 1-leucine (4, 5-^{3}H) (specific radioactivity, 62 Ci/mmole) (Schwarz Mann Co.) was added to the bathing medium and the gland incubated in the radioactive leucine alone for 5 hours before exogenous amylase was added. During this time, secretion was collected at hourly intervals for 3 hours, followed by a 50 minute and then a 10 minute collection period. When exogenous amylase was added, secretion was collected at 10 minute intervals for 1 hour and 15 minute intervals during the subsequent hour. The incorporation of ^{3}H leucine into secreted amylase was determined by purifying amylase in all samples by glycogen precipitation, as described below, and measuring its ^{3}H content.

In a number of experiments, after a 2 hour incubation with chymotrypsinogen, acetyl-β-methylcholine chloride (1.0 mg/100 ml bath fluid) was added to the bathing medium and secretion collected at 10 minute intervals for an additional hour.

V. Secretion Blockage

In some experiments, ductal secretion was first collected from the *in vitro* pancreas for two 1 hour periods, followed by a 50 minute period, and a 10 minute period; then the end of the cannula was fused closed to prevent fluid flow. This blockade was maintained for 90 minutes after which time the tube was reopened. Immediately following the resumption of flow (post-blockage), secretion was collected for 15 minutes at either a 2, 3, and two 5 minute intervals, or three 5 minute intervals, depending on the volume of secretion. Two additional 15 minute collection periods followed. 2 ml of bath fluid were removed every 30 minutes up to blockage; during blockage, 2 ml were removed every 10 minutes. Bath and secretion samples were assayed for amylase.

In other preparations, after a 1 hour equilibration period, a back-pressure was applied to the duct system by raising the end of the cannula in approximately 10 mm increments (0.74 mm Hg back-pressure) starting at the level of the papilla and continuing until secretion was reduced almost to zero (the organ was mounted vertically with the papilla in the most dependent position). The height of the column of fluid was then lowered back to the level of the papilla in steps of approximately 30 to 40 mm. At each level, secretion was collected for 5-10 minutes depending upon flow rate. These sampling periods reflected steady-state flow for all levels at which the cannula was set, i.e., for each setting an additional 5-10 minute collection period produced the identical flow.

Analytic Techniques

Amylase Activity

Alpha amylase activity was measured by following the hydrolysis of a starch substrate labeled covalently with Remazol Brilliant Blue R (Amylose Azure, Calbiochem) (Rinderknecht, 1967). A total volume of 0.5 ml (H_2O + sample) was added to 4.5 ml of a 2% solution of the substrate in 0.02 M sodium phosphate buffer (pH 7.0) containing 0.05 M NaCl. Samples were incubated in a shaking water bath at 37° and the reaction terminated at 15 minutes by the addition of 2 ml 0.1 N acetic acid. Samples were then filtered through #2 Whatman filter paper and the absorbance of the filtrate read at 595 nm. The absorbance was compared to a standard curve made from 2x crystallized porcine alpha amylase (506 I.U./mg) (Worthington Chemical Co.). Since amylase activity relates linearly to absorbance at 595 only at relatively low amylase concentrations, care was taken to dilute samples appropriately. For most samples, 0.5 ml bath fluid was assayed, though smaller volumes were used for samples obtained in the presence of acetyl-β-methylcholine chloride. Ductal secretion samples were diluted and amounts assayed were equivalent to as little as 0.1 λ of undiluted secretion for stimulated samples, or as much as 2 λ of undiluted secretion for basal secretion.

Chymotrypsinogen Activity

Chymotrypsinogen activity was estimated from the initial rate of esterase activity of an activated secretion sample. To activate the enzyme, 10 to 50 μl of secretion in 1 ml H_2O was added to 1 ml 0.1 M Na phosphate buffer (pH 6.0), containing 50 mg% enterokinase (3.4.21.9) (Miles Laboratory) and the mixture incubated in a shaking water bath for 30 minutes at 37°.

Immediately following incubation, the activation mixture was added to 1.0 ml of 0.1 M Na phosphate buffer (pH 7.4) and 3.0 ml of the substrate (80 mM suspension of acetyl tyrosine ethyl ester in 30% methanol) and then the initial rate of liberation of H^+ measured at pH 7.4 at 25° using a pH stat technique. The presence of chymotryptic activity was related to sample size linearly over the range used. Measurement error has been calculated as no larger than 6% for this assay, both from sample to sample and from experiment to experiment (Rothman, 1976).

Lactate Dehydrogenase Activity

Lactate dehydrogenase activity was measured according to the method of Amador, et al. (1963). The enzyme catalyzes the conversion of lactate to pyruvate and NAD to NADH, and its activity is directly proportional to the increase in absorbance of NADH at 340 nm. 0.3 ml of bathing medium was added to 2.5 ml of a solution containing 5.2 mM nicotinamide adenine dinucleotide, 61.9 mM L-lithium lactate, and 110.8 mM tris buffer (pH 8.8). The reaction was followed spectrophotometrically at 340 nm for 4 minutes at 25°.

RNA Assay

The presence of RNA was determined by the method of Schneider (1957), which takes advantage of the selective solubility of nucleic acids in hot trichloroacetic acid. The amount of RNA is then determined colorometrically with orcinol reagent.

1.0 ml of bath sample was mixed with 2.5 ml of cold 10% TCA and centrifuged at 10,000 g for 10 minutes to remove compounds soluble in cold acid. The precipitate was washed with another 2.5 ml of cold 10% TCA. To remove phosphorus containing lipids, the precipitate was mixed

with 5 ml of 95% ethanol and centrifuged at 10,000 g for 10 minutes. The precipitate was then recovered and the extraction procedure repeated. To separate DNA and RNA from tissue protein, the residue was suspended in 2.6 ml of 5% TCA and heated at 90° for 15 minutes with occasional stirring. The solution was then centrifuged at 10,000 g for 10 minutes and the supernatant saved. The residue was washed with 2.5 ml of 5% TCA, which was added to the prior supernatant to form the final nucleic acid fraction.

Orcinol reagent was prepared immediately prior to use by dissolving 1 g Orcinol in 100 ml concentrated HCL containing 0.5 g of $FeCl_3$. 0.2 ml of the nucleic acid extract was diluted to 1.5 ml and heated in boiling water with 1.5 ml of Orcinol reagent for 20 minutes. Samples were read spectrophotometrically at 660 nm and compared to a standard curve made from yeast RNA (Worthington Chemical Co.).

Protein Assay

The protein content of samples was measured according to the method of Lowry, et al. (1951). 0.4 ml of diluted sample containing less than 0.1 milligram protein was added to 2 ml of a freshly made mixture of 50 ml 2% Na_2CO_3 in 0.1 N NaOH and 1 ml of 0.5% $CuSO_4 \cdot 5 H_2O$ in 1% Na tartrate. The solution was mixed and left standing for 10 minutes. Then 0.2 ml of 1 N Folin reagent was added, the sample stirred and left standing again for at least 30 minutes. Absorbance of samples at 750 nm was measured and compared to a standard curve made with bovine albumin.

Purification of Amylase in Secretion

Amylase was purified from secretion in the transpancreatic transport studies by precipitation with shellfish glycogen. The purification procedure is based on the observation that at 4° and in 40% ethanol amylase

binds but does not hydrolyze glycogen (Loyter and Schramm, 1962). According to Levitzki et al. (1964), an insoluble lattice structure is formed in the cold, and the amylase is liberated as the glycogen is digested when the solution is warmed to room temperature. 25 to 50 μl of juice (containing between approximately 25-315 μg of amylase) was added to 1 ml of 40% ethanol at 4°, and then 50 μl of 0.2 M Na phosphate buffer (pH 8.0), 50 μl of 2% shellfish glycogen and 70 μl 95% ethanol were added in sequence and the mixture agitated for 5 minutes at 4°. The mixture was centrifuged at 2,000 g for 6 minutes, and the pellet resuspended in 1 ml of 40% ethanol at 4° containing 0.01 M Na phosphate buffer (pH 8.0). The wash procedure was repeated 3 times and the final pellet suspended in 1 ml H_2O at room temperature. 100 μg of amylase was added to control samples which contained smaller amounts of amylase to roughly equate the amount of amylase being purified between control and experimental samples. Over the range of sample sizes used, the yield of amylase recovered by glycogen precipitation was between 75-100% and no systematic variation in yield was observed (Figure 2). This purification technique increased amylase specific activity by between 5 and 10-fold, depending upon the specific activity of amylase in the original secretory sample.

Repurification of Sigma Amylase

Porcine alpha amylase (Sigma, Type VI-A) was repurified by a similar procedure. 750 mg of amylase was added to 1 L of 0.01 M Na phosphate buffer (pH 8.0) in 40% ethanol at room temperature and then cooled to 4°. 1 g shellfish glycogen was added slowly to the mixture while it was being stirred by a magnetic stirring bar, and this was followed by the volume of 95% ethanol necessary to return the ethanol concentration to

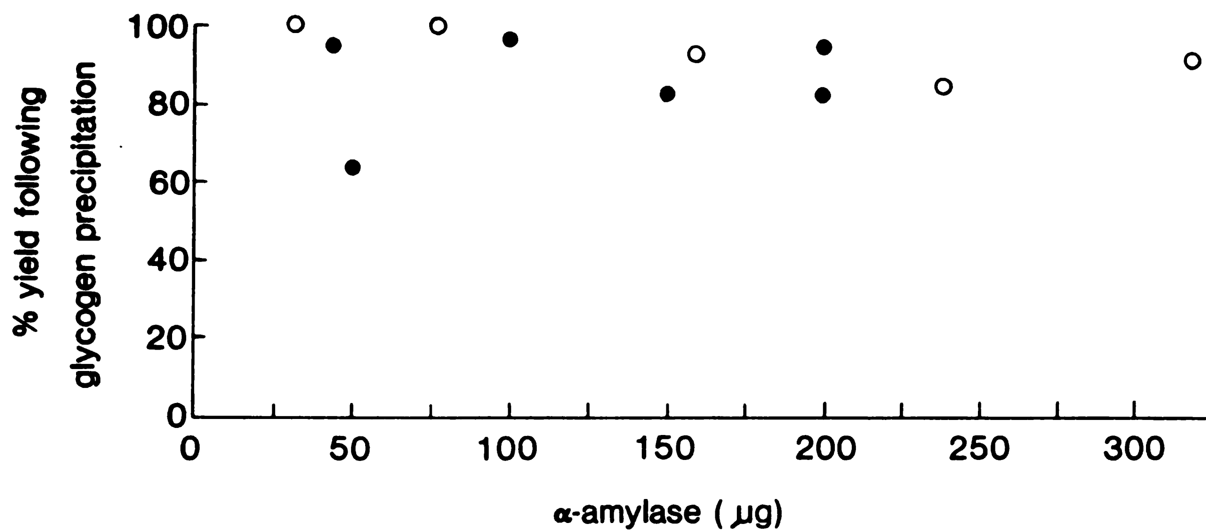

Figure 2. Per cent yield of amylase after glycogen precipitation. 25 to 325 μg of amylase relative to standard (508 U/mg) from either secretion (o) or a commercially purified amylase with specific activity of 700 U/mg (●) were purified and the percentage yield calculated. Each point represents the mean of three separate determinations. No systematic variation in percentage yield was evident in this range.

40%. The mixture was agitated for 5 minutes more and then centrifuged at 5,000 g for 5 minutes. The pellet was resuspended first in one volume of the buffered ethanol solution, respun and then suspended again in 1/2 volume of the same solution for a final spin. The pellet was suspended in 10 ml of Krebs-Henseleit medium and left at room temperature for 1 hour to digest the glycogen. The solution was respun at 10,000 g for 20 minutes to remove undigested glycogen and the larger glycogen breakdown products.

The procedure produced a yield of almost 100% of the added amylase and the specific activity was increased by approximately 5-fold. Amylase after purification accounted for some 20% of the protein in the original material, the rest presumably being primarily other digestive enzymes. We estimated chymotrypsinogen contamination in the original material, and assuming that its specific activity is roughly the same as 5x purified bovine chymotrypsinogen (Worthington Chemical Co.), chymotrypsinogen accounted for about 10% of its protein content. Chymotrypsinogen contamination of the repurified amylase was only about 0.07%.

<u>Radiation Counting</u>

Radiation was determined in a Beckman liquid scintillation counter. The dried millipore filters containing TCA precipitable protein were added with 1 ml of Beckman Bio-Solv Solubilizer BBS-2 to glass counting vials. Purified amylase samples diluted in 1 ml H_2O were added to counting vials with 2 ml of the solubilizer. 20 ml of toluene containing fluor [8 g/liter 2 - (4' - tertbutylphenyl) - 5 - (4" - biphenyl) - 1, 3, 4 - oxdiasole and 0.5 g/liter 2 - (4' biphenyl) - 6 - phenyl - benzoxazale] was then added, the vial closed and shaken vigorously until the solution clarified. Occasionally, it was necessary to add more solubilizer to

clarify the suspension. External standard ratios were of the order of 660, indicating minimum quench and a counting efficiency of approximately 35 to 40% for tritium and of about 90% for ^{14}C.

RESULTS

I. Secretion of Digestive Enzyme From Two Intracellular Pools

The cisternal packaging-exocytosis model suggests that all newly synthesized enzyme moves en masse through a set series of membrane-bound compartments to the duct lumen. To test this hypothesis, the _in vitro_ pancreas was first incubated with ^{14}C leucine for 2 hours and then stimulants of secretion were added to the bathing medium. If the cisternal packaging-exocytosis model is correct, stimulating enzyme output should increase the specific radioactivity (S.R.A.) (dpm/unit protein) of enzyme in secretion towards its maximum level for the system, and in the continued presence of the label in the medium, the S.R.A. of secreted protein should continue to increase towards this steady-state level. Since only one intracellular precursion pool of digestive enzyme is proposed (that is to say that all enzyme must travel to the duct lumen via the same route), over time more and more of this pool would necessarily consist of newly synthesized (labeled) enzyme. Increasing the secretory rate in the continued presence of label, therefore, could only increase the proportion of enzyme in secretion that is newly synthesized; it could not decrease it. When either cholecytokinin-pancreozymin (CCK-PZ) or acetyl-β-methylcholine chloride (MCh) was added to the _in vitro_ pancreas, protein output increased (Figure 3), however, the S.R.A. of secretion, or the proportion of newly synthesized enzyme in secretion, dropped dramatically, indicating that older, unlabeled enzyme was being secreted preferentially (Figure 4). In the presence of CCK-PZ, the mean decrease in S.R.A. was about 78% for the period of maximum depression (10 min post-stimulus). The depression in S.R.A. was even more profound

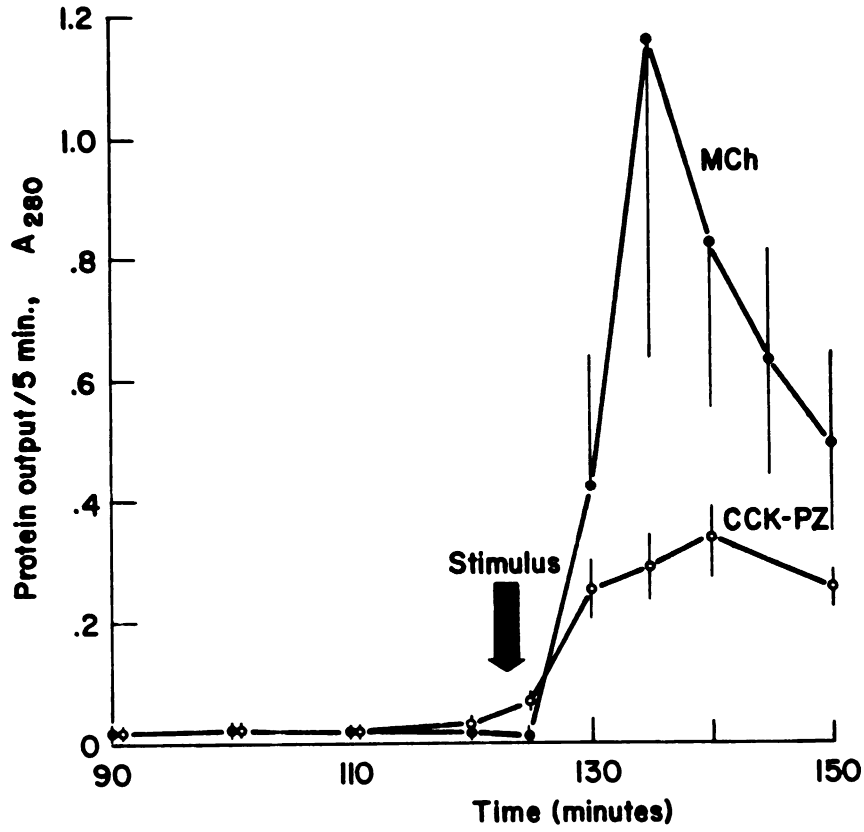

Figure 3. Effect of acetyl-β-methylcholine chloride (MCh) and cholecystokinin-pancreozymin (CCK-PZ) on protein output by the whole rabbit pancreas *in vitro*. Values are means ± S.E.M. for 4 MCh and 3 CCK-PZ studies. Note that augmented protein output occurred in the first 5 minutes juice sample after CCK-PZ, but was delayed with MCh.

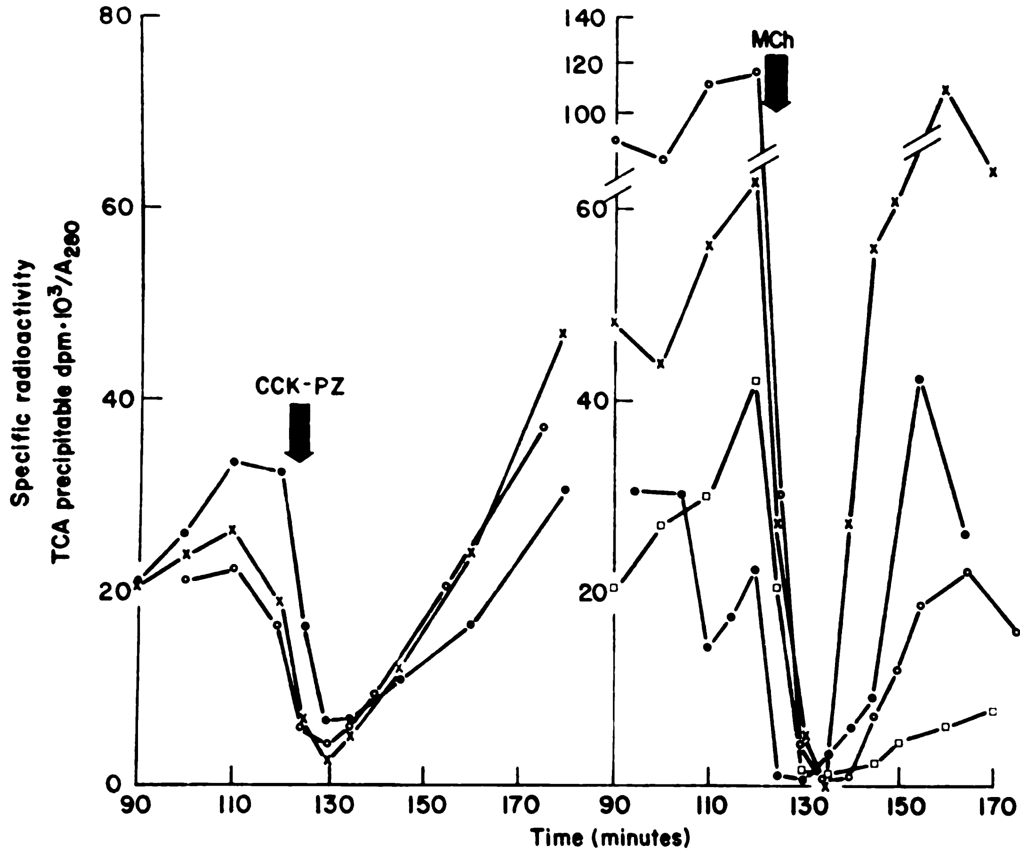

Figure 4. Effect of acetyl-β-methylcholine chloride (MCh) and cholecystokinin-pancreozymin (CCK-PZ) added to bathing medium 2 hours after the addition of ^{14}C-leucine, on the specific radioactivity of secreted proteins. Four MCh and 3 CCK-PZ experiments are shown.

in the presence of MCh; during the period of maximum depression (10-15 minute post-stimulus), S.R.A. was reduced to only 2.4% of the pre-stimulus levels.

The decrease in S.R.A. with MCh was due both to an absolute decrease in the secretion of newly synthesized enzyme (Figure 5), as well as an increase in the secretion of older, stored enzyme. Radioactivity in enzyme in secretion in MCh-treated glands decreased 71% ± 7 S.E.M. in the first 5 minute post-stimulus, whereas in control glands it increased 17% ± 16 S.E.M. during this period (Table III). In addition, the inhibition in the amount of newly synthesized enzyme in secretion was maximum in the first 5 minute post MCh, although the increase in enzyme output was not evident until the second 5 minute period (Figure 3 and Table III).

In contrast, no delay occurred in the output response to CCK-PZ. [Å 280/5 minute for prior control period was 0.026 ± 0.015, whereas in the first 5 minutes after CCK was added it was 0.064 ± 0.015 ($P < 0.025$)]. Furthermore, the absolute amount of newly synthesized (labeled) enzyme in secretion actually <u>increased</u> with CCK-PZ stimulation (Figure 6), even though the secretion of old stored enzyme was disproportionately augmented (Figure 4).

When MCh was added to the bathing medium at the same time as the isotope, an inhibition in the secretion of newly synthesized enzyme relative to controls also occurred. Cumulative DPM for the first hour in the presence of the cholinergic agonist was less than half that for controls (Figure 7).

When the cholinergic agonist was added to the bathing medium after a 4 hour incubation with ^{14}C leucine, the S.R.A. of secretion decreased to 36% of its pre-stimulus level (as opposed to 2.4% when MCh was applied

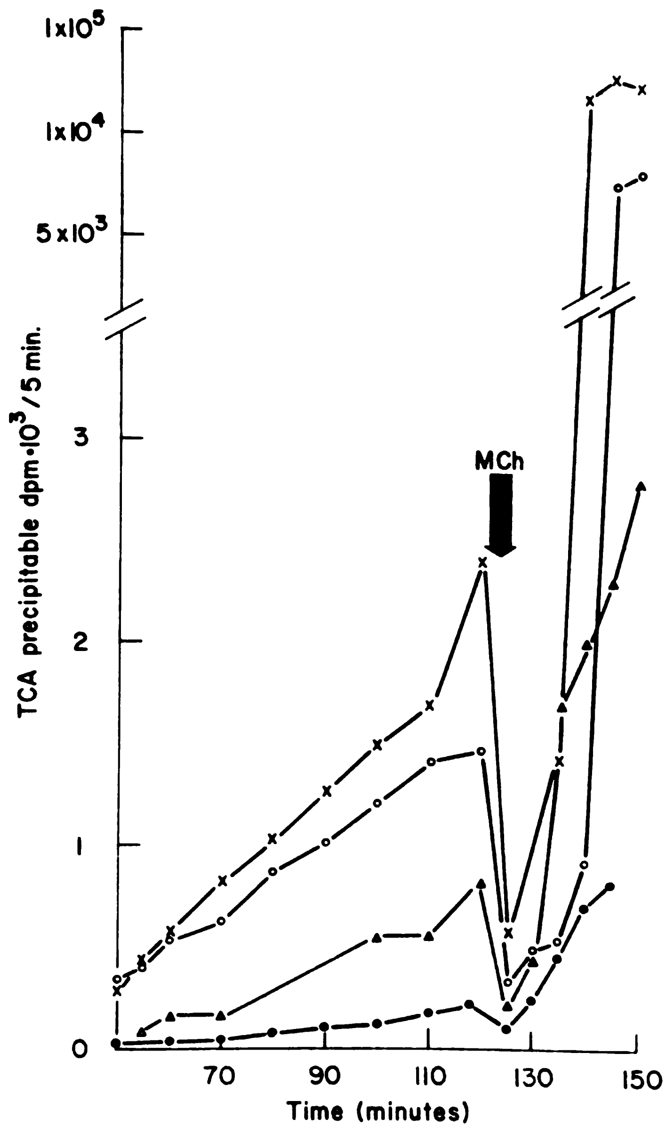

Figure 5. Effect of acetyl-β-methylcholine chloride (MCh) added to bathing medium two hours after the addition of ^{14}C-leucine, on the output of new (labeled) protein. Four experiments are shown.

Table III

Inhibition of Overall Protein Secretion Relative to Inhibition of Newly Synthesized (labeled) Protein Secretion 5 minutes after Acetyl-β-methylcholine Chloride (MCh) was Administered. Values are Means ± S.E.M. and the Numbers in Parentheses Represent the Number of Experiments.

	Percent Inhibition of Total Protein Secretion (120-125 min Compared to Mean Output for Prior 60 min)	(Control)-(+MCh),%	Percent Inhibition of Labeled Protein Secretion (120-125 min Compared to dpm/5 for Prior 10 min)	(Control)-(+MCh),%
Control	18 ± 19(4)		+17 ± 16(4)	
		0		-80
+MCh	18 ± 10(4)		71 ± 7(4)	

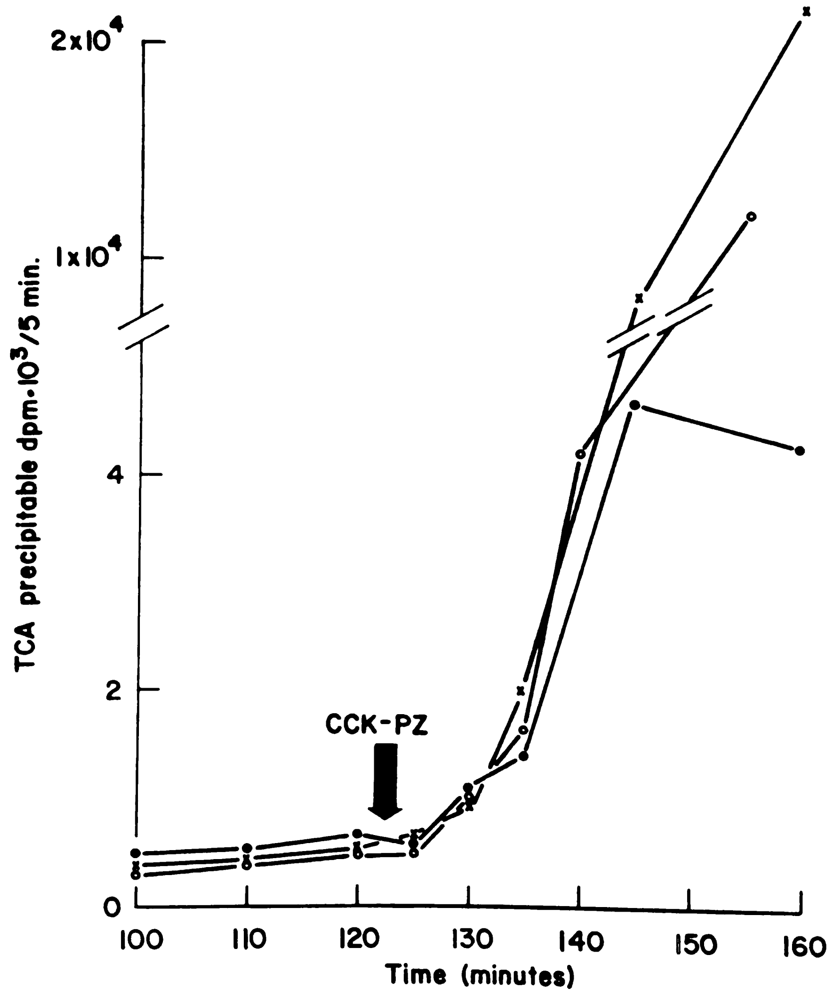

Figure 6. Effect of cholecytokinin-pancreozymin (CCK-PZ) added to the bathing medium 2 hours after the addition of ^{14}C-leucine, on the output of new (labeled) protein. Three experiments are shown.

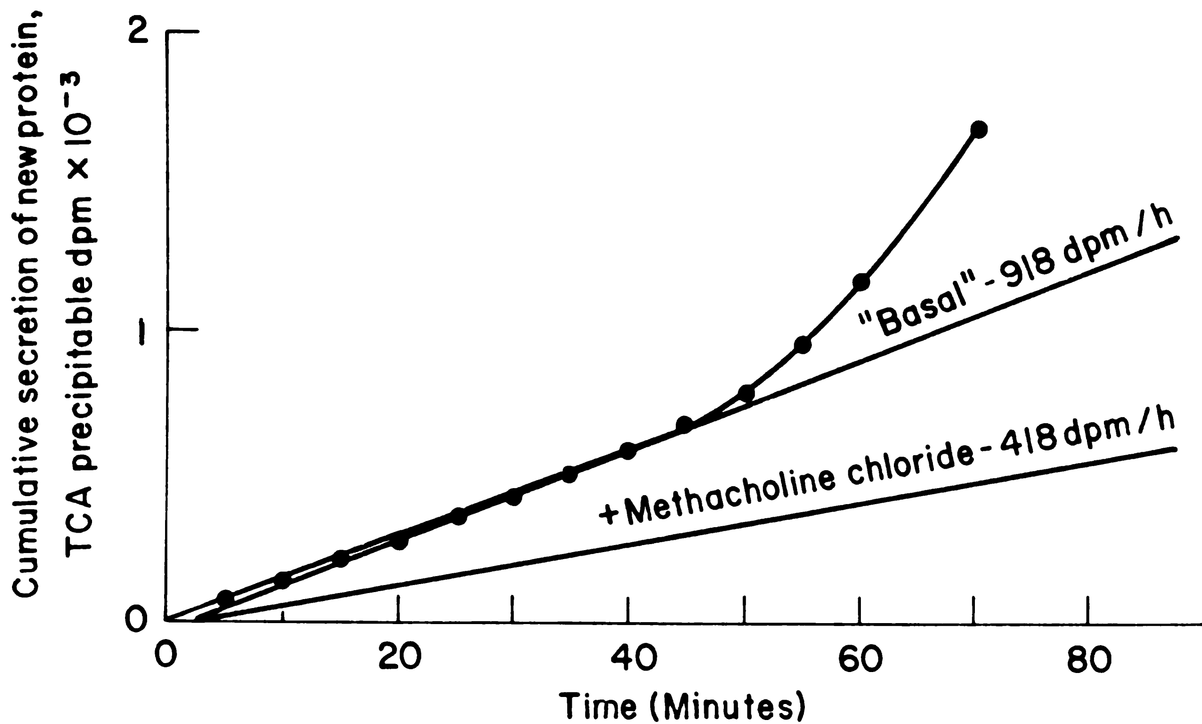

Figure 7. Cumulative early secretion of newly labeled protein in the unstimulated state and in the presence of cholinergic stimulant. Basal data are from 10 preparations and stimulated data are from 4 preparations. A comparison of the two regression lines indicates that the slopes differ with $P < 0.001$.

at 2 hours) (Figure 8). Inhibition in the absolute amount of label in secretion also occurred when MCh was given at 4 hours. It lasted for only the first 5 minutes and bottomed at 45% of the prior control DPM; by 10 minutes the secretion of newly synthesized protein was well above its pre-stimulus level (Figure 9). When MCh was added 8.5 hours after the original addition of isotope and 3 hours after the bath was changed and the label removed, the S.R.A. of secretion increased with augmented enzyme output, indicating now a preferential secretion of older, labeled enzyme (Figure 8).

II. Basolateral Flux of Amylase

Given the hypothesis that digestive enzyme secretion is a bidirectional, concentration-dependent event, the demonstration of a bath to cell flux for chymotrypsinogen in tissue slices by Liebow and Rothman (1974) suggested that a cell to bath flux should also exist. To test for this, the appearance of amylase was measured in the bathing medium of the whole rabbit pancreas in short-term organ culture. In this preparation, apical and basolateral compartments are entirely segregated; cell products secreted across the apical membrane are collected via a catheter inserted into the duct system, whereas cell products secreted across the basolateral membrane appear in the bathing medium.

The amylase content of the bathing medium increased with time and approached an apparent steady-state concentration of 39 nM by 210 minutes (Figure 10). The $t_{1/2}$ of "equilibration" was 50 minutes and the amylase flux at the initial rate was 0.19 nmol/min per gland. In contrast, amylase output in ductal fluid was constant over time (Figure 11). The mean amylase flux in ductal secretion was 0.064 nmol/min per gland (as

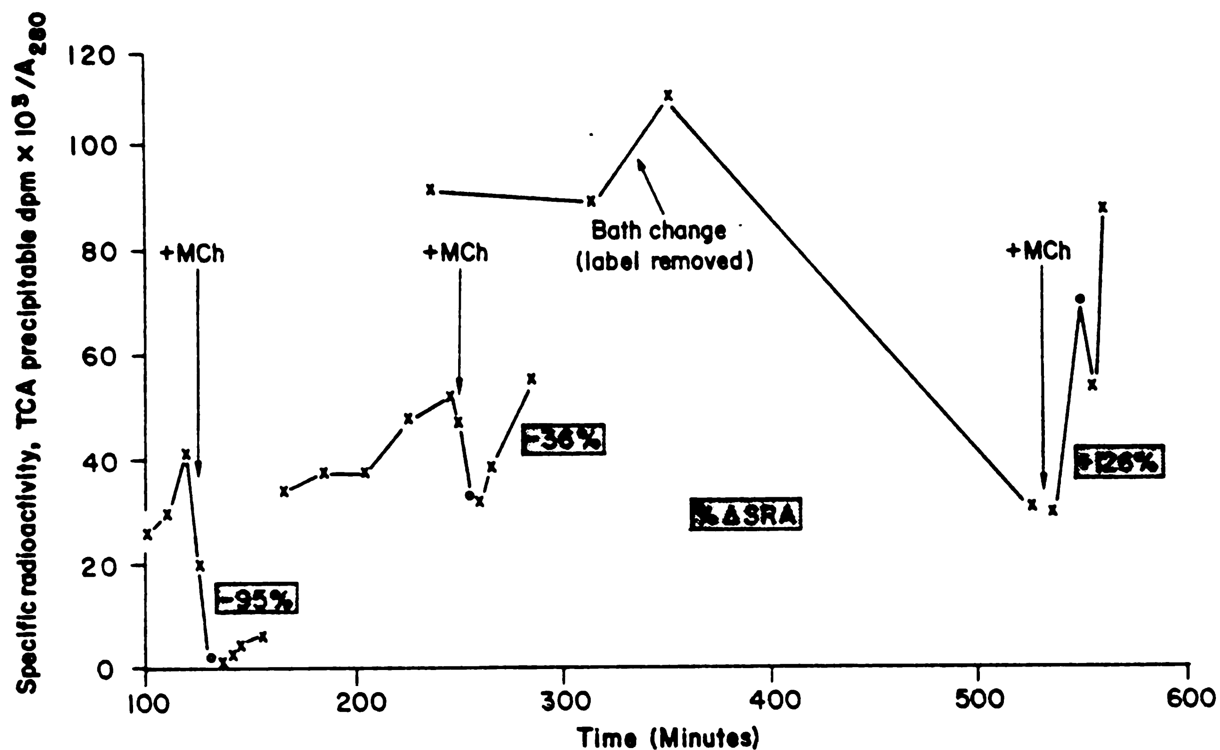

Figure 8. The effect of time on the acetyl-β-methylcholine chloride (MCh)-induced depression in the specific radioactivity of secreted proteins. The number in the stippled rectangle indicates the percentage decrease in the specific radioactivity of secreted protein seen in periods indicated by open circle. From left to right, MCh added to the bath at approximately 2 hours, 4 hours, and 8.5 hours. In the last experiment, label was removed from the bath 3.5 hours prior to the addition of MCh.

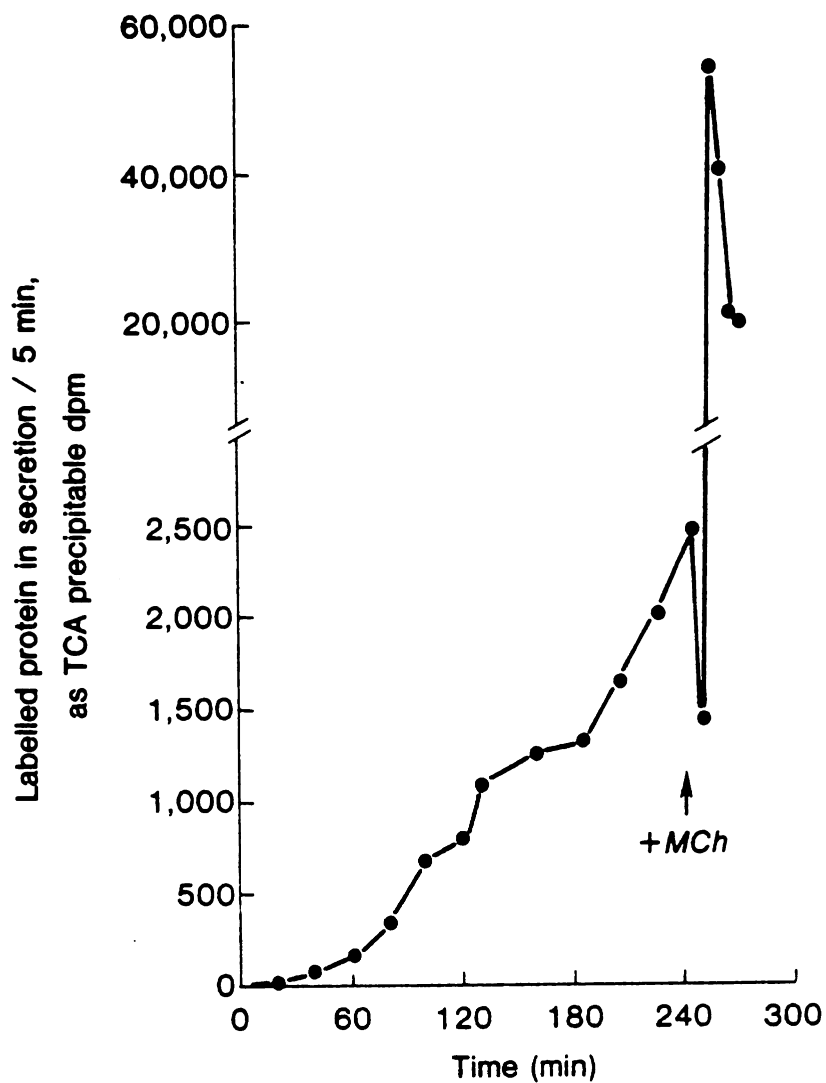

Figure 9. Effect of acetyl-β-methylcholine chloride (MCh) added to bathing medium 4 hours after the addition of ^{14}C-leucine, on the output of newly synthesized (labeled) protein.

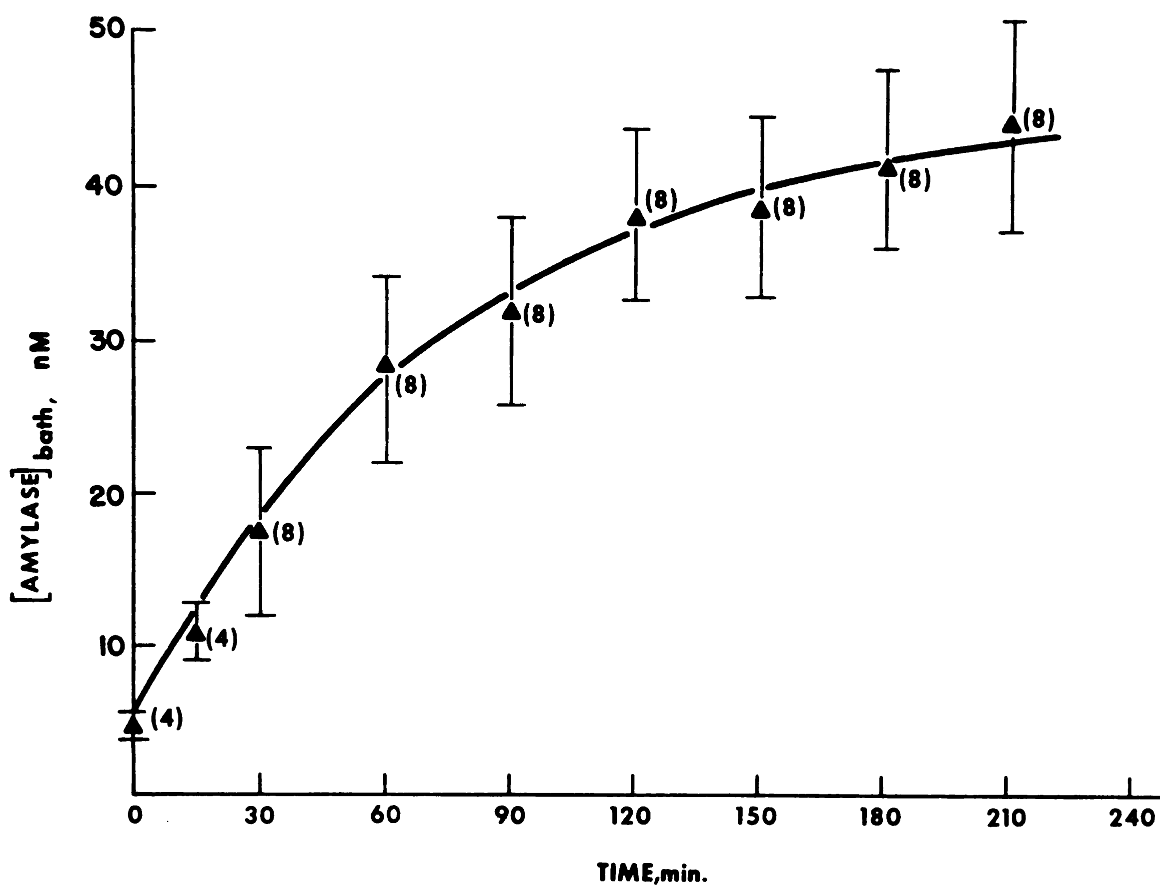

Figure 10. The concentration of amylase in the bathing medium plotted against time. The concentration of amylase in the medium bathing whole rabbit pancreas in culture was monitored for 3.5 hours. Data are the mean amylase concentration ± S.E.M. The number of preparations included for each point is indicated in parentheses.

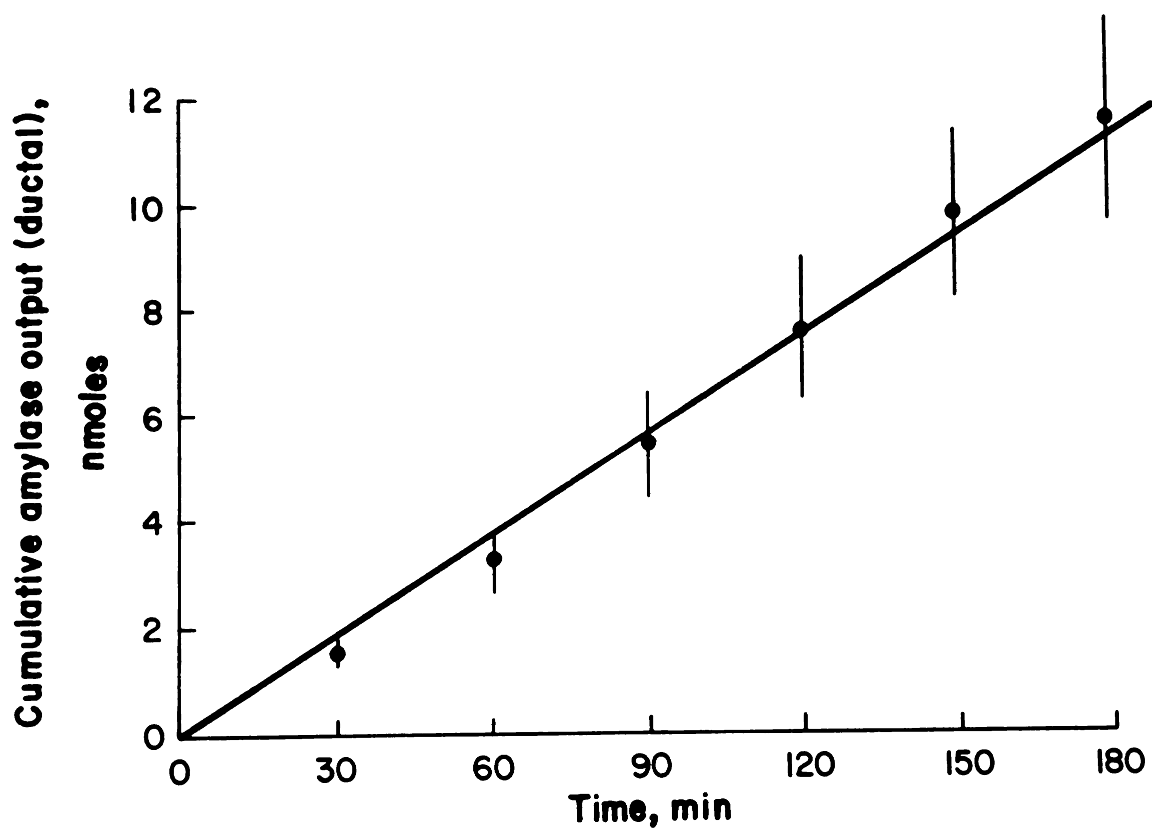

Figure 11. The cumulative amylase output into ductal secretion. Ductal secretion was collected from whole rabbit pancreas in organ culture via a catheter in the pancreatic ducts. The points represent the means of 9 experiments and the verticle bars the S.E.M.

calculated from the slope of the line in Figure 11), which is considerably less than the cell to bath flux. In individual experiments, the ductal concentration of amylase ranged from 59 to 373 times the bath amylase concentration at the steady-state (Table IV).

To determine if bath amylase might reflect release from damaged cells, the rate of appearance of lactate dehydrogenase, a presumably non-transportable cytoplasmic enzyme, as well as RNA were measured over time in the bathing medium. While the rate of release of amylase equilibrated (decreased) over time, the rate of release of lactate dehydrogenase, in contrast, increased with time (Figure 12). The RNA content of the bathing medium varied considerably from period to period, but no general upward trend was evident (Figure 13).

When the bathing medium was changed hourly with fresh solution for 6 consecutive hours, a flux approximately equal to the initial or maximal flux reoccurred with each bath change (Table V). "Equilibration", therefore, was not simply a time-dependent phenomenon, but, rather, was dependent on the amylase concentration in the bathing medium.

Effect of Natural Stimulants on the Basolateral Flux of Amylase

In some preparations, cholecystokinin-pancreozymin (CCK-PZ) was added to the bathing medium at 190 minutes after the beginning of equilibration, and the cholinergic agonist, acetyl-β-methylcholine chloride (MCh) was added 60 minutes later. Both CCK-PZ and MCh increased ductal amylase output dramatically (Figure 14). The ductal response to CCK-PZ peaked at approximately a 12-fold increase in amylase output relative to the prior control period (180-190 min), while the peak response to MCh was roughly 60x the control output. The CCK-PZ values were indistinguishable from controls, while a sharp rise was observed in bath amylase in response to MCh (Figure 15).

Table IV

Amylase Concentration in the Duct and Bath at the Steady-state. Concentrations are Given for Eight Experiments at 180 Minutes.

[Amylase] x 10^{-6} M

Duct	Bath	Duct/Bath
6.4	0.10	59
7.5	0.12	63
6.8	0.053	128
3.3	0.022	165
7.1	0.032	222
17.3	0.063	275
14.9	0.046	324
15.3	0.041	373

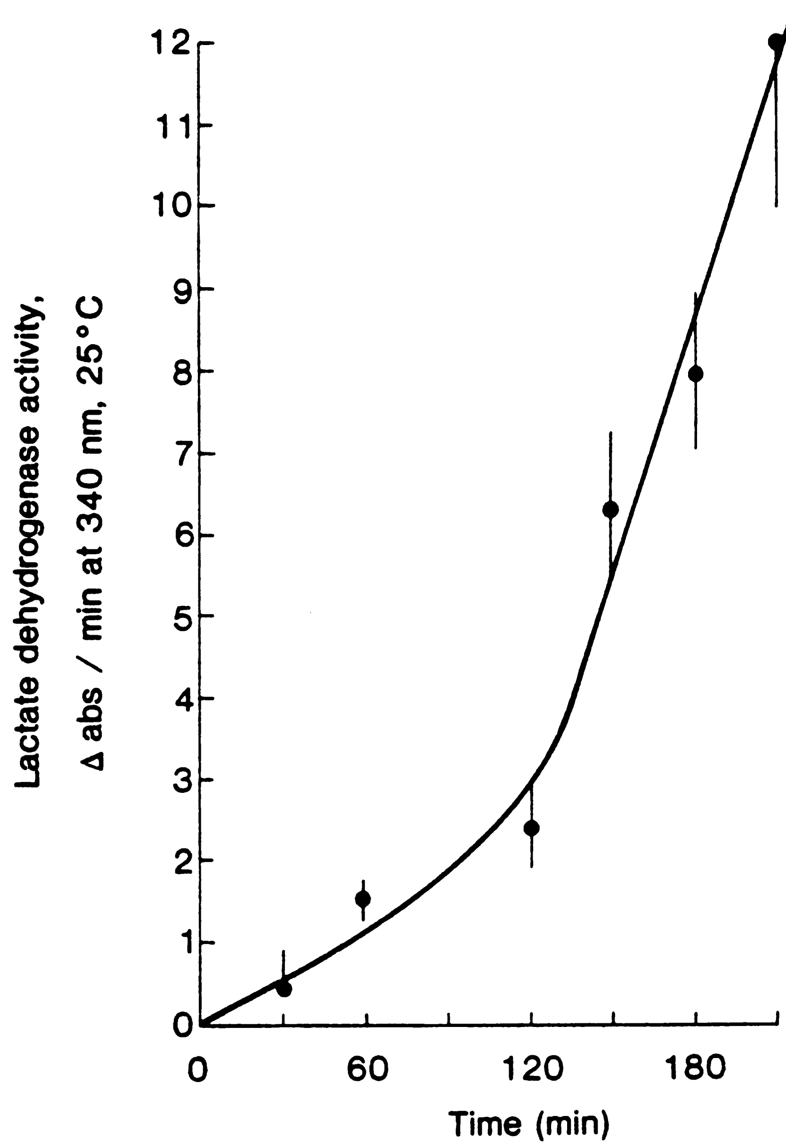

Figure 12. The concentration of lactate dehydrogenase (LDH) in the bathing medium plotted against time. Data are mean LDH concentration ± S.E.M. for 3 experiments.

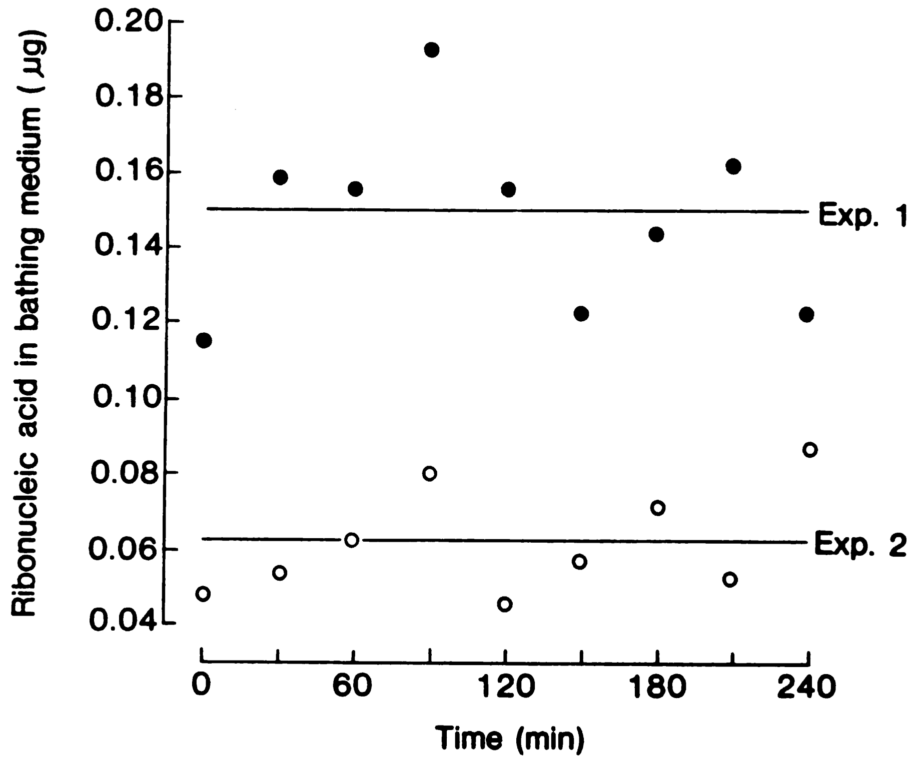

Figure 13. The amount of ribonucleic acid (RNA) in the bathing medium plotted against time. Two individual experiments are displayed and neither demonstrates a change with time.

Table V

Amylase Flux into the Bathing Medium at the Initial Rate with Successive Bath Changes. Values are Means ± S.E.M. for 6 Experiments.

Bath Change, #	Amylase Flux (bath) ng/30 min
1	3.2
2	2.3
3	2.1
4	2.5
5	2.3
6	1.7

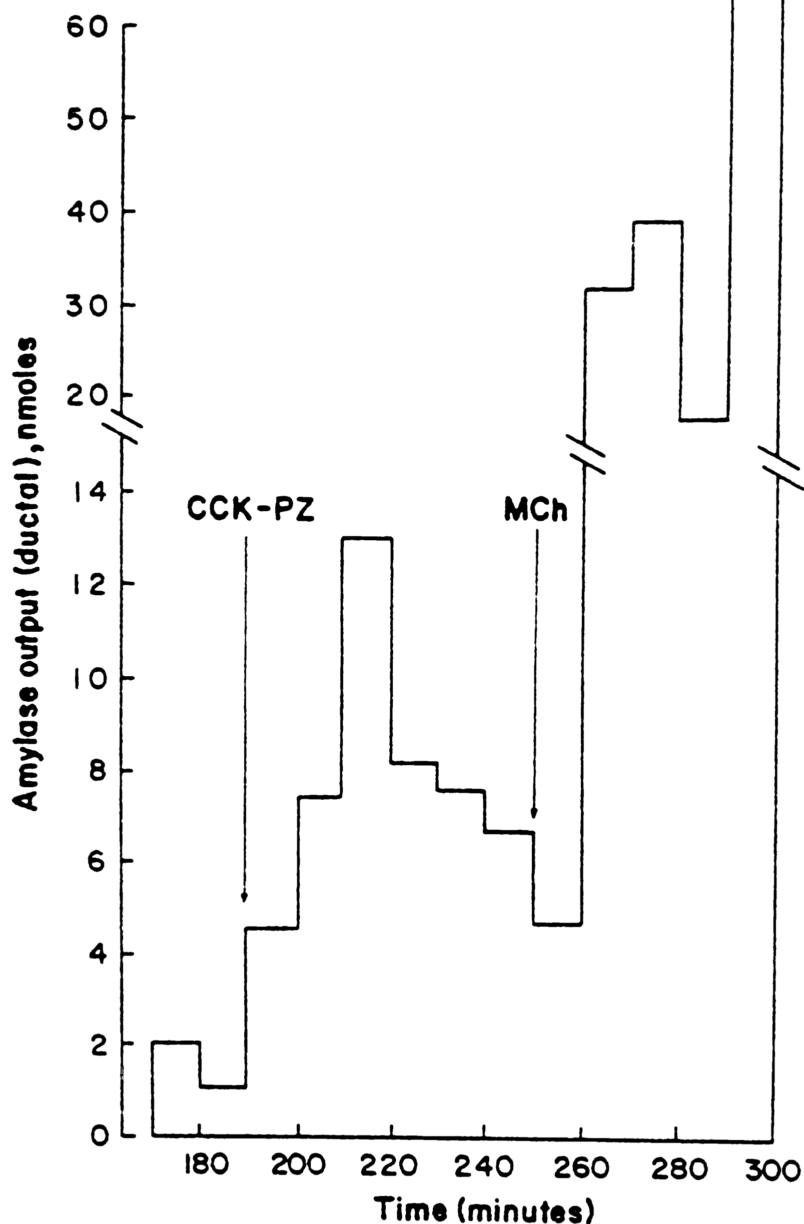

Figure 14. The effect of cholecystokinin-pancreozymin (CCK-PZ) and acetyl-β-methylcholine chloride (MCh) on amylase output in ductal secretion. CCK-PZ was added to the bathing medium at 190 min and MCh at 250 min. n = 5 for all points through 250 minutes; n = 4 from 260 through 290 minutes; n = 3 at 300 minutes. Amylase secretion increased approximately 12-fold at its peak in response to CCK-PZ and approximately 60-fold in response to MCh.

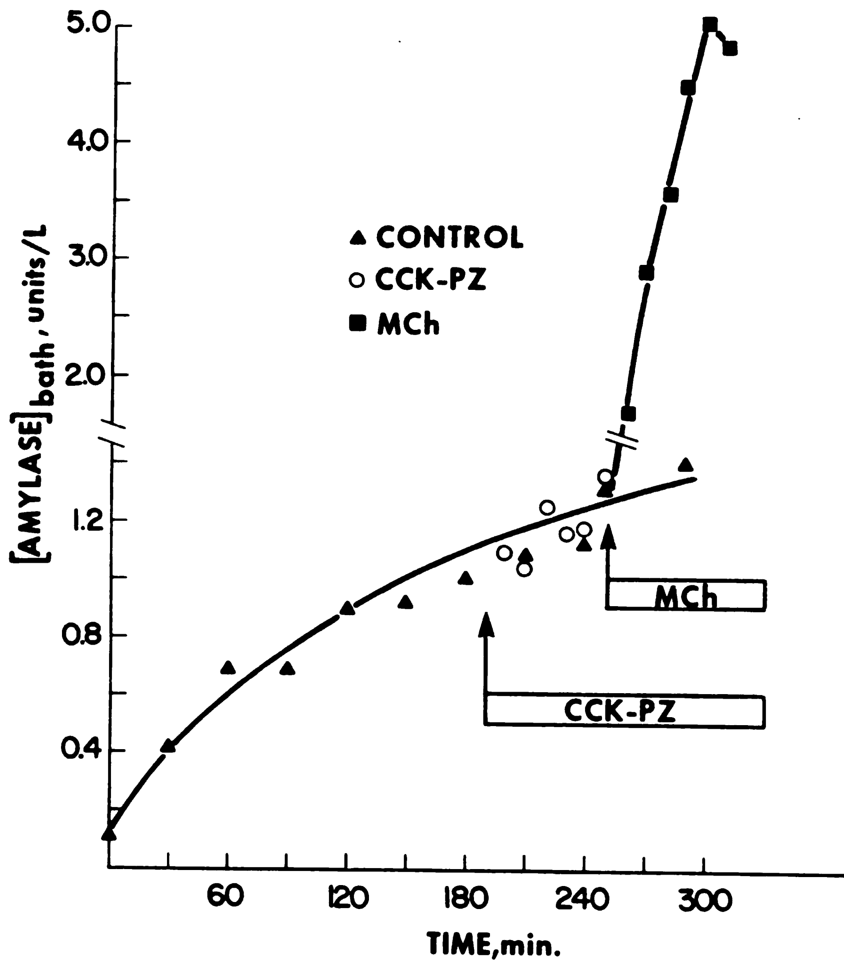

Figure 15. The effect of cholecytokinin-pancreozymin (CCK-PZ) and acetyl-β-methylcholine chloride (MCh) on the concentration of amylase in the bathing medium. Data for each experiment are normalized to the bath amylase concentration at 180 minutes. Control data is indicated by ▲. n = 8 for all control points up to 190 minutes and n = 4 thereafter. CCK-PZ points are indicated by O. n = 4 for all these points except at 250 minutes where n = 3. MCh points are indicated by ■; n = 3 at 260, 270, and 280 minutes, and n = 2 at 290, 300, and 310 minutes. CCK-PZ values were not distinguishable from control data, but when MCh was added, the concentration of amylase in the bath increased greatly.

Comparison Between Flux of Amylase from Cell to Duct and Cell to Bath

The unstimulated, unidirectional flux of amylase into the bath for these experiments is given by the slope of the initial, linear part of the equilibration curve (Figure 10). This can be compared to the net flux from cell to duct (or the rate of ductal secretion) (Figure 11). Using these figures, the unidirectional flux of amylase from cell to bathing medium was some 3 times the net flux from cell to duct (Table VI).

For the stimulated condition, the rate of appearance of amylase in the bath during the second 10 minute period after MCh was added, the period in which amylase release into the medium increased most rapidly, was used as a minimal estimate of the unidirectional flux (Figure 15). (This is a minimal estimate because amylase in the bath was at its steady-state concentration prior to the addition of the stimulant and a significant backflux from bath to cell already existed; see Table VI). Estimated in this way, amylase release into the medium was increased by about 14 times in the presence of MCh. The MCh-stimulated net flux of amylase from cell to duct for this same 10 minute period (Figure 14) was about equal to the cell to bath flux (Table VI).

III. Temperature Dependence of Apical and Basolateral Secretion

The temperature dependence of the amylase flux into the duct and into the bathing medium are shown together in Figure 16. Ductal secretion represents a net flux. The basolateral flux in contrast, was measured at the initial rate since at each temperature the bath was changed prior to the initiation of the measurements. Measurements were made at 5°, 10°, 15°, 20°, 30°, 35°, and 38°, and indicate that both basolateral and apical fluxes have a biphasic, V-shaped relationship to temperature.

Table VI

The Cell to Bath and the Cell to Duct Amylase Flux. The Value given for the Stimulated Cell to Bath Amylase Flux is a Minimum Estimate since Prior to the Addition of the Stimulus, Bath Amylase was at its Basal Steady-state Concentration where a Significant Back Flux of Enzyme from Bath to Cell must Occur.

	Amylase Flux, mol/min/pancreas		
	Net Flux Cell to Duct	Initial Rate Cell to Bath	Duct/Bath
Unstimulated	6.37×10^{-11}	1.93×10^{-10}	0.33
Stimulated (acetyl-β-methylcholine chloride)	3.22×10^{-9}	2.73×10^{-9}	1.18

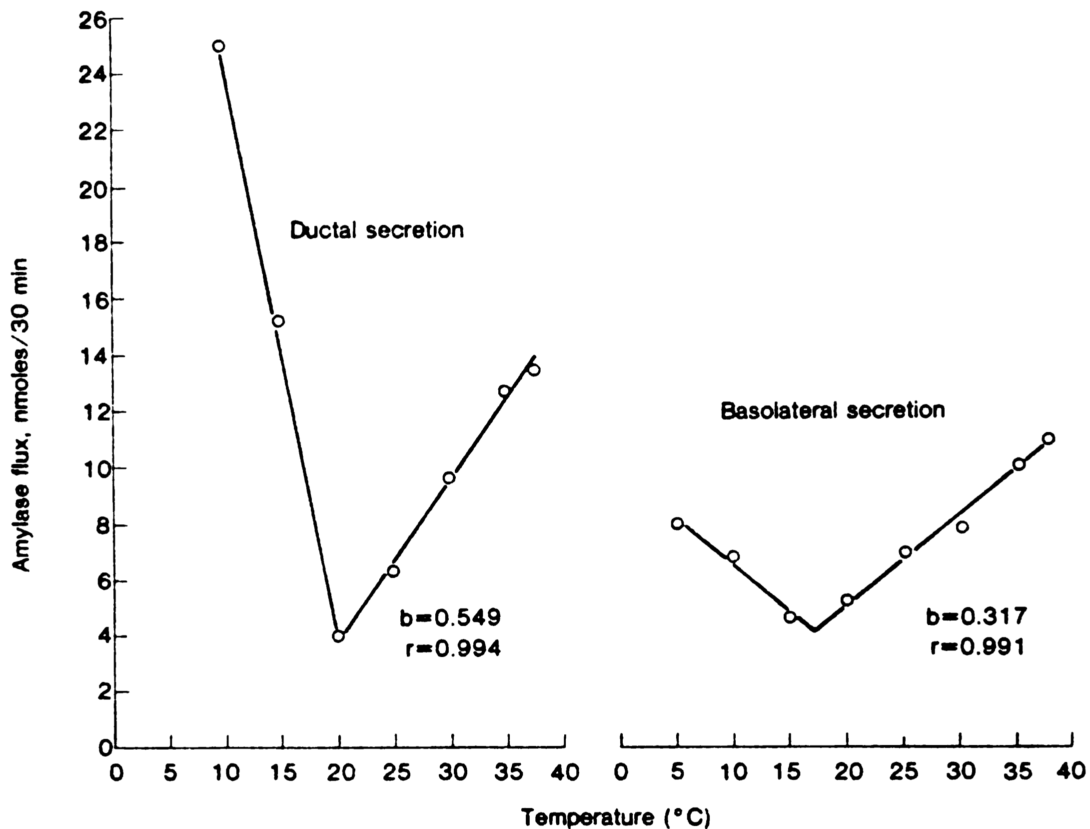

Figure 16. The amylase flux into ductal secretion and basolateral fluid plotted against temperature. The whole rabbit pancreas in organ culture was pre-equilibrated for 30 minutes at each temperature prior to the beginning of the 30 minutes sampling period. For ductal secretion, points from 20° to 38° fit a line with a slope of 0.549 (r = 0.99); n = 3 at 10°, 5 from 5° to 30°, 4 at 35° and 5 at 38°. For basolateral secretion, the slope of the line fitting points from 20° to 38° is 0.317 (r = 0.94); n = 5 for all points.

High rates of secretion occur across these membrane surfaces at both low and high temperatures with the inflection point or minimum falling between 15° and 20°. The function relating temperature to apical amylase output between 20 and 38 is linear with a slope of 0.549 ($r = 0.99$); for basolateral secretion the function is also linear with a slope of 0.317 ($r = 0.99$). Figure 17 demonstrates in greater detail the temperature dependence of the amylase flux into the bathing medium.

Since the viability of the gland declines rapidly after incubation at 37°, flux determinations were made first at 5° and the temperature then was raised in increments. In order to determine if the high secretory rate seen at lower temperatures was a time dependent phenomenon, perhaps related in some manner to the freshness of the preparation, in one experiment the amylase flux was measured first at 25° and then at 20°, 15°, 10°, and 5° consecutively (Figure 18). This data demonstrates rather dramatically that high amylase fluxes occur at low temperatures and low fluxes at intermediate temperatures independent of which range is measured first.

The Q_{10} of the basolateral flux was 1.6 from 20° to 38°. For the apical flux, the Q_{10} was 2.0 from 20° to 38°. The activation energy was 8.3 kcal for the basolateral flux and 12.1 kcal for the apical flux.

The relationship between fluid secretion and temperature was also determined. In contrast to enzyme secretion, fluid secretion only increased as the temperature was raised between 5° and 38° and no inflection point occurred. The rate of fluid secretion related to temperature in a non-linear manner (Figure 19). From 20° to 38°, both enzyme secretion and fluid secretion show similar temperature dependences (Figure 20) and thus in this range the concentration of amylase in secretion remained

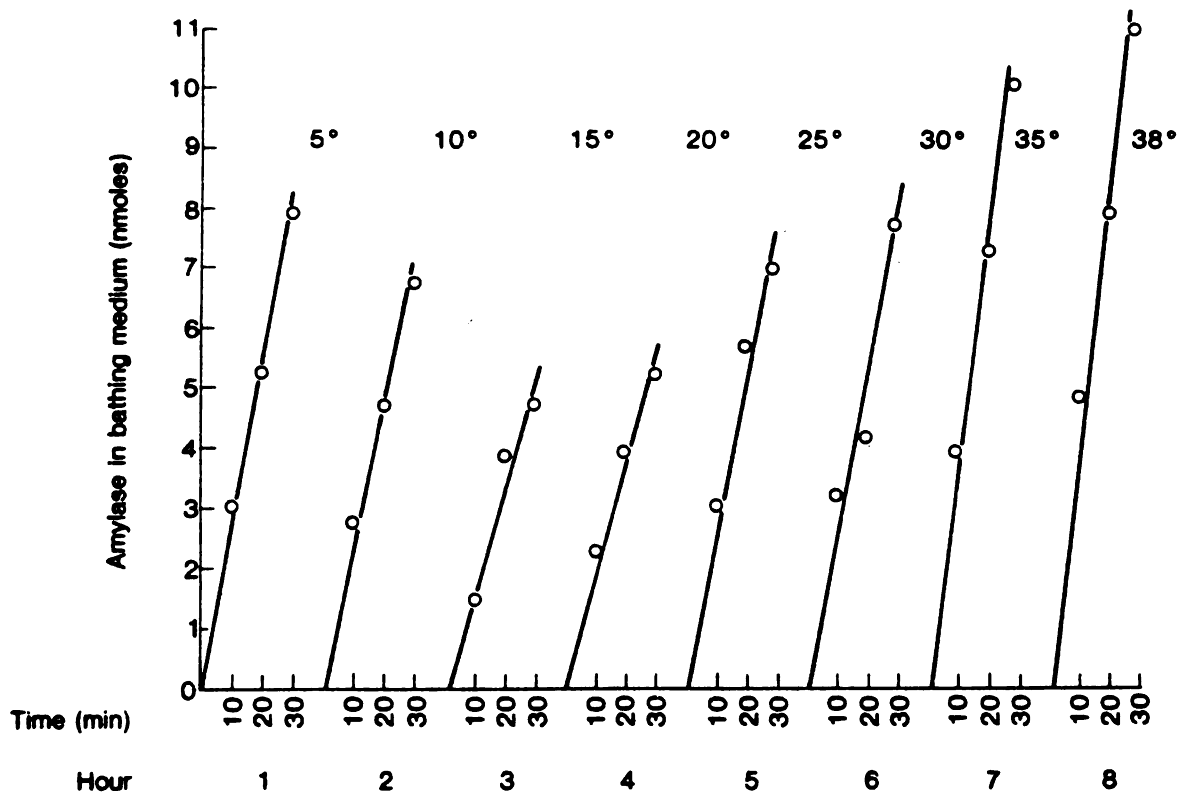

Figure 17. Preparations were pre-equilibrated at a temperature (5°, 10°, 15°, 20°, 25°, 30°, 35 and 38°) for 30 minutes, the bath then changed and bath samples collected at 10 minute intervals for 30 minutes. Points are the means of 6 experiments.

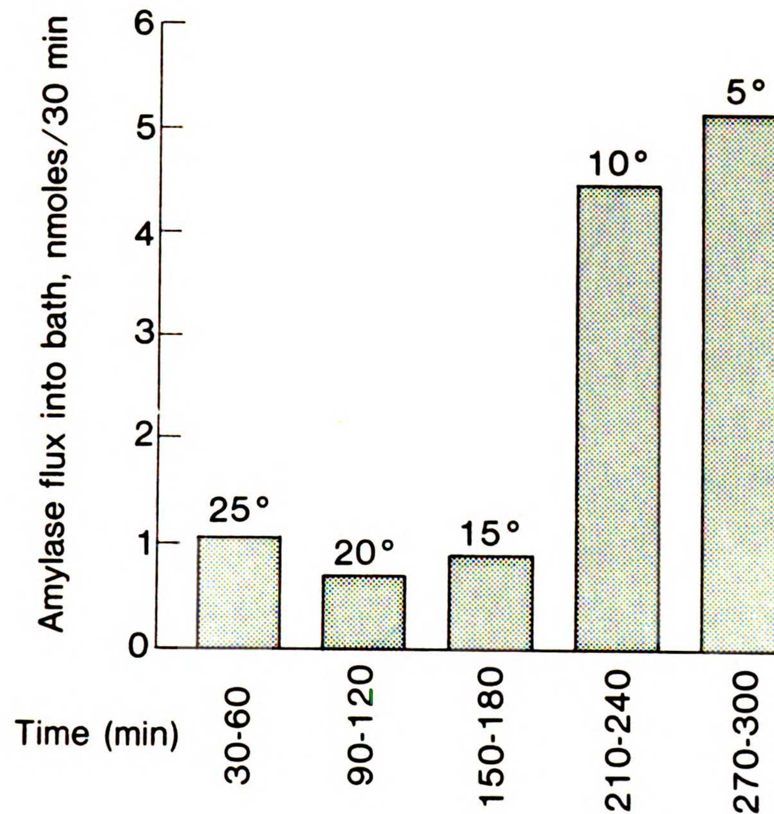

Figure 18. The amylase concentration in the bathing medium vs temperature when the temperature of the preparation was lowered successively instead of raised. After a 30 minute pre-equilibration period at a given temperature (from 25 to 5°), samples of bathing fluid were collected at 10 minute intervals for 30 minutes. The results are similar to those observed when the temperature of the preparation is raised successively with high secretion rates at very low temperature and low secretion rates in the intermediate temperature range.

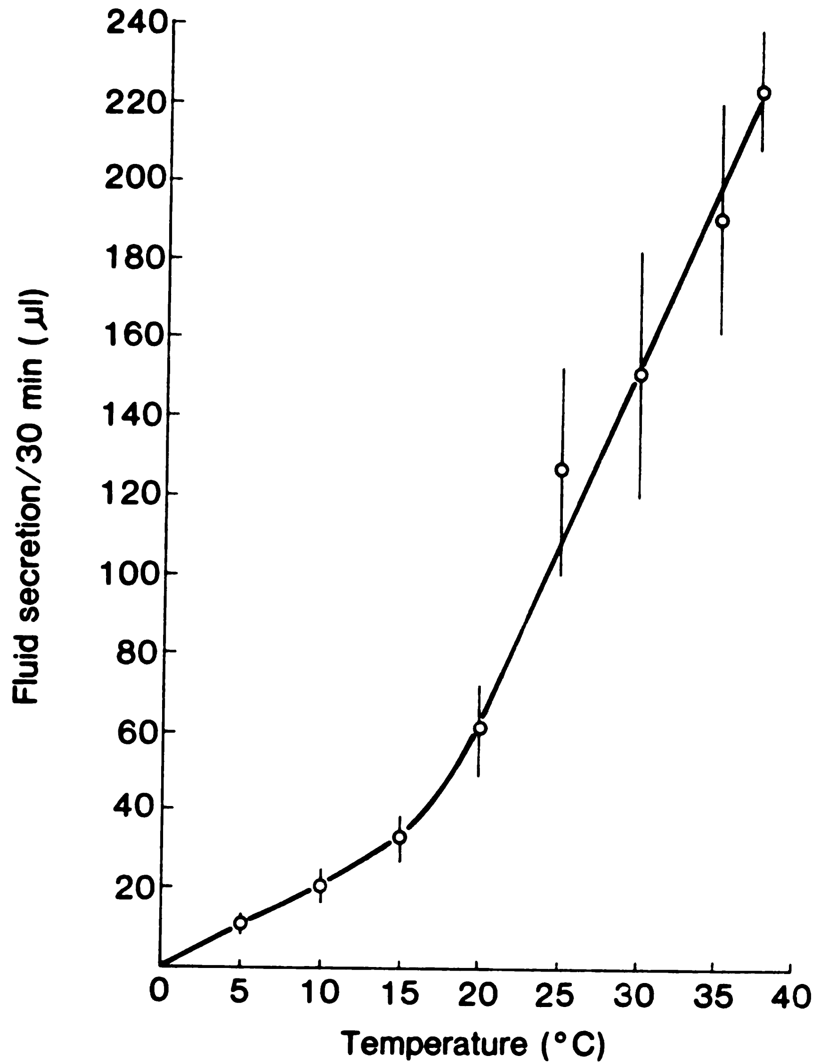

Figure 19. The amount of fluid secretion from the whole pancreas *in vitro* vs temperature. The temperature of the bathing medium was raised in increments from 5° to 38°. The glands were pre-equilibrated at each temperature for 30 minutes prior to a 30 minute collection of ductal secretion. n = 3 at 5° and 10°, 6 from 15° through 30°, 5 at 35°, and 6 at 38°.

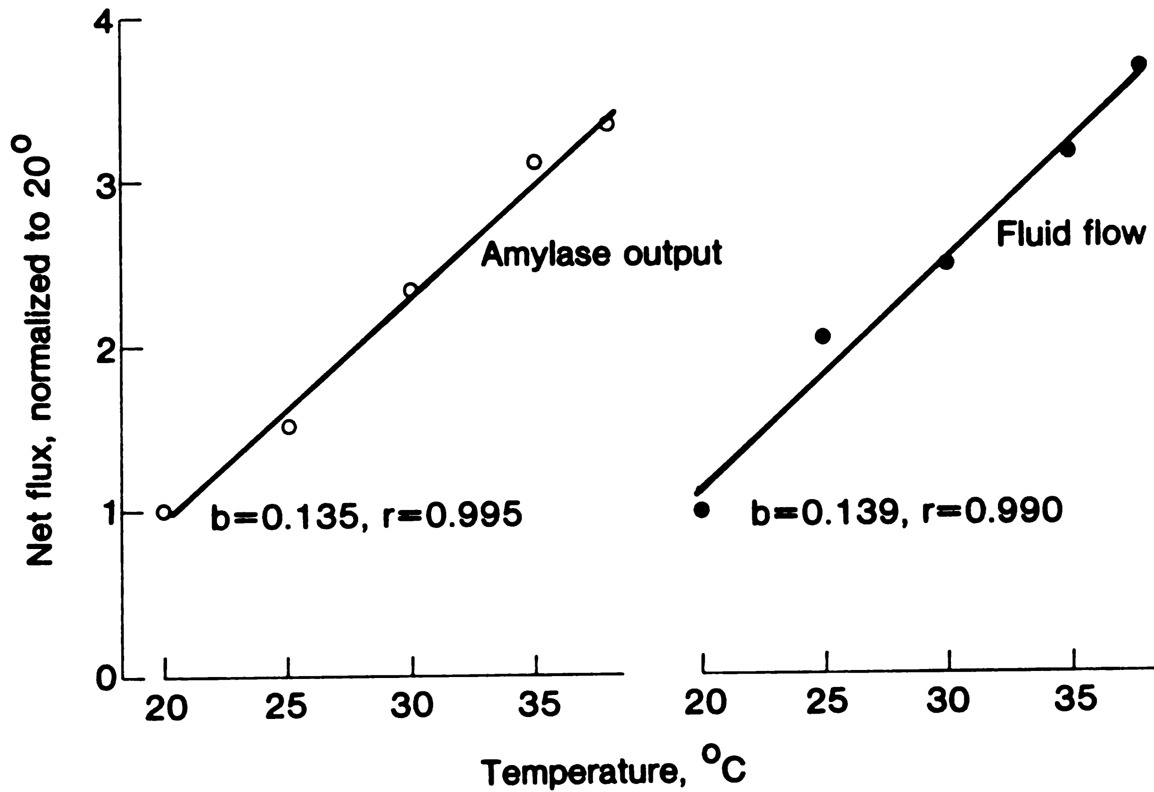

Figure 20. Fluid secretion and amylase secretion vs temperature from 20° to 38°. Both fluid and amylase secretion are essentially identical functions of temperature in this range. For fluid secretion $y = -1.65\ x + 0.139$, $r = 0.99$, and for amylase secretion $y = -1.75\ x + 0.135$, $r = 0.99$. Points are mean ± S.E.M. for 3 experiments.

essentially constant at approximately 4.0×10^{-9} M (Figure 21). In contrast, at lower temperatures the concentration of enzyme in secretion increased with decreasing temperature. At 10°, amylase concentration averaged 9×10^{-7} M.

The high rate of amylase secretion seen in the low temperature range (5° to 20°) could result from an increase in the permeability of the zymogen granule membrane to amylase, from an increase in the amylase permeability of the plasma membrane, or both. To determine if an increase in zymogen granule permeability were involved, the ratio of amylase in the zymogen granule fraction to amylase in the post-microsomal supernatant fraction was measured in both tissue incubated and fractionated at 5° and in tissue incubated and fractionated at 20°. 20° was chosen because the amylase flux is at a minimum at this temperature. If an increase in the zymogen granule permeability to amylase were responsible wholly or in part for the high secretory rates seen at very low temperature, the ratio of amylase in zymogen granules to amylase in the post-microsomal supernatant would be lower at 5° than at 20°. This was not the case, the ratio of amylase in zymogen granules to amylase in the post-microsomal supernatant was consistently higher at 5° than at 20° (Table VII). The data suggests instead that the permeability of the zymogen granule membrane decreases rather than increases as the temperature is lowered from 20° to 5° (approaching the 0.05 significance level with a 2-tailed t test). Therefore, an increase in the permeability of the zymogen granule membrane or an increase in the precursor pool (cytosol) concentration cannot readily explain the augmented rates observed at low temperatures, and the phenomenon probably results instead from the temperature-dependent characteristics of the release mechanism at the apical membrane.

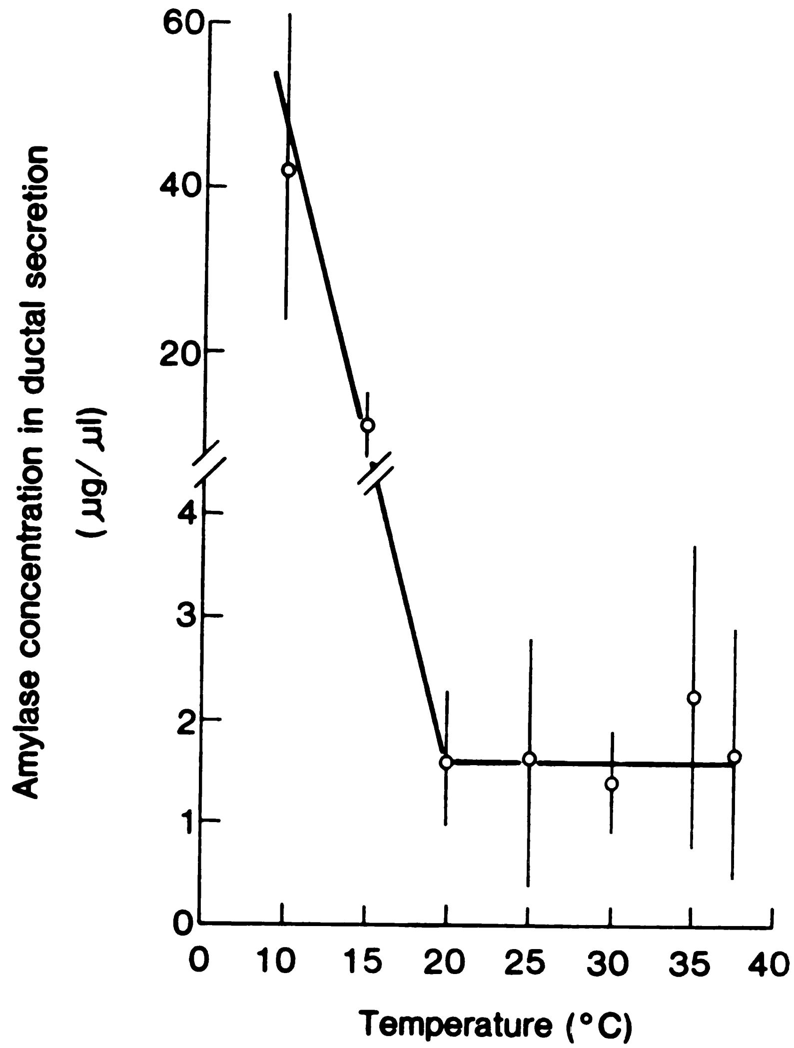

Figure 21. Concentration of amylase in ductal secretion against temperature. Amylase concentration was constant in the 20° to 38° range. From 15° to 5° the concentration of amylase in secretion increased as the temperature was lowered. n = 3 at 10°, 5 through 30°, 4 at 35°, and 5 at 38°.

Table VII

Ratio of Amylase in Zymogen Granules (Zg) to Amylase in Post-microsomal Supernatant (PMS) at 20°, Relative to Same Ratio for 5°.

Experiment #	$\frac{Zg\ 20°/PMS\ 20°}{Zg\ 5°/PMS\ 5°}$
1	.65
2	.36
3	.61
4	.67
	Mean = .57
	S.E.M. = .07

IV. Transpancreatic Transport of Amylase

A transpancreatic transport of digestive enzyme has been shown by Liebow and Rothman (1975) with tracer techniques. To determine the potential capacity of the process, as well as to determine the effect of exogenously added enzyme on the secretion of endogenous enzyme, a large concentration of exogenous amylase was added to the bathing medium of the *in vitro* pancreas. When 1 mg/ml amylase [Sigma, Type VI-A; containing approximately 1/3 protein by weight and amylase at a concentration of 67 μg/mg (relative to a standard with a specific activity of 506 I.U./mg)] was added to the medium bathing the *in vitro* pancreas, the amylase content of ductal secretion increased rapidly and dramatically. The response peaked approximately 20 minutes after the enzyme was added at an average amylase output of 11 times the unstimulated (basal) level (Figure 22), or 15 ± 3 S.E.M. times basal when each preparation was normalized to its own control (Figure 23). Control data indicated no change over this period (Figure 24). The effect was equivalent to approximately 20% of the amylase output at the peak of a maximum cholinergic response for this *in vitro* system. The amylase content in ductal secretion returned towards its original level over time, although bath amylase concentration remained essentially unchanged.

When the dose of exogenous amylase was varied, 0.1, 0.2, as compared to 1.0 mg/ml of the Sigma material, the response did not increase linearly with concentration, as predicted for a non-saturating system, but appeared to reach a maximum value between 0.2 and 1.0 mg/ml (Table VIII).

When repurified amylase was used instead of the crude material, the response was smaller, although still substantial [6.1 ± 1.6 S.E.M. times prior control values (n = 4), or about half the magnitude of the response

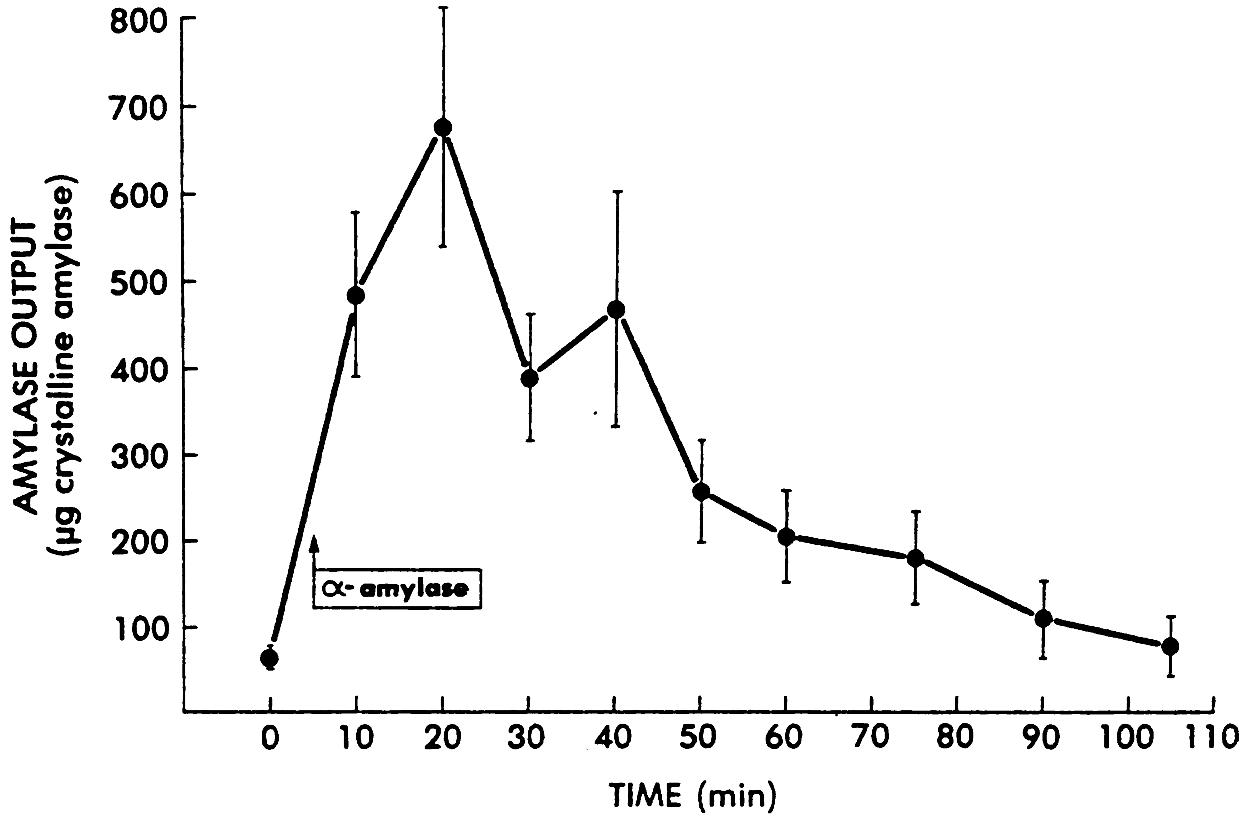

Figure 22. Effect of the addition of 1 mg/ml α-amylase to the bath on the ductal secretion of amylase. Amylase output increased some 11-fold its basal level at the peak of the response. Data presented as mean amylase output/10 min ± S.E.M. Value at time zero is output for 10 minute control period prior to the addition of the amylase to the bathing medium. From 0 to 105 minutes n = 11, 13, 13, 12, 12, 11, 9, 8, 5, and 5 respectively.

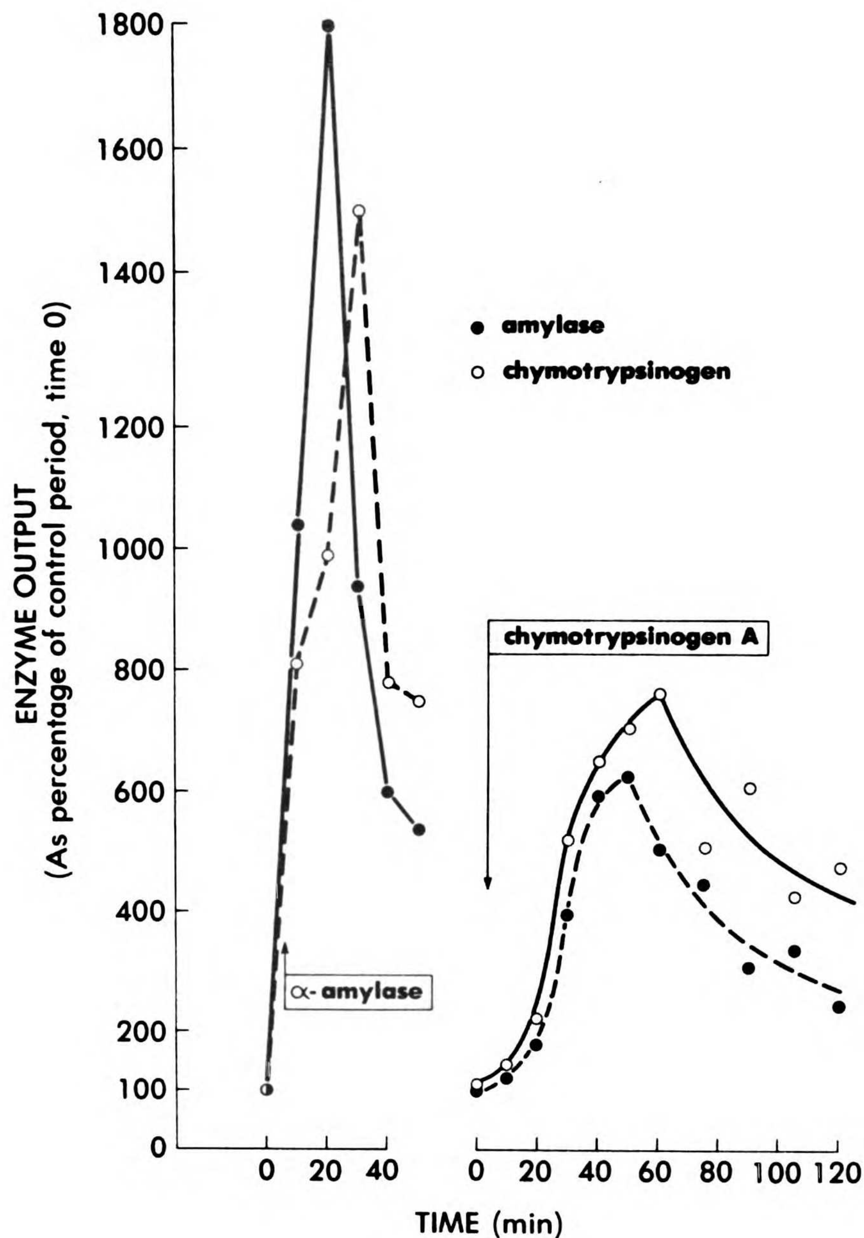

Figure 23. Effect of adding 1 mg/ml α-amylase to the bath on the secretion of chymotrypsinogen and amylase, and the effect of adding 1 mg/ml chymotrypsinogen A to the bath on the secretion of amylase and chymotrypsinogen. Both responses indicate roughly parallel secretion of the two enzymes. Data are normalized for each individual experiment to a 10 minute control period prior to the addition of either α-amylase or chymotrypsinogen A to the bathing medium. Values at this time (time zero) have been set equal to 100. n = 9 to 90 min; 6 at 105 min; and 7 at 120 min.

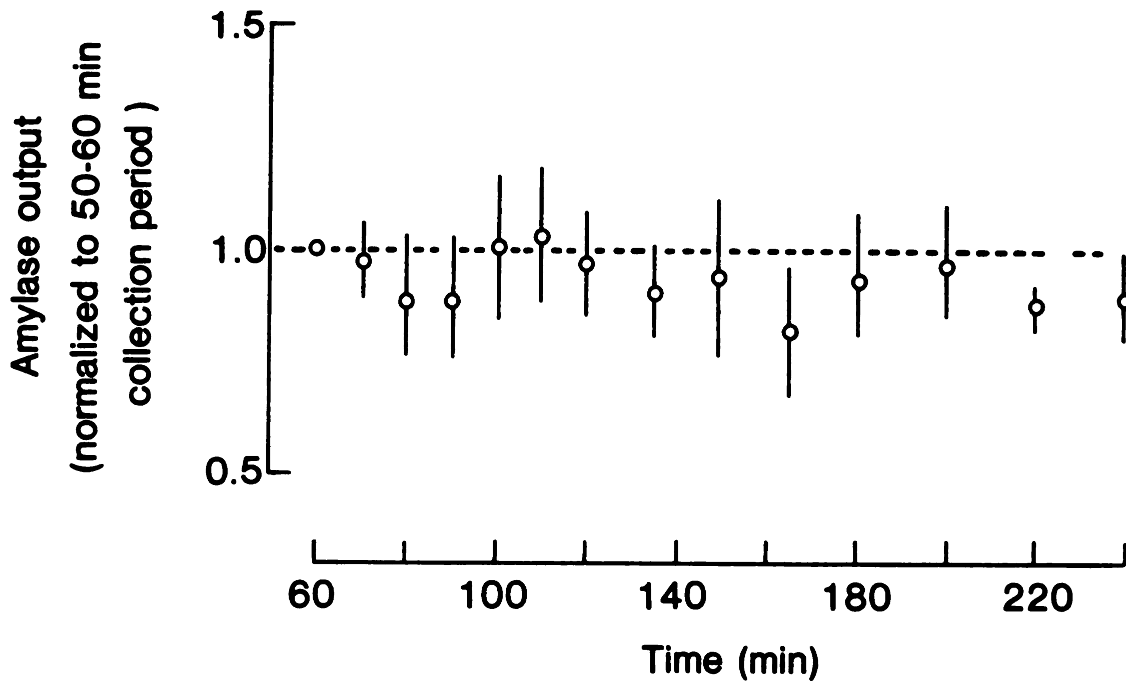

Figure 24. Amylase output in ductal secretion in the basal state. Data for each experiment is normalized to the 50-60 min collection period. (In the experimental condition, enzyme was added to the bath at 60 min). Means ± S.E.M. are shown and n = 3 for all data points.

Table VIII

The Effect of Varying Concentrations of Amylase in the Medium Bathing Rabbit Pancreas *in vitro* on the Secretion of Amylase into the Duct. Basal Amylase Output Averaged 64 ± 12 S.E.M. in the 10 minute Control Period Prior to the Addition of Amylase (n = 16). Sigma Type VI-A Amylase was Added to the Bath.

		Amylase Output at Peak Period (10-20 min), μg/10 min	
$[\text{Amylase}]_{bath}$, mg/ml	n	Observed	Predicted for a non-saturating system
0.1	3	300	(300)
0.2	3	550	600
1.0	10	950	3,000

produced by the crude material]. Thus, amylase secretion apparently was in part stimulated by other molecules in the crude amylase. We tested for the possibility that small compounds were responsible by dialyzing the Sigma material against Krebs-Henseleit medium for 24 hours at 4° before adding it to the pancreas preparation. The secretory response was undiminished as a result of dialysis (Figure 25). Therefore, that portion of the amylase response not elicited by amylase itself was apparently due to the presence of other large molecules, probably other digestive enzymes, in the mixture. This view is supported by the fact that heating the Sigma material at 60° for 30 minutes reduced the output response from 15x basal to 3.1x basal ± 0.78 S.E.M. (n = 3), or about 80%. Further evidence relating to this point will be presented below.

The Effect of Chymotrypsinogen Added to the Bath on the Secretion of Chymotrypsinogen

When 1 mg/ml of a relatively pure chymotrypsinogen (5x crystallized bovine chymotrypsinogen A, Worthington Chemical Co.) [amylase contamination based on an assumed specific activity equal to 2x crystallized porcine alpha amylase (specific activity 506 I.U./mg) was less than .004%] was added to the medium, chymotrypsinogen output in ductal secretion increased. The response developed more slowly than when amylase was added and peaked at 60 minutes (Figure 26). When each experiment was normalized to its own control for the peak period, chymotrypsinogen output was approximately 7.5 ± 2.5 S.E.M. times the unstimulated control output (Figure 23). The magnitude of the response was equal to approximately 30% of the chymotrypsinogen output at the peak of a maximum cholinergic response for this *in vitro* system (Figure 27). In control glands, chymotrypsinogen output remained constant (Figure 28).

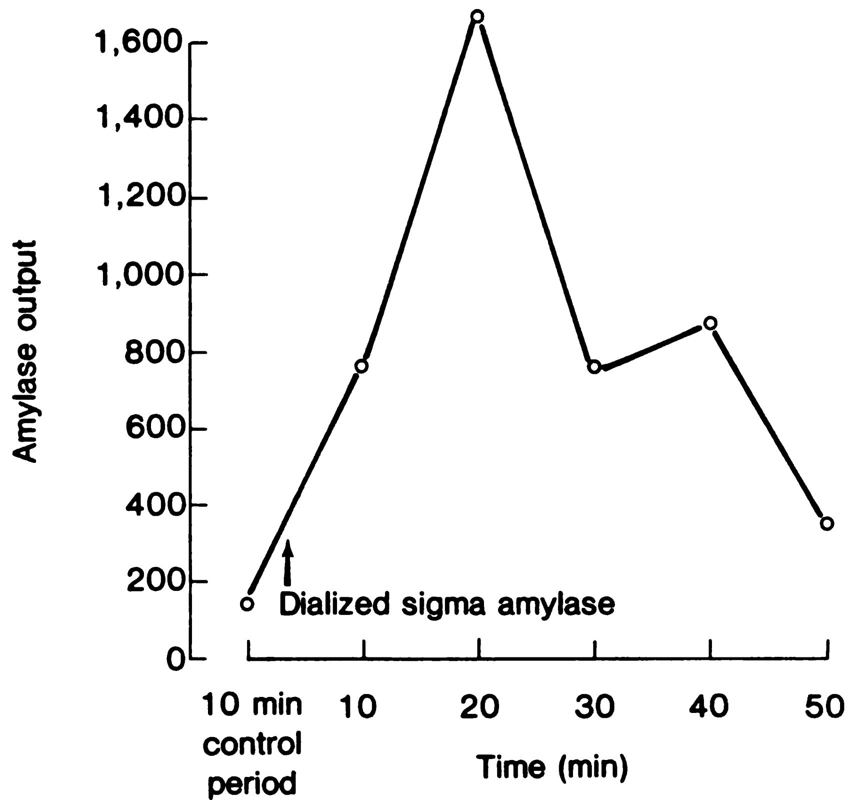

Figure 25. Effect on ductal secretion of adding dialyzed sigma amylase to the bathing medium. Sigma material was dialyzed against Krebs-Henseleit for 24 hours in the cold to remove small molecules and then added to the bath. Removal of small molecules apparently did not alter the response of the _in vitro_ pancreas to the material.

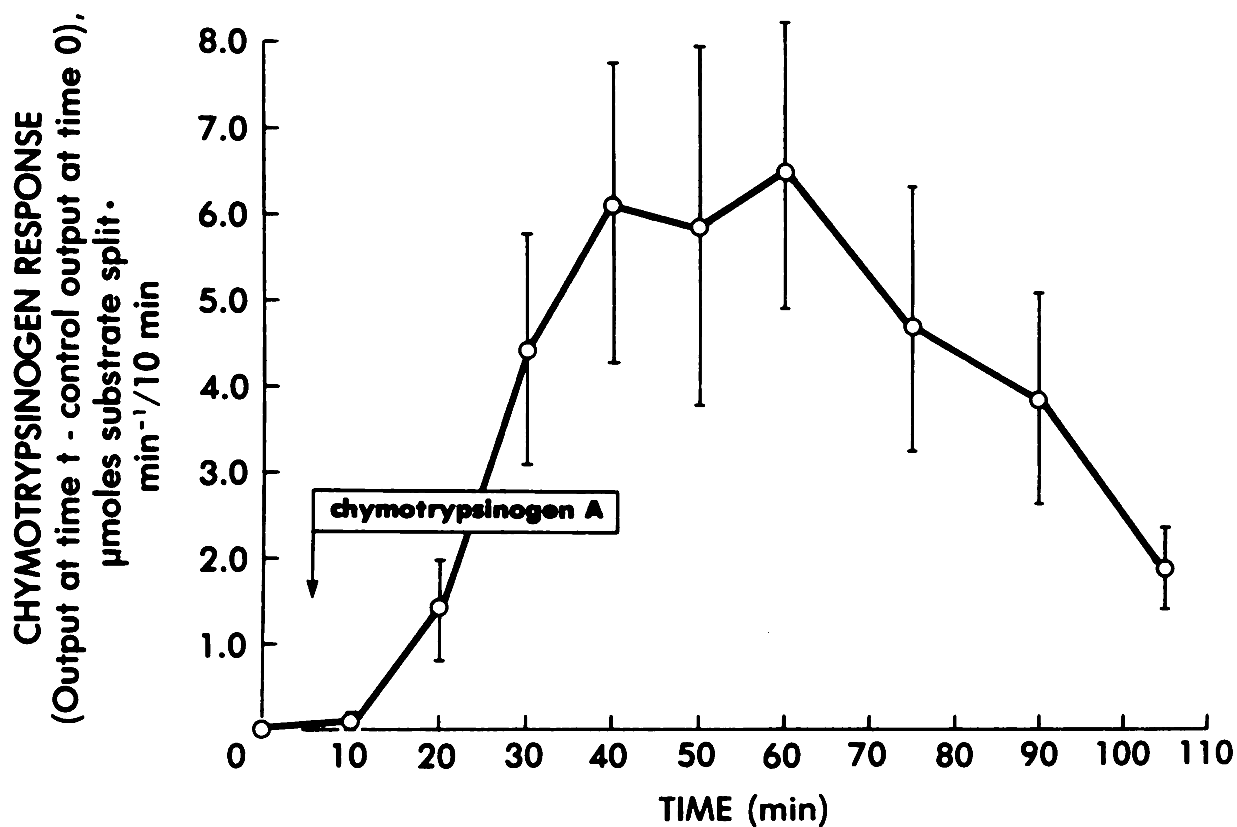

Figure 26. Effect of the addition of 1 mg/ml chymotrypsinogen A to the bath on the ductal secretion of chymotrypsinogen. Data are mean chymotrypsinogen output/10 min ± S.E.M. minus the mean basal secretory output of 2.66 moles substrate split · min^{-1}/10 min [which is the value for the 10 min control period prior to the addition of chymotrypsiogen A (time zero on the graph)]. n = 9 from 0 to 90 min; 6 at 105 min; and 7 at 120 min.

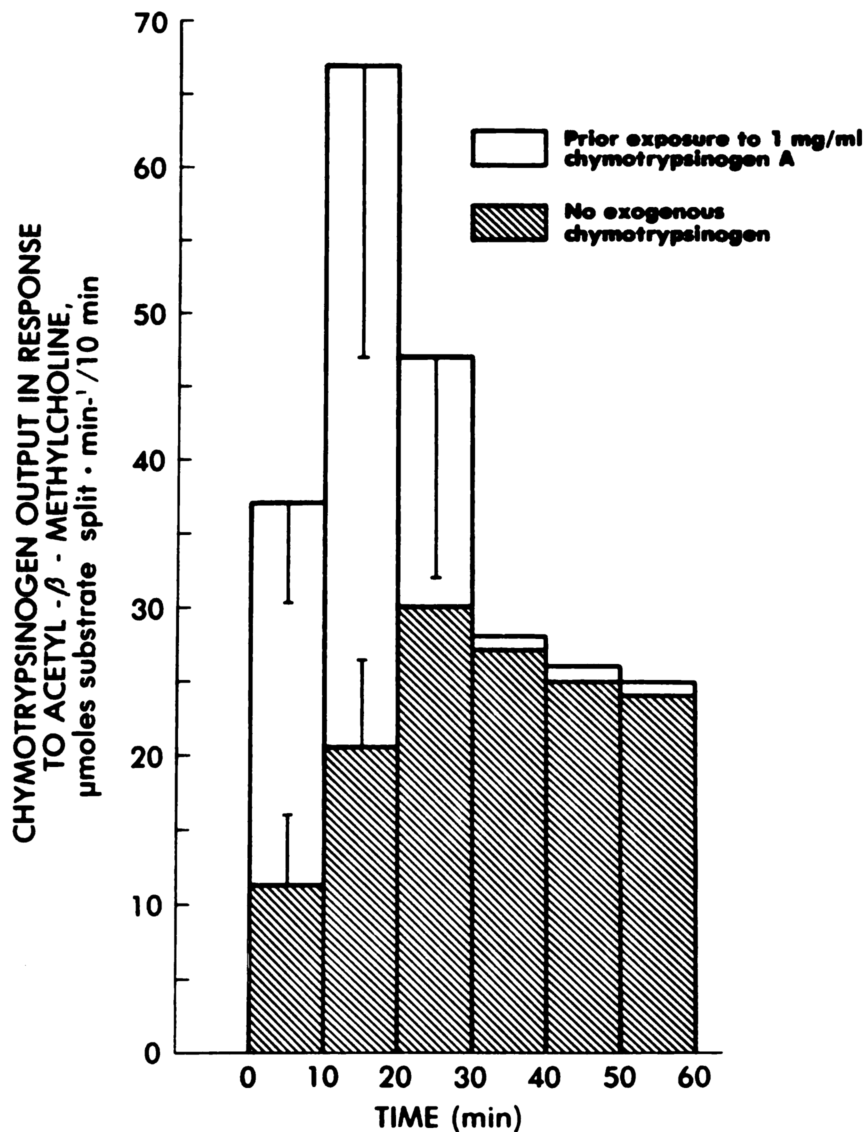

Figure 27. Effect of acetyl-β-methylcholine chloride (MCh) (1 mg/100 ml bath volume) on chymotrypsinogen output with and without prior exposure (2 h) to 1 mg/ml bovine chymotrypsinogen A in bathing medium. MCh was added at time zero. The chymotrypsinogen response to MCh was increased some 3.3 fold at its maximum by the presence of the enzyme in the bathing medium for 2 hours prior to the addition of the secretagogue. n = 5 through 40 minutes; 3 at 40 and 50 minutes; and 4 at 50 and 60 minutes for prior exposure condition. n = 7 for control data. The difference between chymotrypsinogen pre-treated and control groups was $p < 0.01$ at 0-10 min (2-tailed t-test), and $p < 0.05$ at 10-20 min (Mann-Whitney rank test).

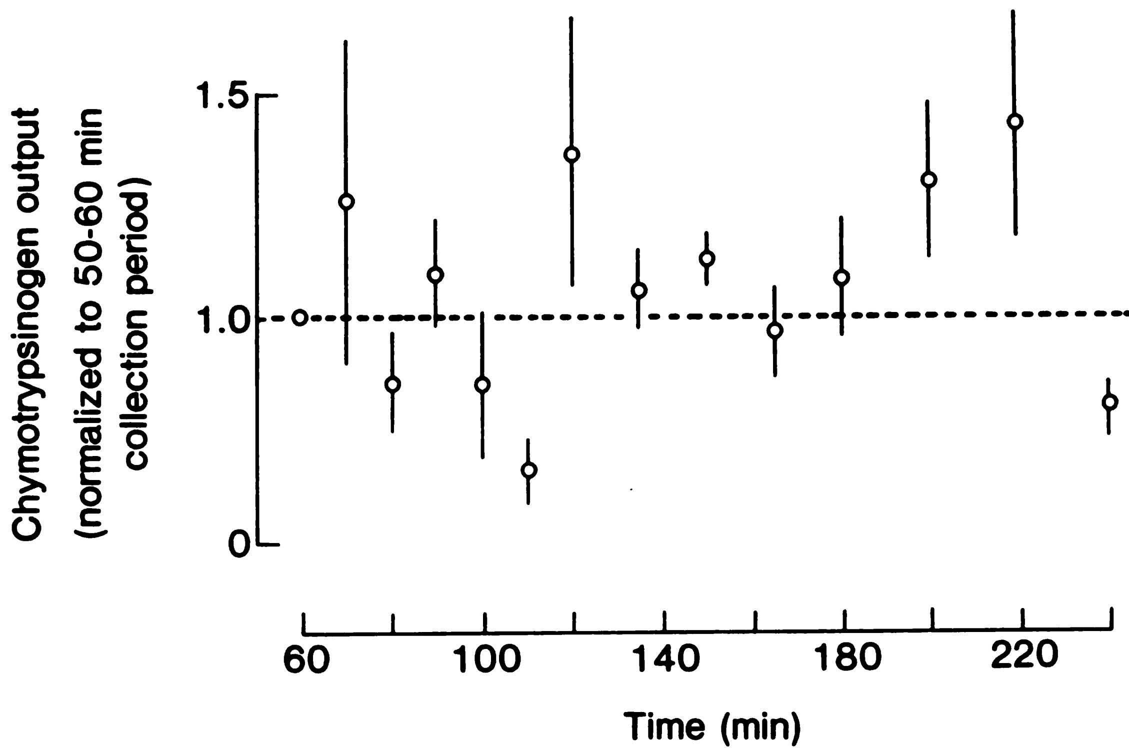

Figure 28. Chymotrypsinogen output in ductal secretion in the basal state. Data for each period is normalized to the 50-60 min collection period. Means ± S.E.M. are shown and n = 3 for all data points. (In the experimental condition, chymotrypsinogen was added to the bathing medium at 60 min). In the control situation, the secretion of chymotrypsinogen remains relatively constant over time.

The Effect of Exogenous Amylase on the Secretion of Endogenous Amylase

To determine if indeed it was the enzyme added to the medium that appeared in secretion, we labeled endogenous digestive enzyme pools with radioactive leucine for 4 hours prior to the addition of the exogenous enzyme and followed the specific radioactivity of secreted amylase. Transpancreatic transport of the unlabeled, exogenous enzyme should lower the specific radioactivity of amylase in secretion. When amylase was added to the bath, the specific radioactivity of this enzyme in secretion did drop precipitously. Ten minutes after the addition of unlabeled enzyme to the medium, the specific radioactivity (SRA) of amylase in ductal secretion was reduced by 82%; and by 60 minutes it was only 7% of control values (Figure 29). The SRA remained depressed for the duration of the experiment, despite the fact that over time amylase output fell back toward control levels (Figures 22 and 29). Thus, even as the response declined, about 90% of the secreted amylase was unlabeled and apparently of exogenous origin.

In addition, the presence of amylase in the bath altered the secretion of endogenous amylase. In most experiments, labeled amylase secretion increased transiently and then was followed by a sustained inhibition (Figure 30). The transient increase averaged 72% of basal amylase output, but was quite variable and ranged from -32% to +250%, accounting for the large standard error seen for these periods (Figure 31). By 60 minutes, amylase radioactivity in secretion had declined to 28% ± 6 S.E.M. of prior control levels where it stayed for the remainder of the experiment (Figure 30). Inhibition of labeled amylase secretion was not seen when either 0.1 or 0.2 mg/ml amylase was used, and only a slight inhibition was observed when amylase was present at a concentration of 0.3 mg/ml (concentrations refer to weight of Sigma, Type VI-A amylase) (Table IX).

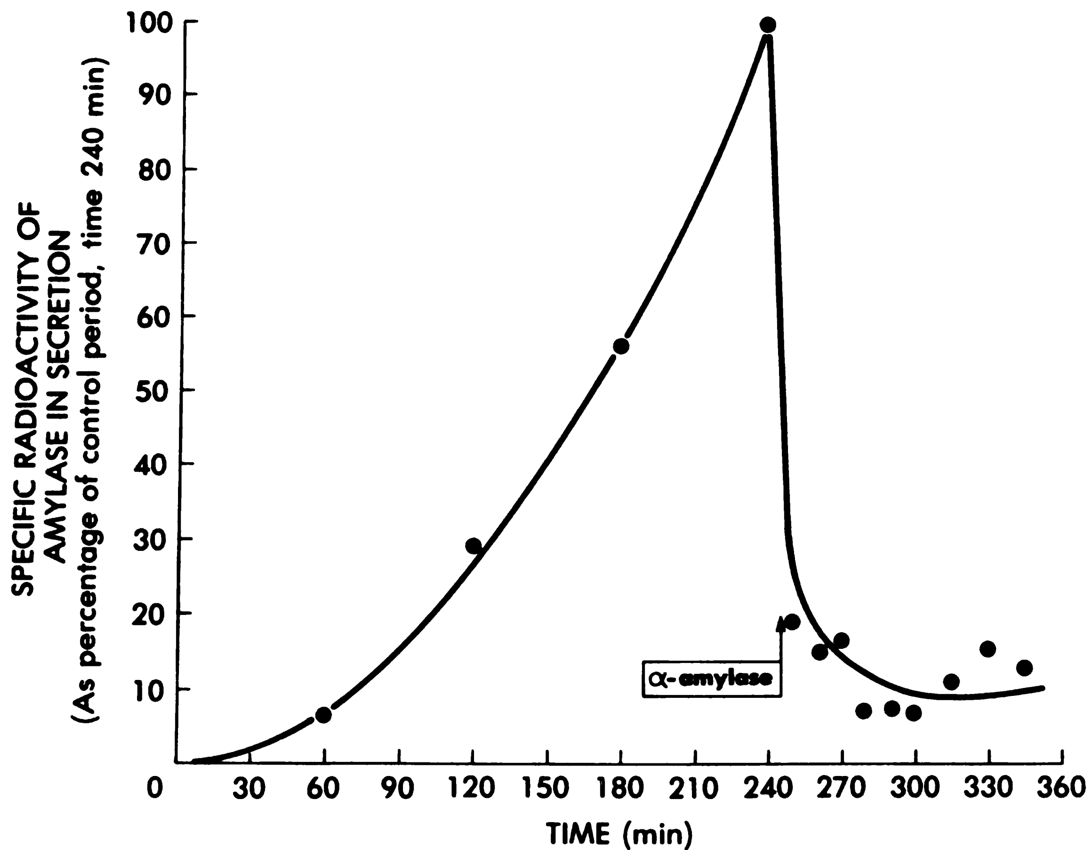

Figure 29. Effect of the addition of 1 mg/ml unlabeled α-amylase to the bath after a 4 hour incubation with ^3H leucine, on the specific radioactivity (SRA) of amylase in ductal secretion. The SRA of secretion dropped dramatically when amylase was added to the bath and remained depressed for the remainder of the experiment. ^3H leucine was added at time zero. Specific activity is expressed as the ratio of mean amylase cpm (n = 8 to 240 minutes, post-stimulus points as in Figure 30) to mean amylase output (n = 8 or greater for all output points except at 330 and 345 where n = 5).

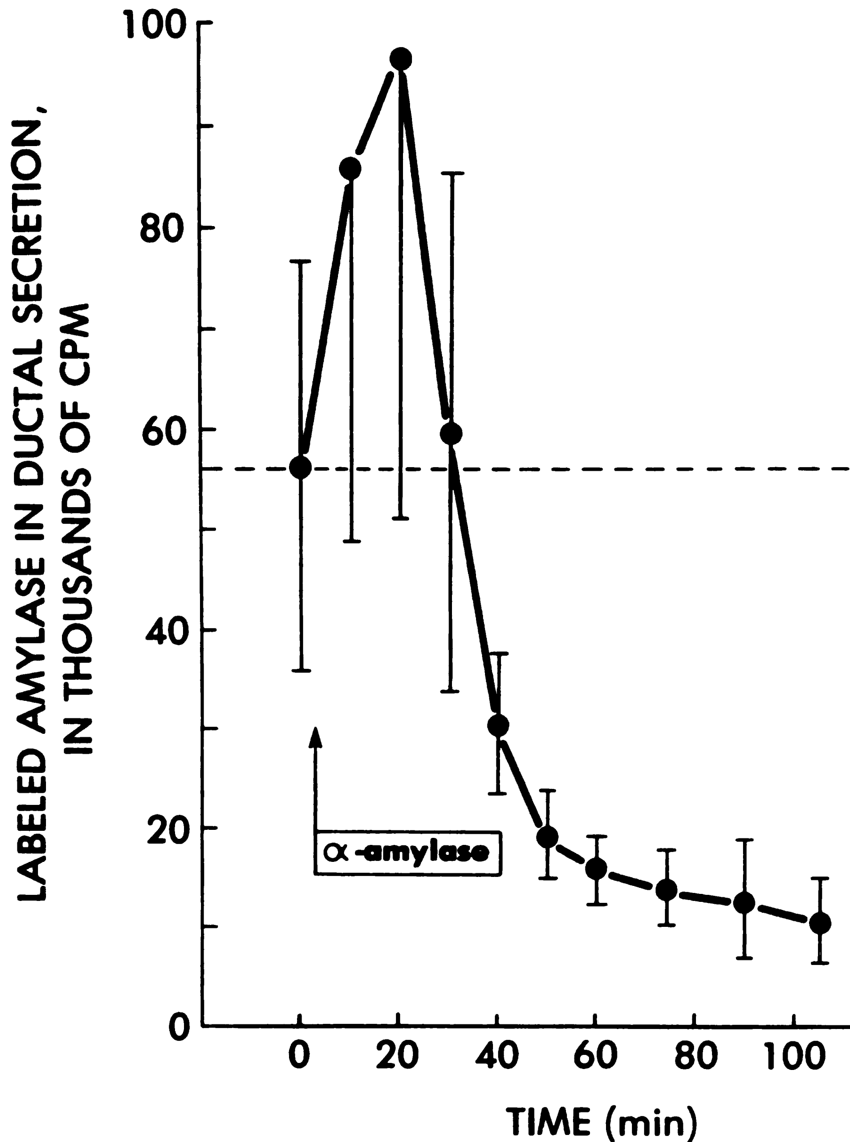

Figure 30. Effect of the addition of 1 mg/ml α-amylase to the bath on the amount of radioactive amylase in ductal secretion. The amount of endogenous (labeled) enzyme in secretion increased transiently when amylase was added to the bathing medium and then was inhibited to about 30% of its pre-stimulus level where it remained for the duration of the experiment. Data are expressed as mean cpm in amylase/10 min ± S.E.M. Value at time zero is cpm in amylase in secretion for 10 minutes control prior to the addition of α-amylase to the bath and 230-240 minutes after the addition of ^{3}H leucine (see Figure 29). n = 8 through 40 minutes; 7 at 50 and 60 minutes; and 3 at 75, 90 and 105 minutes.

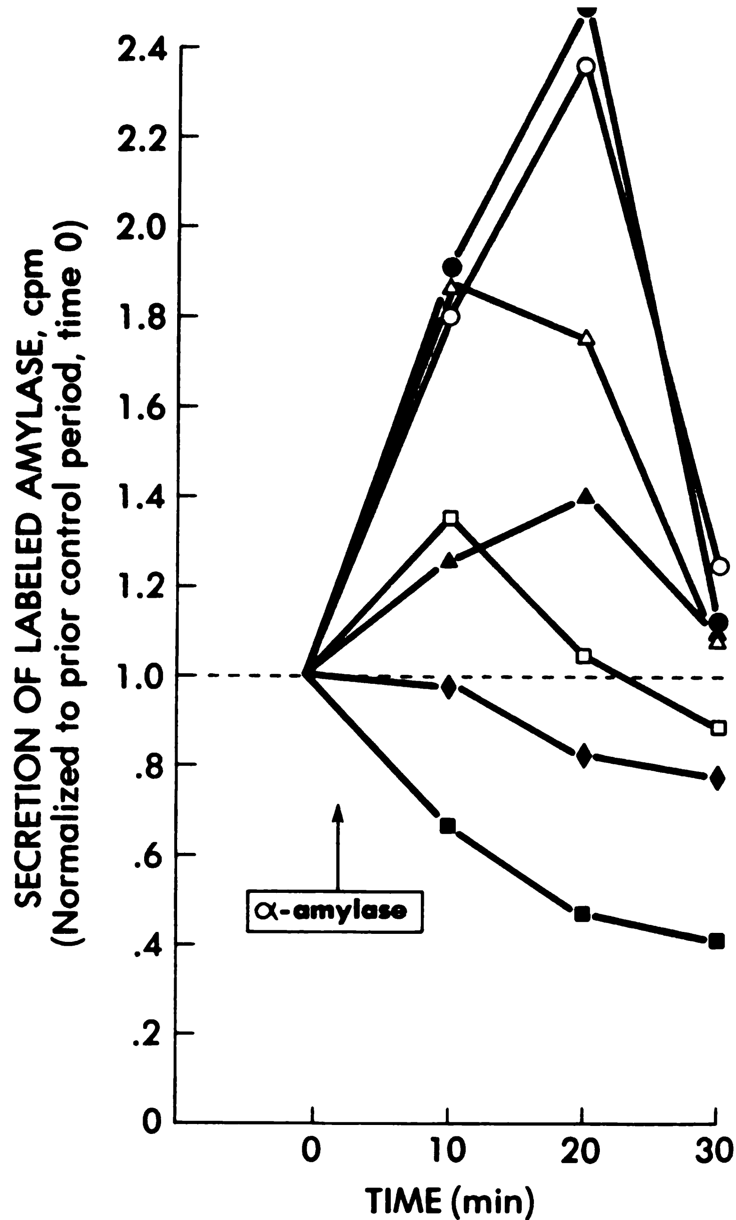

Figure 31. Secretion of labeled amylase in individual experiments for the first 30 minutes following addition of 1 mg/ml α-amylase to the bathing medium. Data is normalized to t = 0, which represents the value for the 10 minute control collection period prior to the addition of amylase to the bath. Labeled amylase in secretion increased transciently in 5 out of 7 experiments and the spread in the magnitude of the response is responsible for the large standard error shown for these time periods in Figure 30.

Table IX

Percentage Change in Secretion of Labeled Amylase 60 Minutes after the Addition of Varying Concentrations of Exogenous Amylase to the Medium (5 h after the addition of ^{3}H leucine). Sigma, Type VI-A Amylase was used. Percentage Change in Label Secretion at 1 mg/ml Bath Amylase is from Figure 4.

$[Amylase]_{bath}$, mg/ml	Percentage change in labeled amylase secretion, +60 min after the addition of amylase to the bathing medium (relative to pretreatment control, -10 min)
0.1	+58
0.2	+41
0.3	-16
1.0	-72

In a previous section, "Secretion of Digestive Enzyme From Two Intracellular Pools", we demonstrated the existence of at least 2 intracellular pools of digestive enzyme in the acinar cell, i.e., when the *in vitro* rabbit pancreas was stimulated with a cholinergic agonist or CCK after a 2 hour incubation with ^{3}H leucine, the specific radioactivity of digestive enzyme in secretion dropped precipitously, indicating the preferential secretion of old or unlabeled protein (Figure 4). A similar but less dramatic effect was seen when MCh was given 4 hours after the addition of label to the bathing medium; the specific radioactivity of total protein was reduced by 35% with cholinergic stimulation at this time, and specific radioactivity returned to pre-stimulus levels by 60 minutes (Figure 8). Thus, it is possible that the results described here are due to a similar phenomenon, i.e., the addition of α-amylase to the bath stimulates the release of less highly labeled endogenous stores. This seems unlikely for the following reasons: 1) amylase added to the bath at 4 hours post-^{3}H leucine produced a much larger decrease in S.R.A. (95%) than a cholinergic drug added at the same time; 2) unlike cholinergic stimulation, the depression in S.R.A. was sustained over time in the continued presence of the enzyme (Figure 29); 3) the *amount* of labeled protein in secretion after cholinergic stimulation at 4 hours only decreased very transiently (Figure 9), and then increased by over an order of magnitude within 10 minutes. In contrast, amylase added to the bath produced a transient increase and a steady-state inhibition of about 70% (Figure 30); 4) incubating a gland for 4 hours with a cholinergic stimulant along with ^{3}H leucine in order to deplete cold pools did not change the pattern of the response when exogenous amylase was added, i.e., sustained inhibition of labeled amylase secretion was still seen (Figure 32).

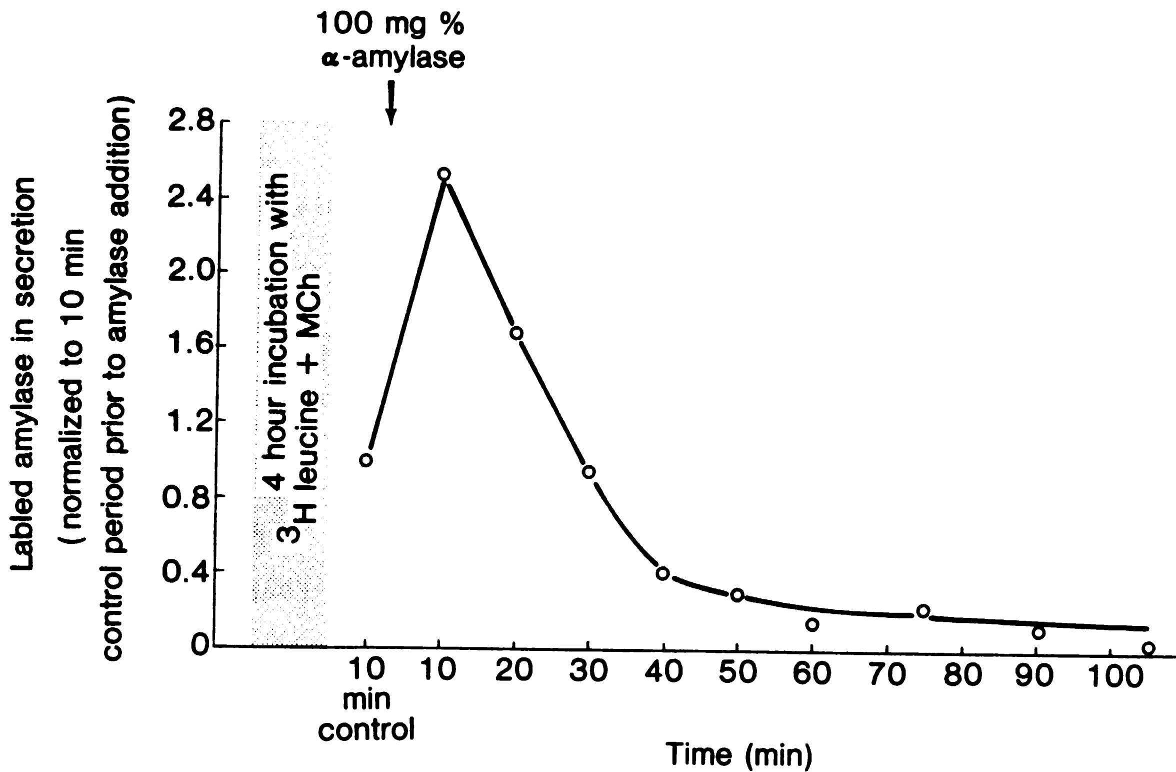

Figure 32. Labeled amylase in secretion in response to the addition of 1 mg/ml amylase to the bathing medium after a 4 hour incubation with acetyl-β-methylcholine chloride (MCh) and ^{3}H-leucine. Data is normalized to time zero, which represents the amount of label in secretion for the 10 min control period prior to the addition of the exogenous amylase and 230-240 min after the addition of MCh and ^{3}H leucine. The pattern of response is similar to that obtained without prior incubation with MCh (see Figure 30).

The inhibition of endogenous amylase secretion by the addition of amylase to the bathing medium suggests competitive transport inhibition between exogenous and endogenous enzyme and hence, suggests that the enzyme moves through the acinar cell. The fact that enzyme movement is through the acinar cell rather than via paracellular shunts is also supported by the observation that the addition of amylase to the bathing medium increased the duct to bath concentration difference for this enzyme. If a diffusional equilibration through paracellular shunts were all that were involved, the concentration difference should have been reduced. At the peak of the response, the amylase concentration in the duct averaged 6,463 I.U./ml (with a range of 1,457 - 18,155 I.U./ml), whereas bath amylase was only approximately 61 I.U./ml (Table X). This concentration difference (6,402 I.U./ml) is 15 times that of the control situation (455 I.U./ml; range 46 - 1,822 I.U./ml), or about 20 times control periods when each experiment was paired to its own prior control.

A cellular route is also suggested by experiments in which a cholinergic drug was added to the bathing medium 2 hours after the addition of chymotrypsinogen A. Under these circumstances, the chymotrypsinogen response to the cholinergic drug was 3.3 times greater than that seen when the cholinergic agent was added in the absence of chymotrypsinogen (Figure 27). Again, enzyme movement likely occurs via a cellular pathway rather than through an intracellular shunt. This is so because the process was stimulated by MCh. In addition, an uphill step, and therefore a cellular route, is suggested by the fact that at the peak of the response, the chymotrypsinogen concentration in secretion averaged 7.47 μg/λ, whereas in the bath, in contrast, it was only 1.0 μg/λ.

Table X

The Effect of 1 mg/ml Exogenous Amylase on $[\text{Amylase}]_{duct} - [\text{Amylase}]_{bath}$. Sigma, Type VI-A Amylase was Added to the Bath.

Experiment	Amylase Concentration Difference (Duct-Bath),* I.U./ml A. No amylase (10 min prior to +amylase)	B. Amylase added (peak values)	C. $\frac{+\text{amylase (B)}}{-\text{amylase (A)}}$
1	364	4,190	11.5
2	131	1,862	14.2
3	506	6,315	12.5
4	430	6,011	14.0
5	556	7,833	14.1
6	273	8,136	29.8
7	1,822	11,577	6.4
8	146	1,457	10.1
9	46	1,558	34.0
10	349	4,043	11.6
11	759	18,155	23.9
12	86	6,416	74.6
$\bar{x}$	455	6,463	21.4
SEM	138	1,374	5.4

* The mean basal steady-state bath amylase concentration, 61 I.U./ml, was subtracted from each ductal concentration value in A. Individual bath values for each experiment were not used.

The Effect of Exogenous Amylase and Chymotrypsinogen on the Secretion of Other Enzymes

The addition of crude amylase (Sigma, Type VI-A) to the bathing medium stimulated the secretion of chymotrypsinogen, as well as amylase (Figure 23), and the increase was of the same magnitude. Indeed, overall protein secretion was also increased in similar proportions, which suggests that the secretion of other enzymes was augmented as well (Figure 33). The removal of other digestive enzymes from the impure amylase by its repurification did not eliminate this effect, and roughly parallel, although diminished, secretion of the two enzymes was still observed, even though only very small amounts of chymotrypsinogen were added to the bathing medium under these conditions (see Methods) (for the period of peak response when repurified amylase was added, the increase over controls was 7.5 times for amylase and 9.5 times for chymotrypsinogen in one experiment, and 7.9 times for amylase and 5.3 times for chymotrypsinogen in another). Similarly, when the relatively pure chymotrypsinogen was added, the secretion of amylase was augmented in a fashion roughly equivalent to that seen for chymotrypsinogen (a maximum output of some 6-7 fold control rates; Figure 23). Thus, the addition of one enzyme to the medium apparently stimulated the secretion of other enzymes and in a grossly parallel manner. Since the Sigma VI-A amylase is contaminated with other enzymes, this is probably the reason why the crude amylase evoked a larger amylase response than the repurified amylase itself.

While the outputs of the two enzymes were more or less parallel when means were compared, a more detailed analysis of the experimental data indicated that the responses were not identical. When their outputs were plotted against each other for individual samples, it could be seen

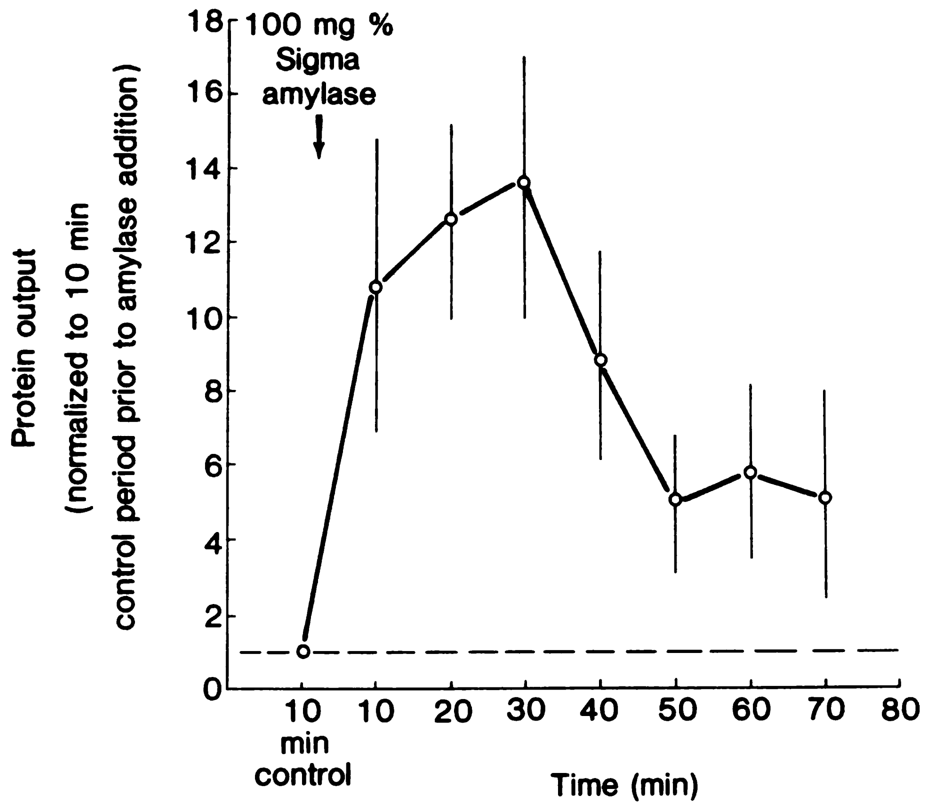

Figure 33. Protein output in secretion in response to addition of 1 mg/ml α-amylase to the bathing medium. Data is normalized to time zero, which represents protein output for 10 minute control collection period prior to addition of amylase to the bath.

that the addition of amylase to the medium altered the distribution of the points, curiously skewing the data towards a more chymotrypsinogen-dominant secretion (Figure 34). The broken line in Figure 34 represents one standard deviation either plus or minus the mean slope for control data and includes approximately 2/3 of the control points. On the other hand, 64% of the amylase treated points were greater than 1 SD above the mean line, i.e., were chymotrypsinogen-dominant relative to controls. The +amylase points (i.e., amylase added to the bath) also show a much greater spread, and when the variance about the two calculated functions (+amylase and control) was compared, the difference was considerable (S^2 amylase = 14.3; S^2 control = 2.7; $F = 5.3$; $P < 0.01$). Thus, the addition of enzyme to the bath greatly _reduced_ the covariance between the secretion of the two enzymes. This change in covariance between the secretion of different enzymes is similar to that reported in other circumstances (Rothman, 1976; Adelson, 1975), and indicates that even though the average response was roughly parallel for the two enzymes, their secretion became more disconnected or independent of each other after amylase was added. Therefore, the parallelism does not indicate linked or obligatorily coupled transport such as in a vesicular release mechanism.

V. Secretion Blockage

In the exocytosis model for digestive enzyme secretion, the net secretory flux is determined by an intracellular event, the rate at which storage granules fuse with the plasma membrane. Since exocytosis is a unidirectional process, the secretory flux would be independent of the concentration of enzyme in the duct system. For an "equilibrium" based system, in contrast, in which bidirectional, concentration-dependent fluxes occur, the concentration of enzyme in the duct system would

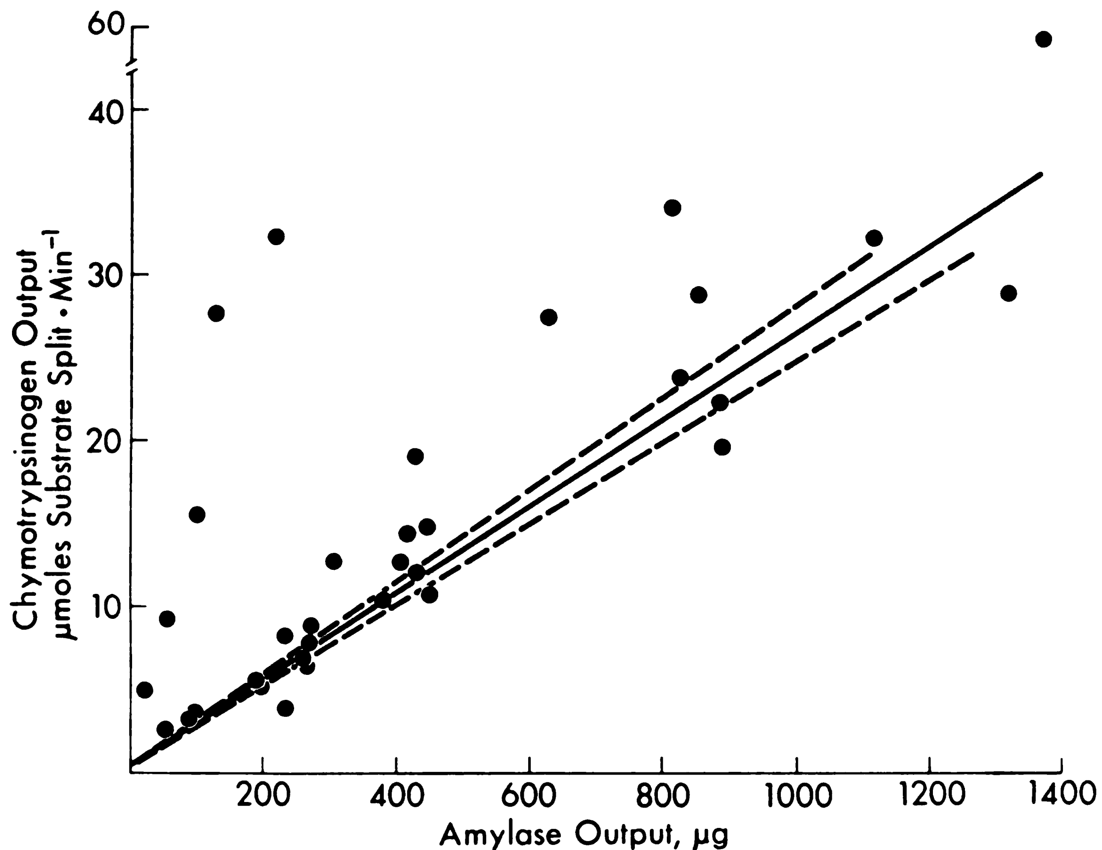

Figure 34. Effect of adding 1 mg/ml α-amylase to the bath on the relative proportions of chymotrypsinogen and amylase in secretion. Amylase output is plotted against chymotrypsinogen output for individual samples. The control line is calculated from 124 individual data points and includes data from both time-paired parallel controls and the first hour of control secretion for the experimental glands. The broken line represents one standard deviation ± the mean slope for control points. Experimental points are from 0 to 50 minutes after the addition of amylase to the bath and are from 7 experiments. All outputs are for 10 minutes sampling periods. Addition of amylase to the bathing medium tended to lead to a more chymotrypsinogen-rich secretion.

be a crucial determinant of the net secretory flux. These differences were used as a further means of distinguishing between the two proposed mechanisms for pancreatic secretion.

When fluid secretion is artificially stopped by preventing its outflow from the duct system (either by closing an indwelling catheter or by applying a backpressure to the column of fluid), enzyme secretion should continue unabated if secretion occurs by exocytosis. Since such a process is not dependent upon the parameters of ductal enzyme concentration, the concentration of enzyme throughout the duct system should simply increase with time as fusion continues to occur. The concentration of enzyme in the ductal fluid of basally secreting glands should be able to increase greatly, certainly to the magnitudinally higher concentrations seen when secretion is driven by stimulants and perhaps as high or higher than the solubility of the enzymes themselves in water.

In contrast, the equilibrium based model predicts that when movement of fluid from the duct system is inhibited, the concentration of enzyme at the site of secretion and throughout the duct system would be limited to the value at which duct to cell and cell to duct fluxes become equal, i.e., the concentration at which a net flux no longer occurs. This limiting value would either be the concentration of the enzyme in the cytoplasm itself, for a simple equilibrium, or, if an active step occurs between cytoplasm and duct, then this limiting concentration would be that necessary to bring the net flux to zero. In either case, secretion should be greatly inhibited if enzyme is not removed from the site of secretion by the fluid flow.

The Effect of Ductal Blockage on Amylase Secretion

Little change was seen in either amylase output (Figure 35) or concentration in secretion collected immediately after a 90 minute duct blockade [amylase concentration 10 minute pre-blockage, 0.89 μg standard/μl ± 0.18 S.E.M. vs 2-5 minutes post-blockage, 0.93 μg/μl ± 0.27 S.E.M. (n = 5)]. Furthermore, amylase concentration in samples collected as long as 45 minutes post-blockage remained relatively unchanged (5-15 minutes, 0.72 μg/μl ± 0.17 S.E.M.; 15-30 minutes, 0.64 ± 0.22 S.E.M.; 30-45 minutes, 0.80 ± 0.23 S.E.M.). Similar results have been obtained *in situ* in anesthetized rabbits (Appendix 1). Thus, enzyme secretion virtually ceased in the absence of fluid secretion [with the minor exception of the filling of an apparent intraductal volume capacitance (see below)]. If enzyme secretion had continued unabated during the 90 minute period, as the exocytosis model would predict, then the output and concentration of amylase should have been increased about an order of magnitude in samples collected immediately after blockage (Figures 35 and 36).

When a graded back-pressure was applied to the column of fluid in the duct, to reduce flow progressively, the results were similar. That is, the concentration of amylase remained relatively constant over the range of flows produced in this manner. Output decreased in proportion to decreased flow, and amylase concentration did not increase as would have been expected if enzyme secretion had continued without decrement as flow was decreased (Figure 36 and 37). For the lowest flows (highest back-pressure), an increase in amylase concentration may not have been seen for a particular period because of a possible time delay in traversing the potential dead space of the duct system. We checked

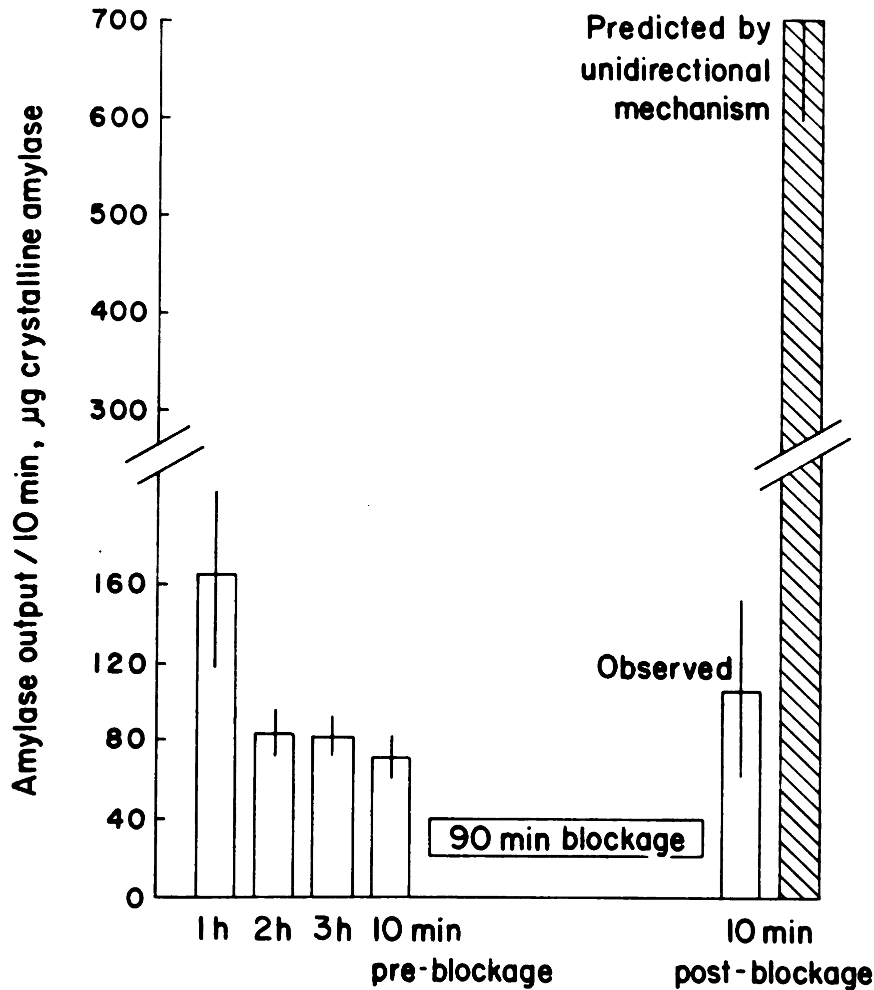

Figure 35. The effect of ductal blockage on amylase secretion. Data are presented as mean amylase output/10 minutes ± S.E.M. (n = 4). Samples were collected at hourly intervals for 3 hours and for a 10 minute period immediately prior to blockade. Post-blockage data are given for the first 10 minute period and compared to the value predicted by a unidirectional transfer mechanism (hatched bar). Secretion during the first period after blockage (10 minute post-blockage) was close to that observed for a similar time period just prior to blockage, indicating that secretion did not accumulate to any degree in the duct system during blockade, as would have been expected if a "vectorial" or unidirectional secretory process accounted for enzyme secretion.

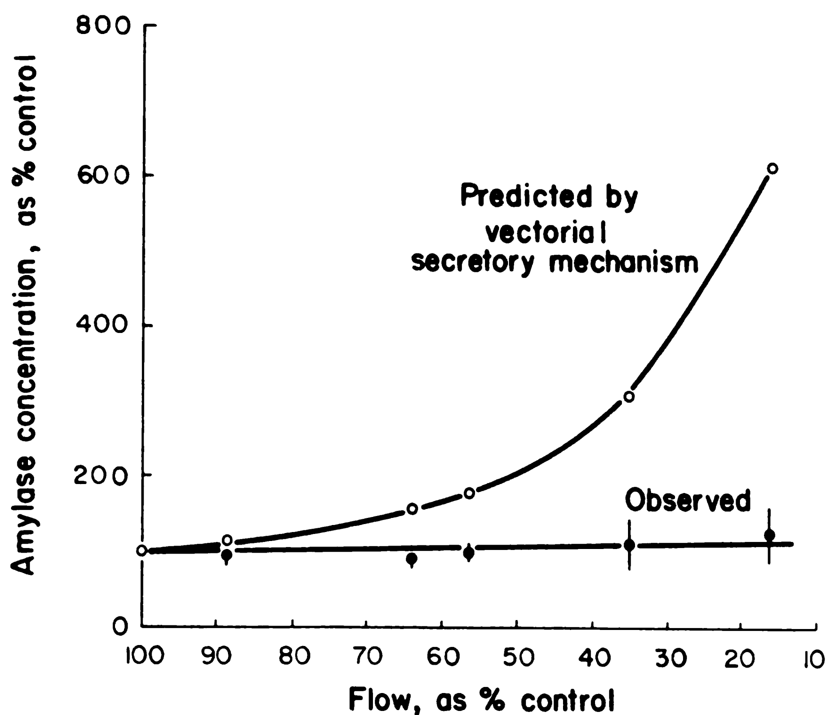

Figure 36. The concentration of amylase in secretion as flow is progressively decreased by the application of a graded back-pressure. The amylase concentration in secretion (closed circles - "observed") remained essentially constant over the wide range of flows studied. Data represent the mean ± S.E.M. for 4 experiments. Open circles (0) represent the increase in concentration that would be predicted if enzyme secretion continued at a constant rate independent of fluid flow, as in a unidirectional ("vectorial") secretory process.

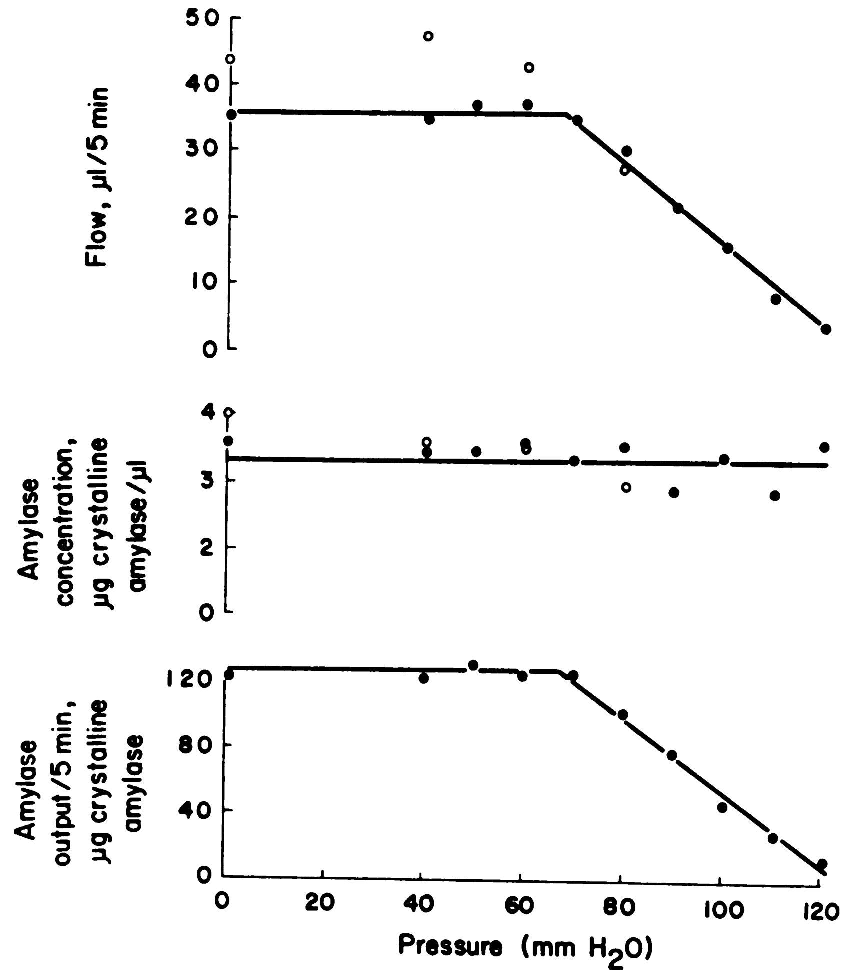

Figure 37. Fluid secretion ("flow"), amylase concentration, and amylase output as a function of back-pressure applied to the column of fluid in the pancreatic duct for a single experiment (closed circles). As back-pressure was increased, both flow and output decreased in tandem leaving concentration essentially unchanged. Pressure is given in mm H_2O above the level of the pancreatic papilla (see text). Open circles represent values for flow and concentration when the back-pressure was reduced back towards zero.

for this by continuing to measure amylase concentration in secretion when the procedure was reversed, and the height of the column sequentially lowered back to the level of the papilla. No increase in enzyme concentration was observed under these conditions (Figure 37, open circles).

The hydrodynamic pressure of secretion (the secretory pressure) is thought to be the consequence of the osmotic movement of water into the duct lumen, and fluid secretion should cease when this pressure is counterbalanced by an equal and opposite pressure such as the applied end-pressure in the present experiments. Thus, the pressure gradient which determines the rate of fluid flow (intraductal pressure minus back-pressure) is altered with changing end-pressure. Increases in column height, however, did not reduce flow at the steady-state until approximately 90 mm H_2O ± 16 S.E.M. (6.7 mm Hg) above the papilla (Figure 37 and 38). Therefore, up to this point, the system compensated in some manner for the diminished driving force. Above 90 mm H_2O, fluid flow decreased in a linear fashion as the applied back-pressure was increased (Figures 37 and 38).

We should consider the possibility that the inhibition of enzyme secretion was a direct effect of the applied hydrostatic pressure, rather than an indirect effect via the reduction in fluid flow. If this were so, then hydrostatic pressure would have had to have altered both fluid flow and enzyme secretion identically; that is, both the pressure at which inhibition begins as well as the rate of falloff after its onset would have to have been exactly the same for the two supposedly independent variables and this seems unlikely. Furthermore, a similar coupling of fluid flow and enzyme secretion has been observed when flow was reduced by means other than an applied back-pressure (see "Discussion").

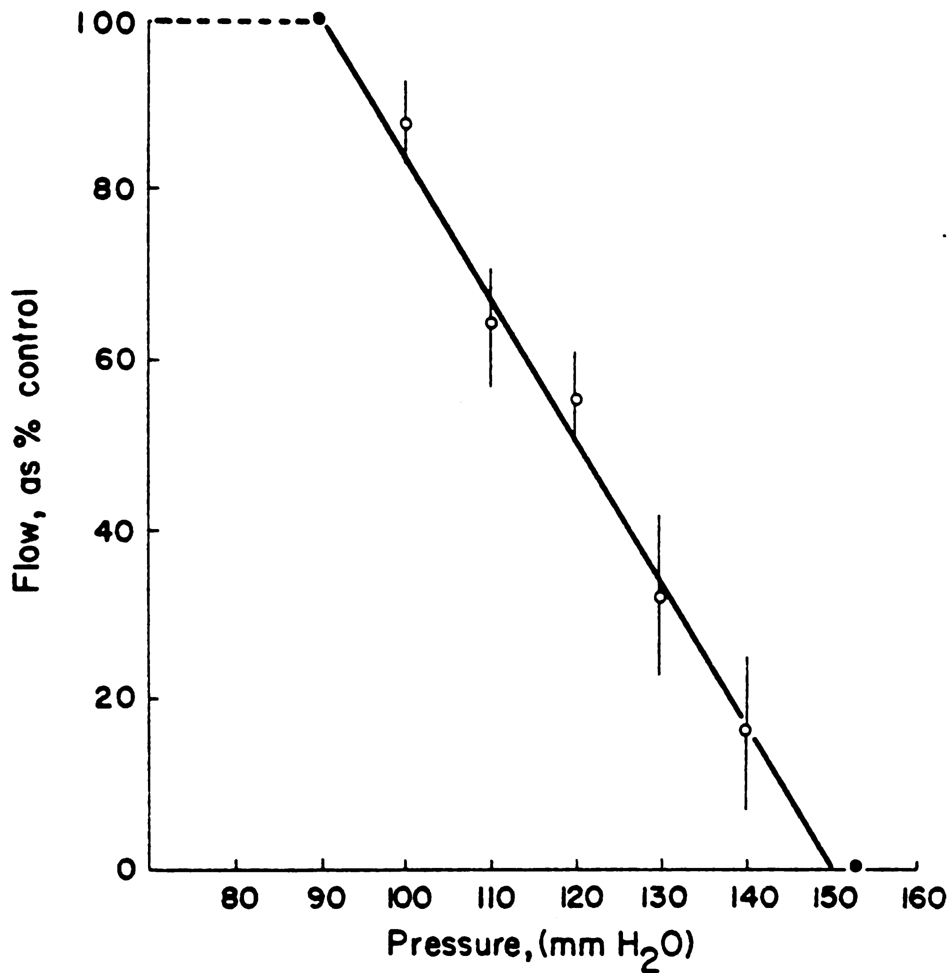

Figure 38. Flow as a function of the applied back-pressure. Data are given as the mean ± S.E.M. for 4 experiments. The mean fall in flow, relative to the period prior to the decline, is shown for each 10 mm H_2O increment in pressure (open circles) and is set to the average value for the beginning of the decline (90 mm H_2O; closed circle). The average zero flow intercept was 154 mm H_2O (closed circle) which is in good agreement with the intercept of 150 calculated from the regression line.

The Ductal Capacitance

Flow during the first collection period after the 90 minute blockade (2-5 min), was substantially augmented relative to prior control flow rates (2.7 times on the average). The total cumulative increase in flow over controls amounted to a mean increase of 95 microliters. This suggests that the duct system has a substantial capacitance, most probably in the larger collecting vessels. The capacitance would be of about the same volume as that of the duct itself, as estimated by extracellular space markers in the free-flow situation (Rossier and Rothman, 1975). The presence of a capacitance in the duct system can also be inferred in another manner. When an open cannula was raised to the column height at which flow just ceased, flow began again in a matter of seconds. The column was continuously raised in this manner, until flow stopped permanently. This behavior is consistent with the gradual filling the capacitance of distensible conduits.

Changes in Bath Amylase During Blockade

Bath amylase concentration increased by about 50% above its equilibrium value during the 90 minute blockade (Figure 39). Average total bath amylase output at 180 minutes was 984 μg, whereas at 270 minutes, it was 1,488 μg. The difference, 504 μg, accounts for 82% of the amylase that would have been secreted into the duct in the absence of the blockade.

At the steady-state in the absence of blockage, the rate of secretion of enzyme into the duct equals the rate of synthesis of new enzyme. Therefore, since secretion of amylase into the bath accounted for only 82% of the amylase that would have been secreted into the duct during the 90 minutes, either synthesis of new enzyme was inhibited by 18%

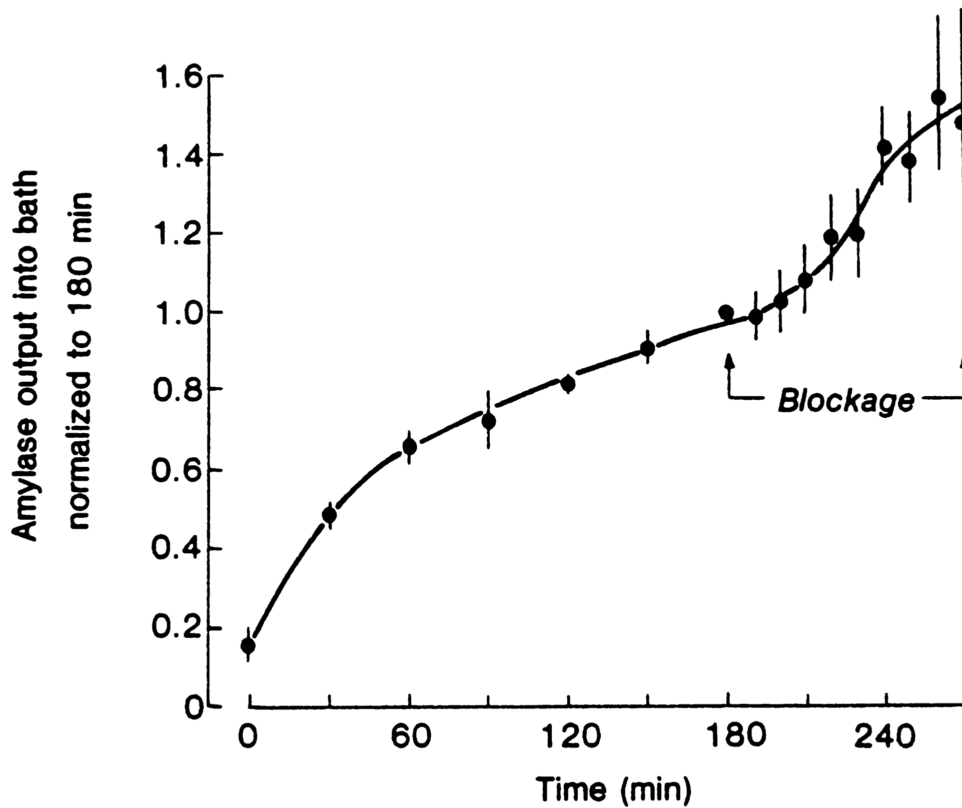

Figure 39. Secretion of amylase into the bathing medium of the *in vitro* pancreas. At 180 minutes, the end of the cannula collecting ductal contents was fused. Inhibiting the outflow of ductal secretion increased the rate of amylase secretion into the bath. Data is normalized to bath amylase value for 180 minutes.

during the 90 minutes blockage period, or alternatively, the zymogen granules accommodated 18% of the newly synthesized enzyme.

Effect of Increasing the Hydrostatic Pressure Gradient for Secretion

In one experiment, the free end of the catheter was lowered below the papilla in increments to determine if increasing the pressure drop between the apical membrane and catheter tip would increase flow. We reasoned that if the driving force for flow was an osmotic force, then no change in rate should occur with the increased pressure drop. No change in flow was observed, suggesting that ion transport rather than hydrostatic pressure generation is the rate limiting process in fluid secretion.

DISCUSSION

I. Secretion of Digestive Enzyme from Two Intracellular Pools

The cisternal packaging-exocytosis model for digestive enzyme transport and secretion in the pancreas proposes that all enzymes move via a fixed sequence of membrane-bound compartments to the duct lumen. This means that only one transport pathway exists and that all digestive enzyme must follow this route (Figure 40, Model 1). If this model is correct, then applying secretagogue to the _in vitro_ pancreas after the addition of labeled amino acids to the bathing medium (and in their continued presence) should increase the amount of radioactivity per unit protein, or the specific radioactivity (SRA) of secreted protein, towards its maximum possible level for the system.

Moreover, if granules containing newly synthesized enzyme move to the duct as an orderly front, that is, approach the lumen in the same order that they are formed, then all granules containing old unlabeled enzyme should be released prior to the secretion of granules containing the newly synthesized enzyme. The SRA of secretion should remain constant after stimulation for a while, as old granules are released, and then increase suddenly to its maximum level, where it should remain in the continued presence of label in the bathing medium. If, on the other hand, granules move in a random fashion to the lumen, that is, new granules mix with old granules, the SRA of secretion should increase gradually to its steady-state value, reflecting a gradual dilution of granules containing older unlabeled enzyme by granules containing newly synthesized enzyme.

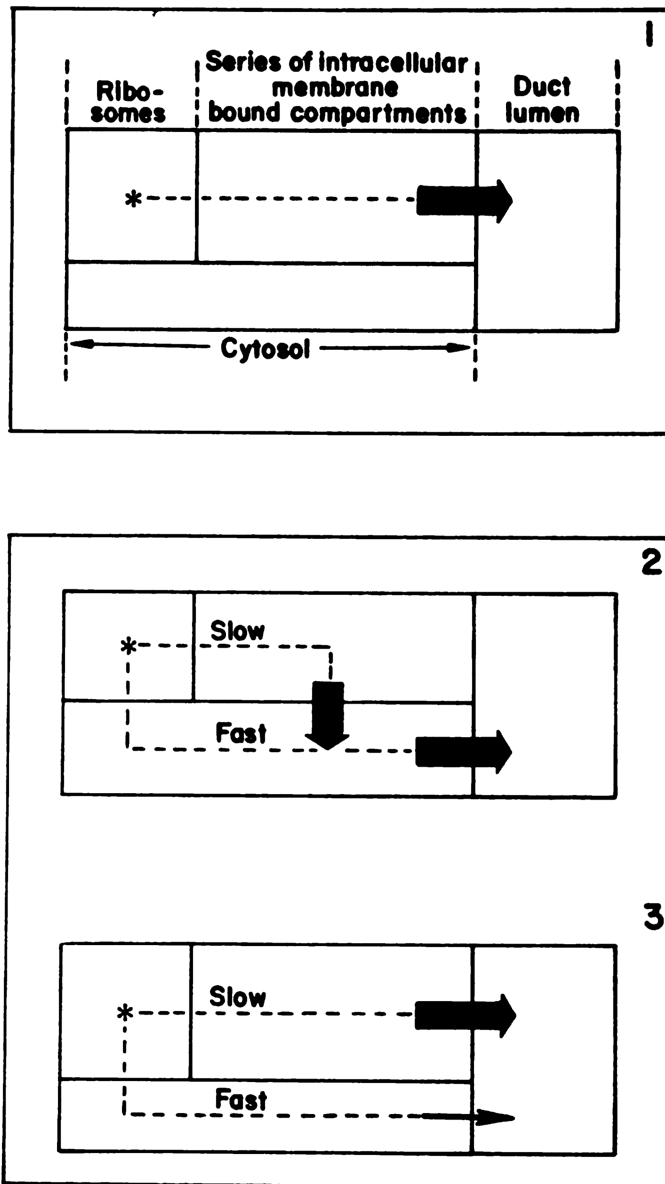

Figure 40. Three models for protein secretion by the pancreatic acinar cell. Model 1 shows a single secretory pathway, as proposed by the Palade model, in which enzyme is transferred in membrane-enclosed vesicles within the cell and released into the duct by exocytosis. Model 3 shows two parallel and independent secretory pathways; one is as in Model 1 while the other represents secretion from a cytoplasmic pool of digestive enzyme. Model 2 is similar to Model 3 except the two pathways are interactive; enzyme from the zymogen granules and cytoplasm mix and exit the cell via a final common transport mechanism. The large arrows indicates the net flux of older, stored protein secreted preferentially in response to acetyl-β-methylcholine chloride or cholecystokinin-pancreozymin.

Of course, if the SRA of secretion has already reached its steady-state value prior to the addition of the secretagogue, stimulation according to the model would leave it unchanged. In no case does the cisternal packaging-exocytosis model predict that secretagogues should decrease the SRA of digestive enzyme in secretion.

When either acetyl-β-methylcholine chloride (MCh) or cholecytokinin-pancreozymin (CCK-PZ) were added to the *in vitro* pancreas, instead, a dramatic decrease in SRA accompanied the increase in protein output (Figure 4). The maximum depression in S.R.A. was approximately 78% for CCK and 97% for MCh. The secretagogues, therefore, mobilized the secretion of a large amount of less highly labeled digestive enzyme, indicating that it was in large part material synthesized prior to the addition of label to the bath. This observation suggests that secretion cannot simply be a process in which all digestive enzyme molecules move en masse through a number of membrane-bound compartments in series with each other, as is proposed by the cisternal packaging-exocytosis model. Instead, the decrease in SRA with stimulation suggests the existence of two pools of digestive enzyme in the pancreas, distinguished by their content of newly synthesized enzyme.

Analogous results are reported by Robberecht (1977) who stimulated human pancreas with caerulein, a compound from frog skin with a carboxy terminal octapeptide sequence similar to that of CCK-PZ. He concurs that the dramatic decrease observed in the SRA of secreted digestive enzyme indicates the existence of two pools of digestive enzyme in the exocrine pancreas. He suggests, however, that such results could be due to the presence of either two granule or two cell types, with basal secretion drawn exclusively from one while the other is tapped only when secretagogues

are present. A similar model has been proposed by Dagorn (1978) to explain his findings. He observed that both the amylase to chymotrypsinogen ratio and the lipase to chymotrypsinogen ratio in secretion changed with stimulation and also that the final ratios were the same with both CCK-PZ and pilocarpine stimulation.

The existence of two intracellular pools of digestive enzyme with basal secretion derived exclusively from one pool while the other pool contributes to secretion only in the presence of secretagogues cannot explain the results reported in this study, since turnover of the "cold" or less highly labeled pool occurred in the absence of stimulation. This is demonstrated by the observation that 2 hours after the addition of isotope to the bathing medium, the depression in SRA with MCh was 97%, while 4 hours post-isotope, MCh caused only a 36% depression in SRA (Figure 8). Moreover, when the secretagogue was given approximately 8½ hours after label was first added, and several hours after the label had been removed as a result of a bath change, the SRA of secretion rose as output increased (Figure 8), indicating the former "cold" pool of enzyme was now indeed the more highly labeled pool.

The data suggest that at least two pre-secretory pools exist and both contribute to basal secretion, with one turning over or equilibrating with newly synthesized enzymes more quickly than the other. One pool will be referred to as the "rapidly equilibrating pool" (REP), and the other as the "slowly equilibrating pool" (SEP). The REP is likely cytoplasmic, and the SEP is likely the storage or zymogen granule pool; evidence supporting this view will be presented below.

Secretion of digestive enzyme across the apical membrane could occur either by totally independent mechanisms from the two pools as suggested by Model 2 in Figure 40, or, alternatively, by interactive mechanisms.

If the secretory mechanisms are independent, then increasing the amount of enzyme released from the SEP with stimulation should have no effect on the secretion of enzymes from the REP. This was not the case with MCh stimulation, which both increased the secretion of cold stored enzyme and also profoundly decreased the DPM in secreted digestive enzyme (the amount of newly synthesized enzyme in secretion) (Figure 5). Similarly, when MCh was added to the bath at the same time as the isotope, output increased and the absolute DPM in enzyme in secretion was substantially depressed relative to controls (Figure 7). Therefore, release of enzyme across the apical membrane must occur by interactive mechanisms from the two pools.

This interaction could be either competitive or noncompetitive in its nature. When MCh was given 2 hours after label addition, the inhibition in label secretion was maximal in the first five minutes after the stimulus was introduced, whereas <u>no</u> increase in output was observed during this period. Rather, a 5-10 minute delay occurred in the onset of augmented secretion (Table III, Figure 3). The fact that the inhibition in the amount of newly synthesized enzyme in secretion occurs prior to the increase in ductal output of enzyme, suggests that two separate events are involved in the response to the secretagogue. One possibility for non-competitive interaction of the two pools, consistent with such a two step response, is that secretion from the rapid pool is turned off before secretion from the slow pool is augmented or turned up. If, however, the decrease in label secretion reflects the shutting off of secretion from the rapid pool, a decrease in enzyme output proportional to the decrease in counts should also occur. This was not observed; output remained constant during this first 5 minutes post-stimulus period.

The other possibility for non-competitive interaction between the REP and SEP, consistent with the data, is that initially MCh causes a decrease in the secretion of enzyme from the rapid pool while at the same time causing a reciprocal increase in secretion from the slow pool. The increase in enzyme secretion from the slow pool would have to be followed approximately 5 minutes later by an additional increase of much larger magnitude in secretion from this pool. This construct is unattractive for two reasons. Not only does it involve a complex adhoc assumption of a reciprocal regulatory event, but in addition, it requires that secretion from the SEP is augmented discontinuously, that is, occurs in two separate steps, with the first step exactly compensating for the rapid pool's contribution to secretion, and only the second augmenting total output.

Since enzyme output did not decrease in the first 5 minutes post-stimulus, it is not likely that the REP is shut off. Apparently, therefore, the interaction of the two pools is of a competitive nature, with enzyme from the SEP inhibiting secretion of enzyme from the REP (Figure 40, Model 3). This indicates that 1) both pools share a common transport system to move the enzyme from cell to duct, 2) sites on the transport mechanism are limited or saturable, 3) MCh stimulation initially makes a large amount of enzyme from the SEP available to "mix" with enzyme from REP and compete with it for sites on the transport mechanism, 4) 5 to 10 minutes later in time, the capacity of the transport mechanism itself is greatly augmented.

A model consistent with these observations is provided by the equilibrium hypothesis. It suggests that the immediate action of MCh is to increase the permeability of the storage granule membrane to digestive enzyme, thereby greatly augmenting the rate of release of the older,

stored enzyme from granule to cytosol. Inhibition of labeled enzyme secretion would occur as cold enzyme floods the "hot" enzyme in the cytosol, mixing with it and competing with it for access to the limited apical transport capacity. Later in time, the secretagogue would act at the level of the plasma membrane greatly, increasing its permeability to digestive enzyme (Figure 41).

The MCh data can also be explained by amending the traditional cisternal packaging-exocytosis model. It would require that we assume the existence of two types of mature granules in the acinar cell. Granules comprising the SEP would have a relatively low affinity for the fusion sites and therefore would turn over slowly, while REP granules would have a higher affinity for the exit sites and therefore would turn over more rapidly. Initially, MCh would be required to increase the affinity of SEP granules in some manner, so that they now would saturate the fusion sites and inhibit the secretion of granules from the REP. 5 to 10 minutes later in time, the secretagogue would have to greatly augment the number of fusion sites. Though this modified model cannot be excluded, there is no evidence to suggest that two pools of granules with these characteristics exist in the acinar cell.

In contrast, the existence of two different intracellular pools of digestive enzyme, one the zymogen granule pool and the other the cytoplasmic pool, is a basic premise of the equilibrium model. There is substantial evidence in support of the view that digestive enzyme exists free in the cytoplasm of the acinar cell and that the cytoplasmic pool is the precursor pool to secretion (see Introduction). Therefore, in contrast to the traditional model, the equilibrium model can explain the present observations in a simple and concise manner, without any additional

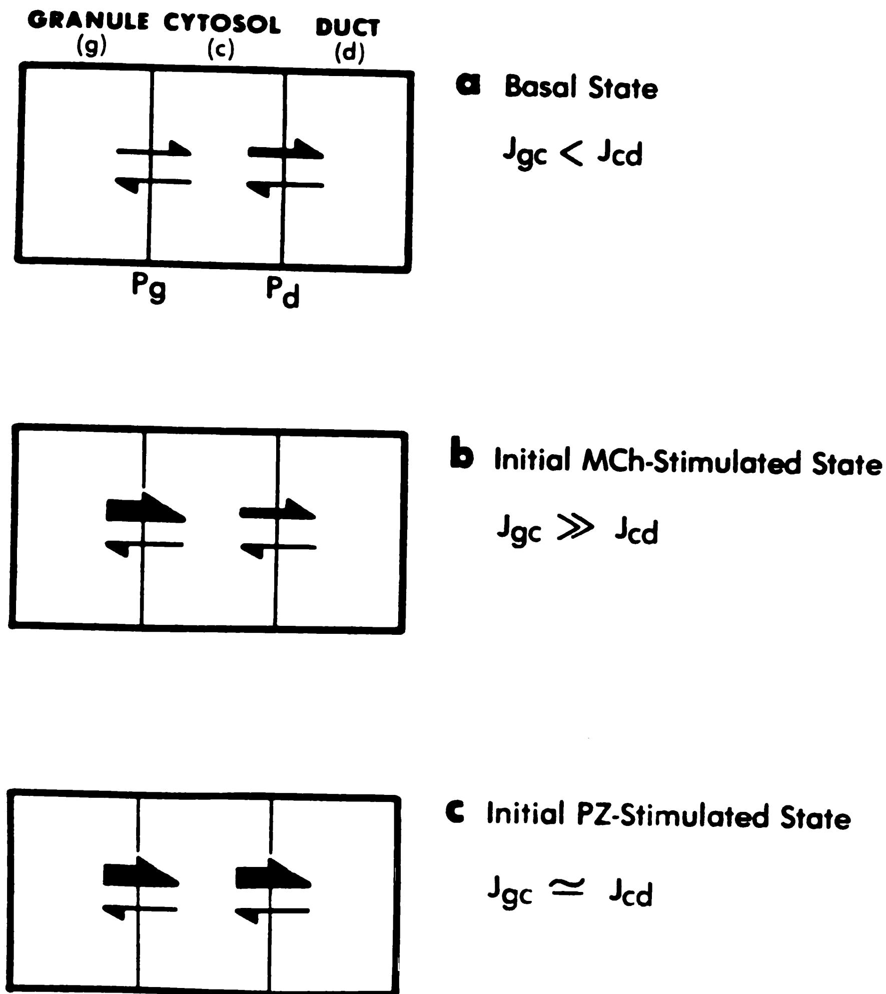

Figure 41. Digestive enzyme fluxes from granules to cytosol and from cytosol to duct, as proposed by the equilibrium theory for the basal state and the initial response to acetyl-β-methylcholine chloride (MCh) and cholecystokinin-pancreozymin (PZ). With MCh stimulation, large scale release of enzyme from granule to cytosol precedes the increase in apical membrane permeability. With PZ, in contrast, apical membrane permeability and zymogen granule permeability increase synchronously.

postulates. Furthermore, evidence supporting the view that MCh first increases the permeability of the zymogen granule membrane, thereby increasing the cytoplasmic concentration of the enzyme prior to augmenting the enzyme flux from cell to duct, has been presented by Ho and Rothman (1979). Their data is consistent with the idea that MCh stimulation initially increases the number of molecules in the cytoplasm of the acinar cell.

Ho and Rothman observed that when MCh is administered, a marked but transient depression occurs in the volume of fluid secreted into the duct. The flow inhibition is maximum at about 3.5 minutes poststimulus, and represents, on the average, 21% of the prestimulus flow. The magnitude of the flow depression for each experiment related to peak MCh output in a highly linear manner, suggesting an intracellular osmotic force. Release of enzyme from granules to the cytosol prior to the increase in apical plasma membrane permeability would increase the osmolarity of the cytoplasm. This could either prevent fluid efflux from the cell, or, alternatively, cause an influx of fluid from the duct. Ho and Rothman have calculated that the number of particles released with MCh stimulation would indeed provide a sufficient osmotic force to account for the magnitude of the observed flow reduction.

If the flow depression is due to an osmotic event, then, changing the osmolarity of the cell should alter the magnitude of the effect, Ho and Rothman reasoned. This phenomenon was observed. When the cellular environment was made hyperosmotic prior to stimulation, MCh produced a flow depression less than seen at normal osmolarily, and conversely, when the cellular environment was made hypo-osmotic, the same protein load released into the cytoplasm produced a larger flow depression than normal.

The gastrointestinal hormone CCK-PZ also causes a large increase in enzyme output which is accompanied by a large decrease in SRA (dpm/unit protein). However, CCK-PZ, in contrast to MCh, causes neither a delay in the output response, nor an inhibition in the secretion of newly labeled enzyme. In terms of the equilibrium model this suggests either that the direct effect of the hormone occurs only at the level of the plasma membrane or, alternatively, that both the storage to cytosol flux and cytosol to duct flux are stimulated simultaneously (Figure 41). Therefore, we would predict that no cytosolic protein build-up should occur with CCK-PZ stimulation.

Indeed no flow depression was observed by Ho and Rothman when the _in vitro_ pancreas was stimulated with CCK-PZ. Moreover, when MCh and CCK-PZ were given together, the flow depression in the first six minutes was somewhat less than when MCh was administered by itself. This reduction in flow depression with CCK-PZ is consistent with the view that the hormone initially enhances the permeability of the apical membrane, thereby preventing the build-up of a cell to duct protein gradient as large as would occur with MCh alone. These flow studies offer strong support for the equilibrium hypothesis, as well as the model it provides to explain the difference in response of the system to the two secretagogues.

II. Basolateral Flux of Amylase

The view that release of amylase into the bathing medium results from a natural permeability of the basolateral membrane of the acinar cell to this molecule is suggested by the following points taken together: i) the cell to bath flux was exponential in nature, suggesting the equilibriation of two fluxes, one from cell to bath and one from bath to cell;

ii) initial rate kinetic reoccurred each time the bath was charged; iii) the time course of equilibration was slow relative to a simple diffusional process; iv) the substantial magnitude of the unidirectional flux into the medium (in these experiments, it was 3 times greater than the concomitant net flux across the apical surface of the cell in the unstimulated condition, or about 75% of the initial total secretory flux); v) the existence of an amylase concentration gradient of substantial magnitude between ductal fluid and bathing medium at the steady-state; vi) the observation that while both acetyl-β-methylcholine (MCh) and cholecystokinin-pancreozymin (CCK-PZ) stimulate ductal secretion, only MCh, but not CCK-PZ increases the amylase flux into the bath; and vii) the large increase in the cell-to-bath flux produced by the cholinergic agent, some 14 times the control rate on the average.

Before we can conclude that such a natural permeability exists, however, there are two alternative explanations for the amylase flux into the bath that must be considered: amylase release into the medium as a result of cellular breakdown or damage, and a shunt flux from the duct system into the bath. Cellular breakdown or damage cannot account for the amylase in the bath. Amylase that might have been released as a result of surgical trauma or related events was removed by a 60 minute incubation prior to the initiation of the experiment. Beyond that, if substantial amounts of amylase were derived from the breakdown of cells during the experiment, then its appearance in the bath should have increased gradually, rather than being maximal initially and decreasing towards zero with time as was observed. Furthermore, the release of lactate dehydrogenase, a presumably nontransported cytoplasmic protein and a commonly used marker for cellular breakdown, did not show the same

pattern of release as amylase. Finally, after several hours *in vitro* and after the steady-state concentration of amylase had been attained in the medium, the system was still responsive to a cholinergic agonist that magnitudinally increased the flux of amylase into the bath. This large increase could not have been the result of a stimulus-induced increase in amylase synthesis from cells compromised in some way to produce an "unnatural" amylase release into the bath, since cholinergic agents do not increase protein synthesis by pancreatic tissue *in vitro* to any substantial degree (Jamieson and Palade, 1971, Morisset and Webster, 1971).

If bath amylase, on the other hand, were derived from duct contents via an irreversible shunt, that is, flow through torn ducts, etc., then the amount of amylase in the bath should have increased linearly over time as did ductal secretion. This did not occur; rather, amylase in the bath approached a steady-state value with time. Alternatively, a shunt flux from duct to bath might be equilibrating, for example, a diffusional intercellular pathway from the lumen. In this case, the amylase concentration in the medium at its steady-state should equal ductal concentration. This also was not seen; the concentration of amylase in the bath was always less than in the duct at the steady-state and by at least an order of magnitude (Table X). Finally, and perhaps most forcefully, if bath amylase were derived directly from ductal contents, then CCK-PZ, a potent secretagogue, should have increased the amount of amylase in the bath greatly [either proportional to the increase in ductal secretion (output) for an irreversible leak or proportional to the increase in ductal concentration for an equilibrating system]. Such an increase did not occur although CCK-PZ greatly increased ductal secretion (approximately 12-fold for the peak response).

Similar results are reported by Kanno (1972), and Saito and Kanno (1973). They demonstrate an amylase flux into the bathing medium in perfused cat pancreas in the basal state. They find, as have we, that CCK-PZ in low concentrations (0.02 to 0.2 mg/ml) increases the apical amylase flux without affecting the magnitude of the amylase flux into the perfusing medium. Surprisingly enough, they also find that at much higher concentrations of the hormone (0.5 to 1.5 mg/ml), the situation is reversed with the basolateral secretion of amylase greatly enhanced while apical secretion of the enzyme remained close to its basal rate.

Moreover, they show data from a single experiment in which a high dose of CCK-PZ led to a large increase in amylase output into the perfusing medium. It was accompanied by a substantial hyperpolarization of the acinar cells, as well as an increase in membrane resistance. They show data from another experiment at the same dose, in which no increase in blood amylase occurred and in which only minor increases in acinar cell hyperpolarization and membrane resistance were evident. Kanno hypothesizes that the increase in membrane resistance that can occur at high CCK-PZ doses, results from an uncoupling of adjoining acinar cells. This leads to a widening of intracellular channels which shunt the enzyme from duct to bath.

He concludes that <u>all</u> amylase found in the perfusing medium moves via a shunt pathway from duct to bath. His conclusion is not justified by the data, however. If the amylase flux into the bathing medium in the unstimulated state, were derived from a shunt flux from duct to cell, the increase in ductal output of amylase at low CCK-PZ concentrations should be accompanied by a proportional increase in basolateral output. However, basolateral amylase output was unchanged, though the apical

amylase output increased as much at 18-fold. The data, therefore, instead supports our view of a natural permeability of the basolateral membrane to amylase in the unstimulated state.

The finding that at high CCK-PZ concentrations little or no change occurs in ductal amylase secretion, while a large increase is evident in the basolateral output, could result, as Kanno hypothesizes, from the opening of intracellular channels. Much firmer evidence, however, is needed to support this hypothesis. For example, if intracellular channels were opened at high CCK-PZ concentrations, so as to divert most enzyme from duct to bath, a major part of fluid flow should also be diverted via these same channels into bath. If Kanno is correct, therefore, ductal fluid secretion should be severely depressed when the basolateral output of amylase is augmented. Meanwhile, the alternative explanation, that high concentrations of CCK-PZ selectively stimulate the natural mechanism present in the acinar cell for transporting amylase from cell to bath, cannot be excluded.

Since there are substantial amounts of pancreatic digestive enzyme in normal blood, it certainly is not surprising that the basolateral membrane is permeable to these proteins. However, in the absence of pathological conditions, it has often been assumed that this blood-facing membrane is impermeable to digestive enzymes, in accordance with the idea that digestive enzyme can only leave the acinar cell by the exocytosis of zymogen granule contents at the apical surface. Moreover, the permeability of the basolateral membrane described here is consistent with the fact that the pancreatic amylase content of blood is not static, but is cleared and replenished with a half-time of about 3 hours (Ryan, 1975). Reinforcing this idea, the average steady-state concentration of amylase in the bathing medium for the present experiments was roughly

similar to that found in porcine blood (Ryan, 1975). [approximately 1.7 μg/ml of bath fluid (as determined by enzyme activity), as compared to approximately 1.4 μg/ml of plasma in porcine blood (as determined by radioimmunoassay)].

Since bidirectional fluxes appear to exist across both mucosal (Liebow, 1976) and serosal surfaces of the acinar cell, the use of tissue slice, minced tissue, or isolated cell preparations to measure secretion must be re-evaluated. Such techniques generally assume that only one flux exists (cell-to-duct across the apical membrane), and therefore consider the exposure of the basal surface of the cell to the medium as irrelevant to the measurements. However, the release of enzyme into the fluid that bathes such preparations can be a composite of as many as four interactive fluxes, depending upon the specific experimental conditions, and cannot be assumed equivalent to measuring secretion collected from the secretory duct alone.

Also, if we are dealing with equilibrating systems, the output of enzyme from tissue into bathing medium will be determined by the volume of that medium; the larger the volume per tissue mass, the larger the overall output. Therefore, reports that it is only possible to get fractional release of previously labeled protein from tissue slices (Heisler, et al., 1972) need not reflect an intrinsic quality of the secretory process, but could simply be the result of a steady-state equilibration in a limited volume. In addition, when enzyme release into the medium decreases with time, or even stops, it cannot necessarily be concluded that the tissue is dying or dead, because such behavior can reflect the equilibration of the system as well. The expectation that secretion should be linear in these nonpolarized systems has led

investigators to reasonably explain observed curvilinear functions as being made up of sequential linear functions [e.g., attributable to washout of enzyme already in the duct system, true secretion, and death (Jamieson, 1971)]. In an equilibrating system, linear secretory rates can only be expected at the initial rate of secretion, that is, before any back flux develops.

III. Temperature Dependence of Apical and Basolateral Secretion

The V-shaped relationship of both the basolateral and the apical secretory flux to temperature, with high secretion rates occurring at both high and low temperatures, was an unexpected finding. In a preliminary experiment testing the rate of basolateral secretion in the 20° to 38° range, we found that amylase release was higher at 20° than at 25°. Since the secretion rate increased steadily from 25° to 38°, it seemed unlikely that this anomalous point was in error; we therefore extended the range downward to 5° and indeed did find that the rate of amylase secretion also increased as the temperature of the gland was lowered from 20° to 5°.

Within the framework of the equilibrium theory, release of stored enzyme into secretion occurs in two stages; the first, transport across the zymogen granule membrane into the cytosol and the second, transport across the plasma membrane into the duct system or extracellular environment. Therefore the net flux of enzyme across the plasma membrane, the secretory flux, can be changed either by an alteration in the digestive enzyme concentrations of the precursor pool (to secretion), or by an alteration in the permeability of the plasma membrane itself to digestive enzyme.

Low Temperature Range

We shall focus first on the low temperature range. Lowering the temperature of the gland from 20° to 5° could increase the permeability of both the zymogen granule membrane and the plasma membrane, of only the zymogen granule membrane, or alternatively of only the plasma membrane. If the change in temperature increased the permeability of both the plasma and the zymogen granule membrane to digestive enzyme (and to about the same extent), then the secretory flux would increase while the concentration of enzyme in the cytosol remained constant. If the change in temperature increased only the permeability of the zymogen granule membrane to digestive enzyme (or increased the permeability of the zymogen granule membrane to a significantly greater extent than it did the plasma membrane), then the concentration of enzyme in the cytosol, the precursor pool to secretion, would increase, and this, via mass action, would increase the net flux of enzyme across the plasma membrane. Finally, if the decrease in temperature increased only the permeability of the apical membrane to digestive enzyme, then the secretion of amylase would increase while the concentration of enzyme in the cytosol either decrease or remained constant.

In an attempt to distinguish between some of these possibilities, we compared the amount of amylase in the zymogen granules (Zg) to the amount of amylase in the post-microsomal supernatant (PMS) in tissue incubated and fractionated at 5° and at 20°. We chose to look at the Zg/PMS partitioning of amylase at these temperatures because 5° is the temperature at which the secretory flux is at its maximum in the low temperature range, while 20° is close to the inflection point where secretion across the plasma membrane is at its lowest point for all

temperatures. If the permeability of the zymogen granule membrane to amylase is increased by lowering the temperature from 20° to 5°, then we would expect the ratio of amylase Zg/amylase PMS to be lower at 5° than at 20°, reflecting increased transfer from storage to the PMS at the lower temperature. This would indicate that in the intact tissue either the permeability of the zymogen granule membrane alone or of both zymogen granule and the plasma membrane were increased below approximately 20°.

Just the reverse was observed. Release of amylase from zymogen granules into the PMS was lower at the lower temperature. Since a relative decrease rather than an increase in the amylase concentration in the PMS was actually observed at low temperature, an increase in the precursor pool concentration clearly cannot account for the augmented secretion seen in intact tissue. Therefore, the permeability of the plasma membrane must increase with very low temperature. Furthermore, since we have shown that zymogen granule permeability does not increase at very low temperature, the increased secretory flux must result from the temperature dependent permeability characteristics of the plasma membrane alone.

<u>High Temperature Range</u>

Liebow and Rothman (1976) found that in the 20° to 30° range the rate of release of amylase from isolated zymogen granules increased with increasing temperature. Therefore, the increase in the rate of amylase secretion seen in the intact tissue as the temperature was raised from 20° to 38° might involve an increase in the cytoplasmic concentration of the enzyme. However, the permeability of the plasma membrane to amylase may also be increasing with increasing temperature in this range, though

currently we cannot determine if this is true. If plasma membrane permeability did increase and this increase were equal to or greater than the increase in zymogen granule permeability, no change or even a decrease would actually occur in precursor pool concentration of enzyme.

Mechanism

An increase in the permeability of the plasma membrane to amylase could reflect either a change in the access of enzyme to transporting sites (that is a change in the affinity of the system for amylase) or a change in the time that it takes for molecules to move through the membrane (a change in apparent V max). A third possibility is that the number of "transport sites" could vary in some manner with temperature (cracks, for example, could develop in the membrane as the temperature was lowered). At present, however, it is only possible to speculate in a general sort of way about the nature of the changes that may occur in the acinar cell plasma membrane as temperature is varied.

With cooling, lipids do become less fluid and more ordered. Adam (1941) demonstrated that monolayer films of lipids contract on cooling within a certain temperature range which is specific for each lipid. Similarly, using electron diffraction techniques, Engelman (1970) demonstrated that as the membrane of *Mycoplasma laidlawii* passes through the temperature range in which it undergoes phase transition, its lipids go from a fluid state to a highly ordered one in which they assume a hexagonal, closest packing configuration. The phase transition temperature of membranes in mammalian systems often does occur around 20°, and it is possible that the increase in membrane permeability that occurs in the *in vitro* pancreas when the temperature is lowered from 20° to 5° results from a decrease in lipid fluidity and lipid surface area which leaves other transport related molecules within the membrane more exposed.

There are other reports in the literature of an increase in membrane permeability with low temperature. Ring (1965), for example, showed that release of α-amino isobutyric acid from pre-loaded Streptomyces hydrogenans increased as the temperature was lowered below a critical point. He finds that this critical point is lower than normal in organisms grown in the cold and higher than normal in organisms grown at high temperatures. He also shows that the influx of ^{14}C thiourea was considerably higher at 0° than at 30°. He suggests that the increased permeability of the plasma membrane to these substances reflects a widening of membrane pores, due to a "crystallization" or "precipitation" of the lipids as the temperature is lowered through the membrane phase transition, and points out that changes in the phase transition temperature represent adaptive changes in the lipid composition of its membrane by the microorganism to temperature.

The Constancy of Enzyme Concentration

The relative constancy of the concentration of amylase in apical secretion from 20° to 38° could indicate that enzyme secretion and fluid secretion are linked processes. It is also possible, however, that the two are separate processes and only by chance have similar temperature dependencies in this range. In support of this latter view is the dramatic change in enzyme concentration that occurs at lower temperatures. Further support for this view, that the two processes are separate, comes from the fact that agents such as CCK-PZ and MCh can alter the amount of enzyme in secretion independent of the amount of fluid secretion, while the hormone secretin can alter the amount of fluid secretion independent of enzyme output.

On the other hand, constancy in enzyme concentration is observed when ductal secretion is either partially or totally blocked (Section V). Similarly, data by Ho and Rothman (1979) suggest that when flow is reduced in a stimulated gland by either ouabain or low Na^+, a parallel reduction in enzyme output occurs with concentration remaining constant. These observations require that _within a given state_, the secretion of fluid and the secretion of enzyme are related processes, linked by concentration-dependent bidirectional fluxes of enzyme. Within this framework, the constancy of enzyme concentration from 20° to 38° provides a further instance of the linkage of enzyme and fluid secretion by concentration-dependent fluxes. In this light, the permeability change that occurs in the plasma membrane below the inflection point in the current experiments, can be likened to a change in state, and indeed may well reflect a phase transition of the membrane lipids.

Absence of Enzyme Pumping at Apical Surface

The constancy of enzyme concentration in ductal secretion as the temperature is lowered to 20° illuminates an additional aspect of digestive enzyme secretion. If transport of amylase across the apical membrane were an energetically up hill process, we would expect it to show a very strong temperature dependence in this range. That is, if the ductal concentration of amylase were maintained higher than the cytoplasmic concentration of the enzyme, then as the temperature of the gland decreased between 38° and 20°, the concentration difference that the cell was able to maintain across the apical surface should also decrease, and the concentration of enzyme in the duct should fall. Since enzyme concentration remained constant in this temperature range, it seems unlikely that an up hill step does occur at this surface.

Apparently therefore, the concentration of enzyme in the duct is either the same or lower than the concentration of enzyme in the cytoplasm of the cell. (If fluid flow maintains enzyme concentration at or close to its initial rate, then the enzyme concentration in the duct will be lower than the enzyme concentration in the cell--see Section V).

Data presented in Section IV of this study, as well as data by Liebow and Rothman (1975) suggest that transpancreatic transport of digestive enzyme involves an active step, but does not indicate whether the up hill step occurs at either the basolateral or apical membrane. By elimination then, the current data suggests that an active step occurs from blood to cell at the basolateral surface.

IV. Transpancreatic Transport of Amylase

Amylase added to the medium bathing the rabbit pancreas _in vitro_ produced a dramatic increase in amylase secretion, and likewise, the addition of chymotrypsinogen to the medium increased chymotrypsinogen secretion. These responses are consistent with the hypothesis that mass action can drive digestive enzyme secretion, as well as the prediction of the enteropancreatic circulation hypothesis of a substantial transpancreatic transport capability.

To determine if it was the enzyme added to the medium that was recovered in secretion, we incorporated labeled amino acids into endogenous amylase pools for 4 hours prior to the addition of exogenous amylase. We reasoned that if the augmented amylase secretion were of exogenous origin, then the specific radioactivity of secretion should decrease substantially and remain suppressed as long as the exogenous enzyme was in the bath. This was observed; the specific radioactivity of amylase in secretion was reduced by about 90% at the apparent steady-state.

If the transport of enzyme across the apical membrane is due to a specific saturable process, and if exogenous and endogenous enzyme mix in the cytoplasm, as the equilibrium hypothesis proposes, then under certain circumstances, it should be possible to observe competitive transport inhibition between these two pools of enzyme. That is, the addition of a substantial amount of exogenous enzyme should inhibit the secretion of endogenous enzyme, if both exogenous and endogenous material are transported into the duct by a common mechanism and if the enzyme is present in the pre-secretory pool (cytoplasm) at concentrations which saturate or nearly saturate the transport system. The present observations are consistent with this hypothesis. The addition of exogenous amylase produced a sustained depression in the absolute secretion of labeled enzyme (by 72% on the average), while total amylase secretion into the duct was increased by about an order of magnitude. Furthermore, as we would expect if endogenous and exogenous amylase compete for exit sites, the inhibition of endogenous amylase secretion was not seen when relatively low concentrations of exogenous amylase were added, even though there was a substantial response in terms of amylase output (Table IX). The presence of a saturable transport mechanism is also shown by the observation that varying the concentration of amylase in the medium did not produce a linear change in transpancreatic transport. That is, at the highest concentration of bath amylase, amylase output was only about 1/3 of that predicted if bath concentration and secretory flux were linearly related (Table VIII). These results are consistent with earlier experiments in which enzyme derived from different intracellular pools appeared to compete for exit in response to the addition of a cholinergic drug (Part I).

The Transient Increase in the Secretion of Endogenous Amylase

The apparent paradox of a transient enhancement in the secretion of endogenous enzyme immediately after the addition of the exogenous material can be explained by the equilibrium model. When unlabeled enzyme is added to the bath and enters the cell, the specific radioactivity of cytoplasmic amylase is proportionately reduced. If the enzyme simply passed through the cytoplasm and did not equilibrate with other storage pools, then the secretion of endogenous enzyme should have either been unaltered (for a process well below its V_{max}), or inhibited (if the transport maximum is approached). It should not increase. If, however, amylase in the cytoplasm equilibrates with enzyme in other storage pools, such as the zymogen granules, then a transient increase or an apparent delayed onset of inhibition should be seen. In this case, the addition of unlabeled enzyme to the cytoplasm would introduce an initial disequilibration between the specific radioactivities of enzyme in different intracellular pools, viz., the specific radioactivity would be less in the cytoplasm to which unlabeled enzyme had been added. Re-equilibration initially would result in a net flux of labeled amylase into the cytoplasm, since influx from other pools into the cytosol would contain relatively substantial amounts of labeled (endogenous) enzyme while efflux into other pools from the cytosol would consist predominantly of unlabeled (exogenous) enzyme. (This is an example of counter-flow.) The result would be to increase the absolute amount of labeled amylase in the cytoplasm, which would in turn increase the flux of labeled amylase into the duct until either total re-equilibration occurred or the increase in the amount of label in the cytoplasm was offset in secretion by competitive inhibition between labeled and unlabeled species for exit from the cell. If in the steady-state a disproportionate amount of

the exogenous enzyme remained in the cytoplasm relative to other storage pools, then there would also be a steady-state increase in the amount of labeled enzyme in the cytoplasm.

The Transient Nature of the Overall Response

Since the overall secretory response was not maintained at maximal levels but decreased over time, despite the continued presence of enzyme in the medium at a constant concentration, the increased net secretory flux must also be a transient, rather than a steady-state phenomenon. The transient nature of the overall response may be the result of a transport-related metabolic substrate becoming depleted over time of a regulatory adaptation of some sort, but it also can be explained in terms of equilibrating fluxes. When exogenous enzyme is added to the medium, the unidirectional influx from bath to cell is increased via mass action, as is subsequently the unidirectional efflux from the cell to duct. At early times this would result in an increase in the net secretory flux or output of enzyme from the duct system. A back-flux from duct to cell could develop over time, however, reducing the net flux across the apical membrane back toward control rates. Efflux from cell to duct would still occur, and indeed might remain maximal due to the continued presence of exogenous enzyme, even though the net flux had returned to control levels. This interpretation is consistent with the observation that the inhibition of labeled amylase secretion was maintained over time despite the declining overall secretory output (Figures 22 and 30), which would be the case if the efflux remained maximal despite a decreased net flux.

<u>The Response of Secondary Molecules</u>

The observation that the addition of one enzyme to the bathing medium stimulated the secretion of other enzymes cannot wholly be accounted for by contamination of the added enzyme with other enzyme species. Relatively pure amylase and chymotrypsinogen both elicited secretory responses for the other enzyme roughly equivalent in time-course and magnitude to that of their own transpancreatic transport (Figure 23). Indeed, the chymotrypsinogen response to the addition of amylase was much greater and occurred more rapidly than the chymotrypsinogen response to the addition of chymotrypsinogen itself. Thus, there appears to be a kind of entrainment in the responses for the two enzymes which is not predicted by the equilibrium hypothesis in its simplest form, i.e., as in diffusion, the transport of different molecular species should segregate independently. On the other hand, this entrainment does not appear to be the result of the linked transport of the enzymes. To the contrary, the addition of amylase decreased the covariance or relatedness of their secretion. Thus, the augmented secretion of secondary endogenous molecules appears to be an independent event, stimulated in some manner by the uptake and secretion of the exogenous enzyme. Understanding why and how this occurs may bring us closer to understanding the secretory mechanism.

V. Secretion Blockage

Traditionally, fluid secretion and enzyme secretion by the pancreas have been viewed as independent phenomena. This bias apparently developed because enzyme secretion can be stimulated by secretagogues without stimulation of fluid secretion, and conversely, fluid secretion can be

stimulated without a parallel stimulation of enzyme secretion. In these experiments evidence is provided that in the basal state, fluid secretion and enzyme secretion are in fact closely coupled phenomena. The traditional viewpoint is consistent with the exocytosis model, while the idea that the two processes are interrelated is consistent with the concentration-sensitive equilibrium model.

Fluid secretion was either artificially stopped by preventing outflow from the duct system, or reduced by applying a back-pressure to the column of fluid in the duct. The bidirectional, concentration-dependent equilibrium model predicts that when fluid flow is restricted, the net flux of enzyme into the lumen should stop as the concentration of enzyme in the duct system reaches its equilibrium value, that is, when the flux from cell to duct equals the flux from duct to cell. The unidirectional, cisternal packaging-exocytosis model, in contrast, predicts that secretion would continue unabated during blockage or flow reduction, since the rate of protein secretion would be determined intracellularly by the rate at which secretion granules fuse with plasma membrane, and would therefore be independent of fluid flow.

The results demonstrate that the outflow of water from the duct system is a critical determinant of the net flux of digestive enzyme across the apical plasma membrane of the secretory (acinar) cell. That is, the net transfer of enzyme across this membrane ceases when the "excretion" of water ceases, and is reduced proportionately when flow is reduced in a graded fashion. Such behavior suggests that the net flux of enzyme across the membrane, i.e., its "secretion", is dependent upon the concentration of enzyme in the duct. The simplest explanation for this behavior is that the flux of protein across the membrane is

bidirectional and depends upon transmembrane concentration gradients as proposed by the equilibrium theory. In contrast, the prediction of the exocytosis hypothesis that the concentration of enzyme would greatly increase in ductal fluid during blockage was not borne out by the evidence.

We cannot exclude the possibility that an exocytosis-like release process is responsible for secretion in the pancreas, however the data cannot readily be explained by the traditional hypothesis. Moreover, even if the model is amended to include "bidirectional" exocytosis or exocytosis linked to endocytosis, the observations still cannot be explained in any simple manner by such a construct. For example, in a bidirectional exocytosis model, the reduced output would presumably result from increased "re-uptake" of enzyme. However, increased re-uptake would have to be regulated relative to flow in order to maintain the concentration of enzyme in the duct system constant over a wide range of flow and during total blockage, i.e., it would have to increase in exact proportion to the reduction in flow. Furthermore, in order to explain the fact that the concentration of enzyme in the duct increases and decreases independently of flow under stimulated conditions, we would have to further hypothesize that the regulated re-uptake could itself be regulated, i.e., the relationship between protein efflux from the cell and its re-uptake could be varied independently of flow.

Other amendments to the model no doubt can and will be proposed by those committed to the exocytosis view. In contrast however, the observations can be explained in a simple manner with no additional assumptions by a diffusion-like secretory process. By the law of parsimony then, we feel that the equilibrium model is the hypothesis that best explains the observations.

Ho and Rothman (Rothman, 1979), using the same biological preparation, have shown a similar constancy in the enzyme concentration of secretion in the stimulated state in the face of decreasing fluid secretion produced by either ouabain or a low Na^+ medium. Therefore, a reduction in water and salt secretion within a given state by whatever means, apparently produces proportional decreases in enzyme secretion. Another example of this phenomenon was demonstrated in Section III. The concentration of amylase in ductal secretion remained constant as parallel changes in fluid secretion and enzyme secretion occurred when the temperature of the bathing medium of the <u>in vitro</u> pancreas was raised in steps from 20° to 38°. The link between fluid secretion and enzyme secretion in all these conditions can only reasonably be explained by the existence of bidirectional concentration-dependent fluxes of enzyme across the plasma membrane.

Enzyme concentration in the duct system remained remarkably constant during both total blockage and reduced outflow conditions. This means that enzyme fluxes across the membrane in the free-flow situation must have maintained the enzyme concentration in the duct system at, or close to its maximum value for the gland's basal or unstimulated state. Thus, either digestive enzymes are in equilibrium across the apical membrane (that is, the concentration of free enzyme in the cytoplasm and the duct lumen are roughly the same), or a steady-state concentration gradient is maintained between cell and duct (presumably by an energy dependent efflux process). Data from Section III showing a similar temperature dependency for amylase output and fluid secretion from 20° to 38° suggests that the enzyme is indeed in equilibrium across the luminal membrane. In both cases, the net secretory flux would be produced by the removal of

fluid from the secretory site. A possible schema for the sequence of events is as follows: active ion transport occurs with water moving as a consequence of an osmotic force; this establishes a concentration gradient favoring the net efflux of enzyme from cell to duct.

Initially our working model for the system envisaged that fluid flow, by removing enzyme from the immediate vicinity of the luminal membrane, kept enzyme secretion close to, or at its initial rate. The current experiments suggest that this is not the case. To maintain an essentially constant concentration in the duct lumen, the equilibrium of enzyme across the membrane must be more rapid than its removal by fluid flow. This model suggests therefore that fluid flow must be the rate limiting step and not enzyme secretion, as envisaged in the "initial rate" model.

These experiments also bear upon another aspect of pancreatic secretion. It is believed by some investigators that cells lining the ducts, as opposed to cells in or near the acinus, are responsible for the addition of a substantial proportion of the secreted fluid (Schulz et al., 1964; Mangos and McSherry, 1977). If this were true, then the acinar secretion of digestive enzyme would be diluted by fluid as it traveled down the ducts, and during a flow reduction produced by blockage or back-pressure, the concentration of enzyme throughout the duct system would increase over time toward that at the site of enzyme secretion. Since the concentration of enzyme did not increase during 90 minutes of blockage, it can be concluded that an admixture of a significant amount of fluid by cells lining the ducts during free-flow does not seem to occur.

Conclusion

Taken together, these observations provide provocative evidence in support of the equilibrium model for digestive enzyme secretion and transport in the pancreas. Certainly, individual findings can also be explained by some amended version of the traditional model, however, the data taken as a whole is explained most simply and most completely by the equilibrium model.

In addition, these observations help describe the acinar cell in more detail. They suggest that the concentration of enzyme in the duct system is close to its equilibrium value. They also suggest that active uphill pumping of enzyme does not occur across the ductal membrane. Therefore, in the basal state, the concentration of enzyme in secretion should about equal the concentration or enzyme in the cytoplasm, and the active step, indicated by the transpancreatic transport study will occur at the basolateral surface. Active pumping of enzyme from bath to cell will then maintain the steady-state concentration of enzyme in the basolateral compartment lower than in the other two compartments.

APPENDIX I

Rothman has found that blockage in the in situ pancreas produces an effect similar to that reported here for the in vitro pancreas. The pancreatic duct of anesthetized New Zealand white rabbits was cannulated. After a short period during which flow stabilized, the cannula was fused closed to block the outflow of fluid from the duct system. This blockage was maintained for 30 minutes. If the secretion of digestive enzyme from cell to duct continued unabated in the absence of fluid excretion, the concentration of amylase in the duct system should rise during blockage to about 3-fold that found for the 10 minute prior control. Instead, amylase concentration remained constant or decreased slightly during blockage in all the animals tested (Table XI). The mean amylase concentration was 71% that of the preblockage 10 minute control. This decrease in amylase concentration is explained by the fact that amylase concentration was falling gradually over time prior to blockage and at about the same rate as that observed during the blockade.

Since the duct system may have a considerable capacitance, it is possible that secretion could continue unabated during blockage without any evident increase in the concentration of amylase in secretion. If secretion of amylase were continuous during blockage, then in the 10 minute collection period post blockage, amylase output should be about 3.6x that of the amylase output for the 10 minutes prior control. (This estimate takes into account the gradual decline of amylase concentration over time in situ.) Amylase output in the 10 minutes collection period following blockage was only 1.37x that of the prior control period (Table XII), or about 37% of that expected for continuous secretion. Apparently, therefore, though some ductal capacity does exist, a ductal

capacitance cannot account for the expected amylase output if secretion had continued unabated during blockage. The data does support the *in vitro* evidence that blockade inhibits enzyme secretion, however the effect could be better shown with a blockage of longer duration.

Table XI

The Concentration of Amylase Post-blockage as Percent of Amylase Concentration Found During 10 Minute Control Period Prior to Blockage. If Amylase Secretion were Continuous During Blockage, the Amylase Concentration in Secretion Should Reach about 300% of its Control Value.

Animal #	Post-blockage Amylase Concentration as Percent of Pre-blockage Control
1	78
2	52
3	105
4	48
	$\bar{n}$ = 71%

Table XII

Amylase Output in 10 Minute Post-blockage Collection Period as Percentage of Amylase Output During 10 Minute Prior Control Period. If Amylase Secretion had Continued Unabated During Blockage, Amylase Output would have been about 360% of Prior Control. The Ratio of Observed Output to Predicted Output was 136/360 or 37%. (Predicted Output Takes into Account Gradual Decline of Output over Time.)

Animal #	Amylase Output in 10 Minute Post-blockage Collection Period as Percentage of 10 Minute Prior Control Output
1	100
2	227
3	66
4	152
	$\bar{n}$ = 136

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