Protein Transport by the Pancreas

The current paradigm is analyzed and an alternative hypothesis is proposed.

S. S. Rothman

"By 1960, the story was quite clear: the digestive enzymes of the pancreas are synthesized in or on the ribonucleoprotein particles (ribosomes) and then sequestered in the cisternae (internal spaces) of the endoplasmic reticulum for transport to storage sites preparatory to secretion," writes Keith Porter about the secretion of digestive enzymes by the pancreas in his scientific biography of George Palade in *Science* soon after Palade was awarded the Nobel prize in part for his studies of this process (*1*). Porter continues, the evidence "left no doubt as to the [means of] ... movement of pancreatic enzymes." These comments and similar ones indicate the general acceptance that the theory proposed by Palade and his colleagues some 15 years ago presently enjoys. The experiments from which the theory was adduced are classics in biology and represent a pioneering amalgam of anatomical and biochemical experimental approaches; and the theory itself has been instrumental in the formulation of many similar hypotheses for a wide range of systems that secrete organic molecules. Notwithstanding the importance of the experiments and impact of the theory, the available evidence is only consistent with their hypothesis; it does not offer conclusive proof for the existence of such a mechanism, nor does it exclude alternative interpretations of the data. Moreover, there are a growing number of observations which do not fit the theory and thus suggest the need for alternative hypotheses that may be able to account for more of the available data.

This article, primarily a derivative of observations and discussion in my laboratory over the past 10 years, was written before the publication of Palade's Nobel lecture (*1a*) and hence without knowledge of its specific contents. In his lecture Palade essentially restated his well-known theory to explain how digestive enzymes are secreted by the pancreas, a theory which he feels "has stood well the test of time." My article addresses itself to this issue by examining the nature of the evidence for the current paradigm and introducing an alternative to this hypothesis.

Briefly, the current form of the theory proposes that new peptide chains grow on ribosomes attached to the membrane of the endoplasmic reticulum and elongate directly into its cisternal space through "pores" beneath the point of ribosomal attachment and are thus separated from the cytoplasm of the cell as they are synthesized. Subsequently, it is hypothesized, the completed secretory protein moves out of the endoplasmic reticulum in small smooth-surfaced vesicles formed as buds from the apical end of the reticular network. These vesicles are thought to travel into the Golgi region of the cell where they empty their contents into larger vesicles called condensing vacuoles or immature zymogen granules. Condensing vacuoles are thought to fill in this manner and become mature zymogen granules, the primary enzyme-containing granule of the pancreas. Finally, it is proposed that, after filling, each granule migrates to the apex of the cell where its contents are secreted by exocytosis. In exocytosis the membrane of the intracellular granule that contains the product to be secreted (a secretion granule) fuses with the cell membrane producing a hole in both membranes through which the secretory product leaves the cell (Fig. 1) (*2*).

Central tenents of the Cisternal Packaging–Exocytosis Theory

As is the case with all relatively complicated theories, it is helpful to try to identify the underlying elements of the cisternal packaging–exocytosis theory. These include the following:

1) *Sequestration.* All new digestive enzyme chains (i) are synthesized on ribosomes attached to the membranes of the endoplasmic reticulum, (ii) elongate directly into their cisternal spaces, and (iii) are released therein when the chain is completed.

2) *The continuous isolation of the secretory protein from the cytoplasm.* Having been sequestered, the new protein is kept exclusively within specialized intracellular membrane-bound compartments for the duration of its stay in the cell. Secretory proteins do not have access to the cytoplasm from these intracellular compartments.

3) *The fusion or fusion-fission of membranes.* Membrane-membrane interactions lead to the transfer of protein from compartment to compartment both within and out of the cell. If both hypotheses one and two are correct, so in all likelihood is this third hypothesis. How else could transfer occur?

4) *A series process.* As in an electrical series, the various intracellular compartments that contain digestive enzymes are in series with each other; that is, the secretory protein moves in sequence from ribosomes to cisternal space, to smooth-surfaced vesicles, to condensing vacuoles, to zymogen or secretion granules, and finally to the duct lumen outside of the cell.

5) *Mass transport.* The movement of enzyme within this system, either in the cell or across the cell membrane is an en masse process in which the variety of secreted proteins, probably between 20 to 30 different digestive enzyme species, are transported at essentially the same rate in constant proportion to each other. As a corollary, the specific molecular structure of a digestive enzyme is for all intents and purposes unrelated to its rate of secretion.

Assumptions

Before considering the experimental evidence, there are two assumptions underlying this theory that need discussion. Hypotheses one and two are often assumed to be correct by proponents of the theory. That is, digestive enzyme is assumed, for two reasons, to be sequestered and continuously separated from the cytoplasm. First, it is argued, if digestive enzyme entered the cytoplasm, then the cell would be vulnerable to hydrolytic attack by these molecules. Second, since all the digestive enzymes are sequestered in cisternal spaces directly upon synthesis (a hypothesis that requires a closer look, as is discussed below) and since molecules of the size and "hydrophilic" character of the digestive enzymes cannot cross biological mem-

The author is professor of physiology at the University of California, San Francisco 94143.

branes in molecular form (that is, pass through the substance of a membrane), such enzyme would not have an opportunity to enter the cytoplasm across intracellular membranes. In any event, even if it did, as the result of synthesis on free polysomes or release from attached ribosomes into the cytoplasm, it could not exit the cell and would simply be trapped in the cytoplasm.

The first reason (vulnerability) ignores what we already know of the cell's devices to protect against hydrolytic destruction; the presence in the cell of pancreatic trypsin inhibitor, chymotrypsin inhibitor, an elastase binding protein, and the fact that the proteases and perhaps other enzymes are present in an inactive form (so-called proenzymes). It also ignores the possibility that we may not know all there is to know about the cell's potential defense mechanisms, for example, the presence of other inhibitors or the degradation of accidentally activated enzymes. But perhaps more important, it presumes without evidence that reticular membranes are safe from hydrolysis while other components of the cell are not protected from this danger.

The second reason (membranes are impermeable to proteins) is a derivative of the classical membrane model which proposes that biological membranes contain a continuous lipid layer (or bilayer) at the core, which proteins cannot penetrate. While the evidence for this view has always been relatively fragile, although the view itself was quite imposing, today we are aware that the bilayer does not appear to be continuous but rather is broken by a mosaic of penetrating membrane proteins. Proteins not only apparently penetrate the membrane, but completely transect it in some cases, exist immersed in a hydrophobic milieu, and may well be able to enter this environment in intact membranes from the cytoplasm (*3*). Whether digestive enzymes can behave in a similar way to cross membranes is not now known, but this possibility certainly cannot be rejected out of hand. In any event, since the bilayer does not appear to be continuous, macromolecules might be able to cross the membrane by any of various mechanisms which would not require that they first be "dissolved" in a lipid layer.

To conclude this point, neither danger of hydrolytic destruction from digestive enzymes in the cytoplasm nor the exclusion of these proteins from the cytoplasm on the basis of permeability can be presumed a priori. To do so not only ignores evidence to the contrary, but assumes one hypothesis (vulnerability or impermeability) correct to prove another (the cisternal sequestration and continuous isolation of digestive enzyme from the cytoplasm).

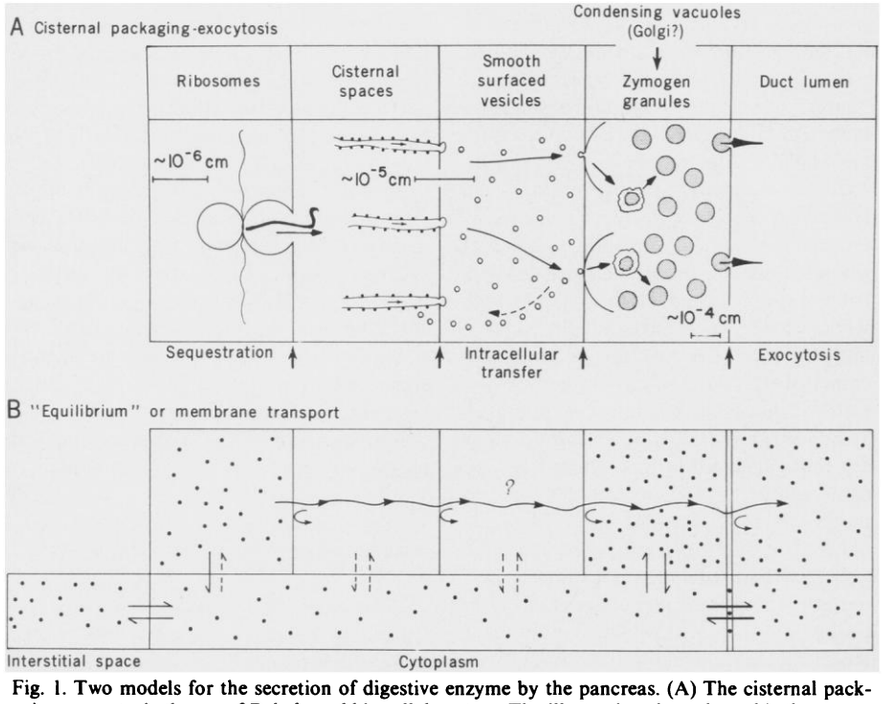

Fig. 1. Two models for the secretion of digestive enzyme by the pancreas. (A) The cisternal packaging–exocytosis theory of Palade and his collaborators. The illustration shows how this theory accounts for the intracellular movement and secretion of digestive enzyme. The illustration is not to scale, but shows an approximate decrease of two orders of magnitude in magnification from the compartment on the left (the approximately 100-angstrom ribosome) to the zymogen granule compartment on the right (the approximately 1-micrometer zymogen granule). The solid horizontal arrows indicate the vectorial transfer of the secretory proteins through a series of compartments as proposed by this theory. The digestive enzymes would be transferred sequentially through five compartments (ribosomes, cisternal spaces, smooth-surfaced vesicles, zymogen granules, and duct lumens) and across four compartmental boundaries [the sequestration boundary, two intracellular transfer boundaries (into and out of smooth-surfaced vesicles), and the exocytosis boundary]. The location of each boundary is indicated by the vertical arrows below the figure. (B) An alternative hypothesis—an "equilibrium" or membrane transport system. In this model, digestive enzymes move bidirectionally through specialized membranes, so that digestive enzymes in various compartments can be in "equilibrium" with each other. The solid arrows indicate processes for which there is evidence. The cytoplasm of the cell is proposed as an important enzyme-containing compartment that acts both as a mixing chamber for digestive enzyme derived from other pools and as a direct precursor pool to secretion. The dots represent individual protein molecules and the varying density indicates that some compartments probably contain digestive enzymes in high concentrations (namely, the zymogen granules), while others may be dilute in this regard (perhaps the cytoplasm). The wavy line indicates that the "equilibrium" model does not preclude the possibility of a parallel cisternal packaging–exocytosis pathway.

Nature of the Evidence

The experimental evidence in support of the cisternal packaging–exocytosis theory can be found in great part in a single type of experiment. For the ensuing discussion it is called *the prototypical experiment*. It deserves to be singled out because experiments of this type have been central not only to tne development of the current concepts of digestive enzyme secretion, but also to the secretion of organic molecules in general. In this experiment, the newly manufactured secretory product is labeled with a marker (such as a radioactive amino acid for a protein product) ideally for a short, relatively defined period (a pulse), and the molecules thus tagged are then followed through the cell as a function of time, usually by one of two techniques thought to localize the product at specific subcellular loci. The first of these techniques involves cell fractionation, the separation of various more or less well-defined subcellular parts based on their relative density and size by means of centrifugal forces. The second technique is electron microscopic autoradiography in which the location of radioactively labeled compounds in thin tissue sections is estimated from the location of a trace (grain) produced by emissions from the radioisotope on a film emulsion which overlies the section.

For the secretion of protein by the pancreas, the prototypical experiment (autoradiographic variety) demonstrates that new enzyme moves from the relatively ribosome-rich basal part of the cell to the api-

cal secretory surface (*2*). Also, grains can be found at least under certain conditions to be accumulated over condensing vacuoles at earlier times than over zymogen granules. This technique does not have the resolution to tell us, for example, whether or not the source of the trace in the basal part of the cell is molecules within the cisternal space or the cytoplasm—or both; or whether the source in the Golgi region of the cell is smooth-surfaced vesicles, the Golgi cisternae, or the cytoplasm. The prototypical experiment (cell fractionation variety), at least as usually reported (*2*), demonstrates that certain subcellular fractions accumulate new protein in a pattern wherein different pools reach peak specific radioactivities (labeled protein/total protein) at different times (Fig. 2). This has been interpreted as evidence for the filling of these pools sequentially.

Experiments of this type must be interpreted cautiously, not simply because of the considerable technical difficulties involved—for example, in the redistribution of enzyme from one compartment to another as a result of tissue homogenization (*4, 5*)—but because caution is required in constructing dynamic functional hypotheses on the static, as well as often fragmentary, information obtained by these methods. There are three general shortcomings of the prototypical experiment, at least as usually done, which should be of concern.

1) The measure is cumulative. Both autoradiography and cell fractionation techniques, as used, identify sites within the tissue where the product is accumulated. These measures of accumulation relate to the overall flux of molecules or transport rate in a complicated and uncertain manner. That is, the rate at which a molecule is moved through a given compartment may be relatively independent of the compartment's size and is probably unrelated to its ability to concentrate the product.

2) The measure is compartmentally discontinuous. Since only the content of some of the cell's compartments can be or are assessed, it cannot be determined how molecules move from place to place. For example, if the cytoplasm of the cell is not accounted for in the measurement, then the observation that a peak of label moves from condensing vacuoles to zymogen granules as a function of time does not tell us whether the label moves directly from condensing vacuole to zymogen granule (two compartments) or whether it moves via the cytoplasm. Moreover, the more general possibility that each compartment is derived from a common third compartment, such as the cytoplasm, and simply fills at different rates is left open.

3) The measure is usually qualitative.

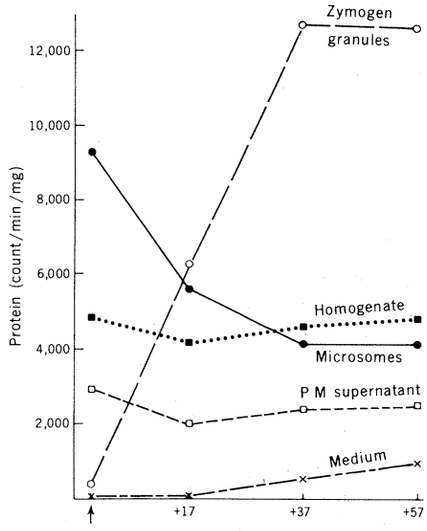

Fig. 2. The specific radioactivity of proteins in cell fractions isolated from slices of guinea pig pancreas after a 3-minute treatment with radioactive leucine plus varying periods of incubation in unlabeled medium (*24*). Abbreviation: PM, the supernatant from which microsomes had been removed. The specific radioactivity of the PM supernatant fraction remains relatively constant over the course of the experiment as the peak specific activity moves from microsome to zymogen granule.

The absolute amount of protein made during the pulse is often not determined and hence its quantitative distribution within the cell cannot be followed during the course of the experiment, and therefore the actual fluxes cannot be assessed. Quantifying these experiments is certainly possible; but it is not usually done.

These limitations indicate that the prototypical experiment cannot alone provide the necessary and adequate evidence to test the theory proposed by Palade and his coworkers. Their theory is simply one explanation for these observations.

The five tenets of the cisternal packaging–exocytosis theory are considered individually as follows.

Sequestration

Do nascent peptide chains pass into the cisternal space of the endoplasmic reticulum? And, if so, do all digestive enzyme proteins follow this pathway? The answers are a cautious "yes" to the first question and "no" to the second. Several observations are consistent with the hypothesis that new protein enters the cisternal space, but at least some of these observations have interpretive problems. There are two general lines of evidence. First, new protein associates with nonribosomal elements of the endoplasmic reticulum (more specifically nonribosomal elements of microsomal vesicles derived from the rough-surfaced endoplasmic reticulum). This has been demonstrated in cell fractionation versions of the prototypical experiment (*2*) and also with isolated microsomes (*6, 7*). New protein associated with nonribosomal elements of the microsome is usually assumed to be in the intravesicular space (*2, 6, 7*). However, the fraction of microsomes generally used in these studies is a detergent-soluble material that contains not only the contents of the intravesicular space but the contents of the microsomal membrane and material adsorbed to its surface. Clear compartmental distinctions have not to my knowledge been made, and therefore these experiments cannot provide conclusive proof for the sequestration of enzyme in intravesicular spaces.

The second line of evidence is the anatomical demonstration of "digestive enzyme" in the cisternal space. This demonstration consists of evidence for intracisternal granules, electron-opaque, presumably enzyme-containing, particles found within the cisternal spaces of guinea pig pancreas (*8*), and the fixing of ferritin-labeled antibodies to certain digestive enzymes over cisternal spaces (*9*). Intracisternal granules are apparently rather rare and have not been observed in most species; and, even in guinea pig pancreas where they were first described, they are seen with uncertain frequency. The antibody data may be the most convincing, but problems of the redistribution of protein during the preparation of the tissue sections requires further study. In any event, even if enzyme is concentrated in the cisternae this does not mean that it is sequestered; that is, it cannot enter more dilute pools such as the cytoplasm.

While some enzyme may well enter the cisternal space, enzyme also appears to be released from the ribosomes into the cytoplasm. Direct evidence for this comes from experiments by Redman, Siekevitz, and Palade (*7*) in which they studied the "vectorial" transfer of new digestive enzyme after its synthesis on isolated microsomes. New or labeled protein was recovered in three compartments: the ribosomes, the detergent-soluble material discussed above, and the suspending medium. Presumably, if all of the enzyme was sequestered in cisternal spaces, then no labeled enzyme would be recovered in the medium. This was not the case. After the addition of radioactive amino acids, substantial amounts of labeled protein were recovered in the suspending medium (Fig. 3). Redman *et al.* (*7*) dismissed this as being due to the leakage of enzyme out of broken microsomal vesicles. However, the kinetics they observed are not consistent

with this interpretation. If enzyme had leaked out of broken microsomes, then the amount of protein in the medium should have increased with time as did the labeled protein content of the detergent-soluble material (cisternal space?): more time, more synthesis, more leakage since the pattern of labeling should mirror the behavior of the detergent-soluble material. This did not occur. Rather, the concentration of labeled protein in the medium reached a constant or steady-state value with a half-time of equilibration, moreover, that was similar to that seen for the incorporation of label into ribosomal protein. This suggests that labeled protein entered the medium as the result of natural equilibria between protein on ribosomes and in the medium (Fig. 3).

If some of the enzyme released in this manner has access to a secretory process at the ductal surface of the cell (by diffusion) then small quantities of labeled protein should be recovered in secretion soon after labeled amino acids are added to the tissue. This is the case. Small amounts of labeled protein are secreted within 5 minutes of the addition of radioactive amino acids (the shortest period of time for which measurements could be made with any precision) (*10*).

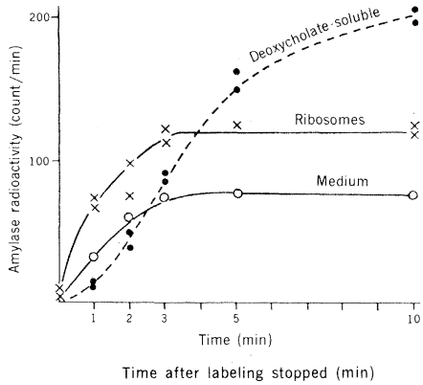

Fig. 3. Radioactive amylase recovered in ribosomes, a detergent-soluble material extracted from microsomes (soluble in 0.5 percent deoxycholate), and the medium obtained after incubating microsomes for varying periods of time in a medium containing radioactive leucine (*7*). The amylase content of the medium is substantial relative to the other fractions and reaches a steady-state value in about the same time as the labeled amylase content of the ribosomes. Conversely, the amylase content of the deoxycholate-soluble material continues to increase with time. These kinetics suggest that amylase may be released into the medium as the result of equilibrium events rather than as the result of the leakage of labeled amylase out of broken microsomes.

Complete and Continuous Isolation of Digestive Enzyme from Cytoplasm

If new peptides are released directly into the cytoplasm, then obviously not all secretory protein can be isolated from it. Experiments by Liebow and myself indicate another site of particulate-cytoplasm interaction (*11*). We found that digestive enzymes in zymogen granules are in equilibrium with enzyme in the surrounding medium across the granule membrane. Digestive enzyme in zymogen granules also equilibrate with labeled enzyme added to the medium suspending tissue slices with isotopic equilibration apparently occurring across the cell membrane, through the cytoplasm, and across the granule membrane (*11*). Therefore, isolation does not seem to be either complete or continuous and digestive enzyme in the cytoplasm is probably derived from at least two sources, ribosomes and zymogen granules.

There is a wide range of other experimental evidence consistent with the idea that there is a cytoplasmic compartment of digestive enzyme (*12*) which includes the following observations:

1) For certain enzymes and animal species a large percentage of the total enzyme content of pancreatic tissue is recovered in the supernatant fraction of homogenates (*13–15*).

2) The percentage of the total enzyme content of tissue recovered in the supernatant can be different for different enzymes (*5, 14, 15*). This is true even for structurally homologous enzymes such as trypsinogen and chymotrypsinogen with similar tertiary forms, isoelectric points, and amino acid sequences which might be expected to distribute equivalently in a supernatant fraction whose enzyme contents were derived solely from the indiscriminate lysis of parent fractions.

3) Reciprocal changes occur in the relative amounts of specific enzymes in the supernatant fraction when the secretion of one enzyme is favored over another and this is in the absence of similar changes in zymogen granule and microsomal fractions (*5, 16*).

4) Exogenously added labeled chymotrypsinogen equilibrates (isotopically) most rapidly with chymotrypsinogen which is apparently cytoplasmic, and more slowly with the chymotrypsinogen content of other intracellular compartments (*11*).

5) Digestive enzyme added to the serosal face of acinar cell either in vitro or in vivo is secreted through the cell, apparently mixing with intracellular digestive enzyme en route (*17–19*).

6) Augmented secretion of digestive enzyme is maintained in the absence of zymogen granules (*20, 21*).

7) Adaptive increases in digestive enzyme storage by the acinar cell can almost quantitatively be accounted for by increases in the enzyme content of the supernatant fraction of tissue homogenates (*22*).

8) There is competition for exit (secretion) between enzymes derived from different intracellular compartments (*18, 23*) (see below).

It may be that all intracellular digestive enzyme-containing compartments are in equilibrium with the enzyme content of the cytoplasm and hence with each other. This is suggested by an experiment by Jamieson and Palade (*24*) in which they followed the "movement" of a new protein through various isolated subcellular fractions of guinea pig pancreas as a function of time. They observed that during the course of the experiment the specific radioactivity of protein in the supernatant fraction from which the microsomes had been removed (PM fraction) remained relatively constant (Fig. 2). They assumed, in accordance with their views, that the enzyme content as measured of this fraction was an artifact, the labeled protein having come from the cisternal spaces of the endoplasmic reticulum, zymogen granules, and the like during homogenization and fractionation. While some of the content of the supernatant may well have reflected a redistribution of protein relative to the natural state [the term redistribution is intentionally chosen since digestive enzymes associate well with at least certain membranes in the acinar cell (*4*)], the content of the PM supernatant was in all likelihood not derived simply as the result of such a process. If it was, then the labeled enzyme content of the supernatant fraction should have reflected the labeled protein content of the parent fractions. Since different fractions would contain the labeled enzyme at different times in a pulse experiment, and since they would presumably lose different amounts during homogenization and subsequent fractionation, labeled protein in the supernatant would be expected to reflect these differences and vary from time to time as labeled enzyme moved from intracellular compartment to intracellular compartment. In order to explain the relative constancy of the PM supernatant specific radioactivity in terms of the cisternal packaging–exocytosis theory, the percentages of the digestive enzyme content lost from each compartment during homogenization and centrifugation would have to have been roughly equal. While such an equal fragility is conceivable, it is not likely. It seems more reasonable to assume that the relatively constant specific radioactivity of the supernatant fraction reflects a steady-state equilibrium between the enzyme contents of the cytoplasm and the other compartments which contain digestive enzymes.

Fusion and Fusion-Fission

If digestive enzyme is found in the cytoplasm, then we must apply a more stringent test to hypotheses which suggest an important physiological role in the normal secretion process for membrane-membrane processes such as exocytosis. We cannot, as has been done, simply assume the need for such processes to produce secretion. According to the cisternal packaging–exocytosis theory, membrane-membrane interactions of the fusion or fusion-fission type occur in two places: first, in the transfer of secretory protein from the endoplasmic reticulum to condensing vacuoles and, second, in the final or secretion step by the fusion of zymogen granule and cell membrane.

The transfer of secretory protein from endoplasmic reticulum to condensing vacuoles is proposed to start with the budding-off of apical segments of the endoplasmic reticulum to form enzyme-containing smooth-surfaced vesicles. The primary evidence for this is the appearance of occasional narrowings in ribosome-denuded apical portions of the endoplasmic reticulum and the existence in the cytoplasm of small smooth-surfaced vesicles which may or may not be derived from the endoplasmic reticulum and may or may not contain digestive enzymes (*2*). These vesicles presumably fuse with the membrane of the condensing vacuole and deposit their contents within the vacuolar lumen. Since condensing vacuoles appear to be of roughly constant size (that is, they do not seem to grow from very small vacuoles to large ones), the membranes of the smooth-surfaced vesicles must also be presumed to undergo fission from the membranes of the condensing vacuoles and find their way back to the parent membrane within the endoplasmic reticulum, thus starting a new cycle. While this is an interesting hypothesis, worthy of investigation, there is at present no substantial body of evidence to indicate that these processes occur, no less account for the movement of digestive enzyme into the apical region of the acinar cell.

The evidence for the exocytosis of zymogen granule contents is hardly more substantial. It is based primarily on microscopic profiles of the acinar lumen which have the appearance of a neck or narrowing (omega forms) and could be geometric sequelae to exocytosis (*2*). Even if we accept these microscopic images as adequate proof for the existence of this process, we still cannot assume that it accounts quantitatively for the natural secretory events without adequate quantitative proof. This is lacking. In fact, one observation places doubt on the importance of exocytosis in normal digestive enzyme secretion. Under conditions in which the formation of new granules is insignificant relative to their rate of disappearance (secretion), the loss of granules should be proportional in a linear fashion to the secretion of granule contents; the more granules lost per unit time, the greater the rate of enzyme secretion. Under these circumstances, the relation actually observed between these two measures is highly nonlinear and granule depletion is not followed pari passu with decreased enzyme secretion, but enzyme secretion is maintained in face of a declining number of zymogen granules (*20*) (Fig. 4). The exocytosis construct cannot easily explain these kinetics. Rather, this is the kinetic pattern we would expect if the zymogen granules first discharged their contents into the cytoplasm of the cell, and secretion was derived from the cytoplasm (Figs. 1 and 4b).

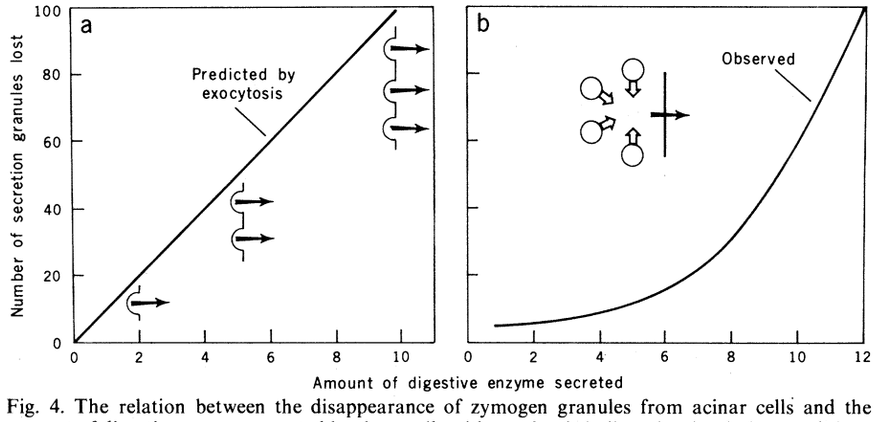

Fig. 4. The relation between the disappearance of zymogen granules from acinar cells and the amount of digestive enzyme secreted by these cells with maximal cholinergic stimulation conditions under which the formation of zymogen granules is slow relative to their rate of disappearance (secretion) (*20*). (a) If secretion occurs by exocytosis, then under these conditions the loss of granules would be expected to be proportional in a linear manner to the secretion of digestive enzyme. (b) The observed function is highly curvilinear and enzyme secretion is maintained even as the number of zymogen granules in the acinar cell approaches zero. The illustration shows a model consistent with the observed kinetics of granule depletion during active secretion. In this model zymogen granules discharge their contents into the cytoplasm prior to secretion across the cell membrane (*20*).

A Series Secretory Process

The cisternal packaging–exocytosis theory proposes that the various intracellular compartments through which secretory proteins travel are arranged in a functional series; that is, the secretory product moves from compartment to compartment in a unique sequence, namely from ribosome to cisternal space to smooth-surfaced vesicle to condensing vacuole to zymogen granule to duct lumen. This sequence is immutable; that is, digestive enzyme goes through each of these compartments in order before entering secretion. [This hypothesis has been recently modified by Jamieson and Palade (*21*) to account for the secretion of digestive enzymes in the absence of zymogen granules in the acinar cell. They have proposed that another smaller vesicle takes the place of the zymogen granule in the sequence and accounts for exocytosis under these conditions (*10*).] If such a series exists, then a sudden increase in enzyme secretion in the continuous presence of a radioactive amino acid precursor should hasten the rate at which isotopic equilibrium is attained for secretory protein throughout the compartments of the system (if intracellular transit time is increased or because of the more rapid depletion of unlabeled enzyme stores or both). At equilibrium the specific radioactivity of digestive enzymes in each compartment would be equal to that of new protein being synthesized on the ribosomal complex. The specific radioactivity of digestive enzymes in secretion should reflect this by approaching the steady-state specific radioactivity more rapidly. This is not observed. There is a striking and rapid decrease in the specific radioactivity of secreted protein (*23*) (Fig. 5). This means that secreted digestive enzyme cannot be derived from a single intracellular precursor pool, namely, the zymogen granule, but must come from at least two functionally parallel intracellular pools, one which adds relatively more protein to secretion in the absence of the stimulant (more rapidly labeled) and the other which adds more in response to stimulation at least at the onset (more slowly labeled). Furthermore, in the presence of a cholinergic stimulus, there is an initial absolute decrease in the secretion of newer (labeled) protein indicating that newer and older digestive enzyme actually compete for exit from the cell. Similar competitive effects have been seen in three other situations so far.

1) Under unstimulated conditions, the amount of newly labeled protein which is

secreted within 45 minutes of the addition of radioactive amino acids is inversely related to overall protein secretion (*10*).

2) The flux of labeled chymotrypsinogen across the acinar cell is transiently inhibited by the addition of unlabeled chymotrypsinogen to the bathing medium (*17*).

3) The secretion of endogenously labeled amylase is inhibited by the addition of unlabeled amylase to the bathing medium (*18*).

There have been attempts to explain such complex kinetic behavior in this (*21*) and other systems on the basis of multiple populations of granules which can be secreted at preferential rates relative to each other under certain conditions. While this may be true in other systems, there is no substantial evidence for a multiplicity of secretion granules in the exocrine pancreas (see below). Both the competition and parallel pool behavior are most readily explained if we hypothesize the mixing of digestive enzyme derived from various intracellular pools (and even transcellular pools) to one degree or another in the cytoplasm prior to secretion across the luminal plasma membrane (Fig. 1). In any event, the idea that digestive enzyme must move from the site of its synthesis through compartments set up in a unique series seems to be incorrect.

Mass Transport

Secretion by exocytosis is a mass transport process. The whole contents of secretion granules are released en masse and therefore at the same rate. The rate at which a particular molecule is secreted is not related either to its molecular structure or its concentration within the granule or outside of the cell. Rather, it is determined by the rate of fusion (or fusion and fission) of granule and cell membrane. In such a system, the secretion of different molecules (independent of their synthesis) could only occur at different rates if they were packaged in separate granules, and if the character of granule membranes enabled their fusion to be separately regulated. If each granule contains the whole of the secretory products, in our case all of the digestive enzymes, then the secreted mixture would be of invariant or fixed "stoichiometry" or content at least for intervals during which alterations in the rate of synthesis of the secretory products could not substantially alter the composition of secretion (presumably of the order of at least 1 to 4 hours for the digestive enzymes of the pancreas).

While we cannot say that each zymogen granule contains exactly the same complement and amount of enzyme, there is no evidence for multiple ordered populations of zymogen granules with different mixtures of digestive enzymes no less for granules which contain only a single molecular species. On the contrary, most of the available evidence suggests that there is only one population of zymogen granules which contains the whole array of digestive hydrolases (*25, 26*). If this is indeed the case, then the secretion of digestive enzyme by exocytosis could only be nonspecific or nonselective for the different enzymes; that is, it would produce a secretion of constant enzyme composition in the short run. This pattern was called parallel secretion some 40 years ago by Babkin when he was challenging Pavlov's theory (*27, 28*) that the enzyme content of pancreatic secretion varied from meal to meal as a function of digestive need and the content of the meal. Babkin and his co-workers (*28*) measured some of the enzymes in pancreatic secretion under a variety of conditions, and observed that the secretion of one enzyme appeared to be roughly parallel to the secretion of others regardless of the stimulus used to elicit secretion. They concluded that the secretory process had no *transport* selectivity and that, if secretory selectivity existed at all, it must be derived from the independent regulation of the synthesis of the various enzymes.

During the past 10 years there have been some attempts to reexamine this issue (*5, 14, 16, 29–33*). As a result, the potential for parallel transport has been reaffirmed (*26, 34, 35*), but not its uniqueness. That is, a nonparallel transport of digestive enzymes can occur; a stimulus can produce unequal changes in the rate of secretion of one enzyme relative to another, changes which cannot be ascribed to altered synthetic rates (*36*). Moreover, the cell's ability to secrete a specific digestive enzyme can be adaptively enhanced by altering the transport capacity of the system for that enzyme as well as by increasing its rate of synthesis (*30*). Nonparallel transport appears to be both regulatory—that is, transport selectivity is necessary to meet specific digestive needs—and situational—that is, selectivity is merely a kinetic consequence of the secretory mechanism apparently of no physiological importance in the regulation of digestion (*37*). Regulatory selective transport seems to be concerned with the molecular regulation of digestion; namely, the separate regulation of specific digestive hydrolyses in the intestine, as opposed to the mass or batch regulation of digestion (namely, parasympathetic control of gastrointestinal function) in which activity is increased or decreased in general as a function of the overall digestive load. For example, the hydrolysis of lysine-containing peptide bonds appears to be independently regulated and this is at least in part the result of altera-

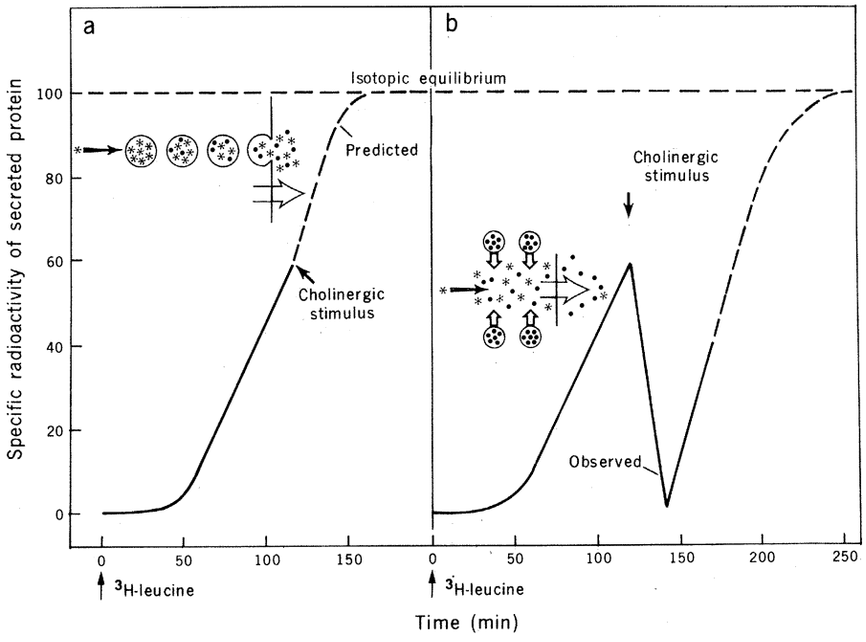

Fig. 5. The effect of a cholinergic stimulus (which increases the rate of protein secretion) on the rate at which the specific radioactivity of secreted protein approaches isotopic equilibrium in the continuous presence of radioactive leucine. (a) The cisternal packaging–exocytosis model proposes that enzyme moves through a single unbranched series of compartments (from ribosome to duct lumen) (Fig. 1). In such a system the attainment of isotopic equilibrium should occur more rapidly when the rate of enzyme secretion is greatly enhanced as nonradioactive enzyme stores are depleted. (b) A profound depression in the specific radioactivity of secreted protein, as well as an absolute decrease in the secretion of labeled protein, is what is actually observed. This is consistent with the hypothesis that zymogen granules discharge their (largely unlabeled) stores into the cytoplasm at the onset of augmented secretion prior to secretion across the cell membrane (*23*).

tions in the transport (secretion) of trypsinogen, which favors lysine-containing bonds, relative to other digestive enzymes (*31*). Another, and perhaps more dramatic example, is found in "chymodenin," a duodenal peptide which selectively enhances the secretion of chymotrypsinogen while hardly, if at all, altering the secretion of other digestive enzymes (*32, 33*). These and numerous other examples of nonparallel or selective digestive enzyme secretion (*5, 14, 16, 29–33*) cannot be explained by a solely exocytotic secretory process if zymogen granules are, as seems to be the case (*25, 26*), of mixed enzyme composition.

Summary

Contrary to the cisternal packaging–exocytosis theory:

1) Digestive enzyme secretion by the pancreas does not appear to be a simple mass transport process.

2) Digestive enzyme secretion does not result from the movement of enzyme from its site of synthesis through a single unbranched series of intracellular compartments.

3) Digestive enzyme is neither completely nor continuously isolated from the cytoplasm of the cell.

4) The direct transfer of digestive enzyme from ribosome to cisternal space, if it occurs, only appears to account for a portion of the secretory protein released from ribosomes.

Thus, this theory cannot be said to have been proved true by the evidence in any of its essential aspects, no less in all of them. The direct evidence for important elements of the process (namely, fusion) is minimal, and the substantial kinetic evidence can almost invariably be explained in other ways and often with more justification. Moreover, there appears to be another secretory process at work in which the cytoplasm of the cell plays an important role both as a mixing chamber for digestive enzyme derived from other intracellular pools and as a direct precursor pool for the transport of digestive enzyme across the plasma membrane (Fig. 1).

The cisternal packaging–exocytosis theory is in great part a derivative of the idea that vesicular transport processes, that is, the transport of molecules by their movement *with* membranes, rather than *through* them, are the sole means by which large molecules such as the digestive enzymes can cross biological membranes. On this basis, vesicular transport mechanisms of one type or another have been proposed to account for the transfer of various macromolecules across membranes even in the absence of experimental evidence. Current views of the structure of biological membranes (*3*) make such a priori assumptions unsatisfactory. We are obliged to distinguish experimentally between vesicular transport and transport by the movement of molecules through membranes regardless of the size of the molecule moved. Indeed, the proposal that a vesicular mechanism is responsible for the transport of digestive enzymes by the pancreas or any other molecule or group of molecules should be substantiated with direct and unambiguous evidence, not only that vesicular transport occurs, but that it accounts in fact for the kinetics of the transport process either wholly or partially.

References and Notes

1. K. R. Porter, *Science* **186**, 516 (1974).
1a. G. E. Palade, *ibid.* **189**, 347 (1975).
2. For a recent review of the theory and the experiments that suggested it, see J. D. Jamieson, in *Secretin, Cholecystokinin, Pancreozymin, and Gastrin*, J. E. Jorpes and V. Mutt, Eds. (Springer-Verlag, Berlin, 1973), p. 195.
3. For a recent review on the structure of membranes, → S. J. Singer, *Annu. Rev. Biochem.* **43**, 805 (1974); and, for a discussion of "the synthesis and degradation of membrane proteins," see R. T. Schimke, in *Cytopharmacology of Secretion*, B. Ceccarelli, J. Meldolesi, F. Clementi, Eds. (Raven, New York, 1974), vol. 2, p. 63.
4. A. M. Tartakoff and J. D. Jamieson, *Methods Enzymol.* **31**, 41 (1974).
5. S. S. Rothman, *Am. J. Physiol.* **218**, 372 (1970).
6. C. M. Redman and D. D. Sabatini, *Proc. Natl. Acad. Sci. U.S.A.* **56**, 608 (1966).
7. C. M. Redman, P. Siekevitz, G. E. Palade, *J. Biol. Chem.* **241**, 1150 (1966).
→ G. E. Palade and P. Siekevitz, *J. Biophys. Biochem. Cytol.* **2**, 671 (1956); G. E. Palade, in *Subcellular Particles*, T. Hayashi, Ed. (Ronald, New York, 1959), p. 64; ———, P. Siekevitz, L. G. Caro, in *The Exocrine Pancreas*, A. V. S. de Reuck and M. P. Cameron, Eds. (Little, Brown, Boston, 1962), p. 23.
9. R. G. Painter, K. T. Tokuyasu, S. J. Singer, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1649 (1973).
10. S. S. Rothman, *Am. J. Physiol.*, in press.
11. C. Liebow and S. S. Rothman, *Nature (Lond.)* **240**, 176 (1972); *Am. J. Physiol.* **226**, 1077 (1974).
12. For a review of earlier evidence for an alternative secretory route, see L. E. Hokin, in *Handbook of Physiology*, section 6, "Alimentary canal," C. F. Code, Ed. (American Physiological Society, Washington, D.C., 1967), vol. 2, p. 935.
13. L. E. Hokin, *Biochim. Biophys. Acta* **18**, 379 (1955); A. K. Laird and A. D. Barton, *ibid.* **25**, 56 (19[illegible] → C. M. Redman and L. E. Hokin, *J. Biophys. Biochem. Cytol.* **6**, 207 (1959); A. J. Morris and S. R. Dickman, *J. Biol. Chem.* **235**, 1404 (1960).
14. S. S. Rothman, in *The Exocrine Glands*, S. Y. Botehlo, F. P. Brooks, W. B. Shelley, Eds. (Univ. of Pennsylvania Press, Philadelphia, 1969), p. 169.
15. E. Hansson, *Acta Physiol. Scand. Suppl.* **161**, 1 (1959).
→ S. S. Rothman, *Nature (Lond.)* **213**, 460 (1967).
17. C. Liebow and S. S. Rothman, *Science* **189**, 472 (1975).
18. L. D. Isenman and S. S. Rothman, *Physiologist* **18**, 259 (1975).
19. H. Götze and S. S. Rothman, *Nature (Lond.)* **257**, 607 (1975).
20. S. S. Rothman, *Am. J. Physiol.* **228**, 1828 (1975).
21. J. D. Jamieson and G. E. Palade, *J. Cell Biol.* **50**, 135 (1971).
22. S. S. Rothman, *Am. J. Physiol.* **219**, 1652 (1970).
23. ——— and L. D. Isenman, *ibid.* **226**, 1082 (1974).
24. J. D. Jamieson and G. E. Palade, *J. Cell Biol.* **34**, 597 (1967).
25. For information concerning the enzyme content of zymogen granules, see J. M. Marshall, Jr., *Exp. Cell Res.* **6**, 240 (1954); K. Yasuda and A. H. Coons, *J. Histochem. Cytochem.* **14**, 303 (1966); J. P. Kraehenbuhl and J. D. Jamieson, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1771 (1972); C. Liebow and S. S. Rothman, *Am. J. Physiol.* **225**, 258 (1973); I. Nadelhoft, *Biophys. J.* **13**, 1014 (1973); S. S. Rothman, S. Burwen, C. Liebow, in *Cytopharmacology of Secretion*, B. Ceccarelli, J. Meldolesi, F. Clementi, Eds. (Raven, New York, 1974), vol. 2, p. 341.
26. A. M. Tartakoff, J. D. Jamieson, G. A. Scheele, G. E. Palade, *J. Biol. Chem.* **250**, 2671 (1975).
27. A. A. Walther, thesis, St. Petersburg (1897); I. P. Pavlov, *The Work of the Digestive Glands* (Griffin, London, 1910).
28. B. P. Babkin, *Secretory Mechanisms of the Digestive Glands* (Hoeber, New York, rev. ed., 1950), p. 54.
29. S. S. Rothman, *Fed. Proc.* **25**, 564 (1966); H. Sarles, C. Figarella, G. Prezelin, C. Souville, *Bull. Soc. Chim. Biol.* **48**, 951 (1966); J. Christophe, J. Camus, J. Deschodt-Lanckman, M. Rathé, P. Robberecht, M. C. Vandermeers-Piret, A. Vandermeers, *Horm. Metab. Res.* **3**, 393 (1971); K. G. Wormsley and D. M. Goldberg, *Gut* **13**, 398 (1972); D. M. Goldberg, J. K. Sale, K. G. Wormsley, *Digestion* **8**, 101 (1973); J. F. Sullivan, R. E. Burch, D. G. Magee, *Am. J. Physiol.* **226**, 1420 (1974); J. P. Felber, A. Zermatten, J. Dick, *Lancet* **1974-II**, 185 (1974).
30. S. S. Rothman and H. Wells, *Am. J. Physiol.* **216**, 504 (1969).
31. S. S. Rothman, *Am. J. Physiol.* **226**, 77 (1974).
32. J. W. Adelson and S. S. Rothman, *Science* **183**, 1087 (1974).
33. ———, *Am. J. Physiol.* **229**, 6 (1975).
34. A. M. Tartakoff, L. J. Greene, J. D. Jamieson, G. E. Palade, in *Cytopharmacology of Secretion*, B. Ceccarelli, J. Meldolesi, F. Clementi, Eds. (Raven, New York, 1974), vol. 2, p. 177.
35. G. A. Scheele and G. E. Palade, *J. Biol. Chem.* **250**, 2660 (1975).
36. There has been continuing confusion concerning both the meaning of the terms parallel transport and nonparallel transport and implications concerning the mechanisms of these phenomena. Parallel transport simply means the secretion of different molecules in constant proportions one to the other over time even in the face of varying conditions or stimuli. Nonparallel transport is the absence of such constancy. A parallel rate of response to a stimulus should not be confused with a parallel quantitative response, that is, proportional increments in the amounts of two molecules secreted. The fact that there are increases in the secretion of several molecules in response to a stimulus does not mean that the secretion of each molecule has been augmented in parallel amounts; it means only that the secretion of each molecule has been augmented to one degree or another. Also, a response is not parallel simply because the proportions of different molecules in secretion remain constant over time in the presence of the particular stimulant. The proportions of the products secreted after the application of a stimulus should of course be compared to the proportions of the same molecules secreted prior to the change in conditions. Probably the most important source of confusion in determining whether or not a response is parallel derives from the practice of taking the mean values for the secretion of a molecule (or the ratio of the secretion of two molecules) from different times and experiments in order to test for differences in the proportion of different molecules in secretion as a result of the applied conditions. When differences are observed, the technique has worked and is therefore satisfactory. However, in the absence of differences between means, one has to look further. This is so because—at least for the case of digestive enzyme secretion—there can be a large natural variance in the overall rate of protein secretion from period to period and animal to animal, which tends to obscure differences in the rate of secretion of one enzyme relative to another, rather than point them out when averaging techniques are used. When this (the general level of protein secretion) is considered in the analysis of the data, then nonparallel effects can be more readily seen (*31, 33*). There also seem to be two misconceptions about the mechanistic implications of parallel transport. First, the parallel secretion of two or more molecules is not tantamount to proof of their cotransport (that is, linked transport to mechanism; in the case discussed here, represented by the exocytosis of zymogen granule contents). Molecules can be secreted in parallel in a wide range of situations, such as at the initial rate of transport or at the transport maximum for the different transported substrates, even if they are secreted by different cells. Second, the existence of parallel secretion and its uniqueness are not mutually inclusive. That is, secretion may well be parallel under one set of conditions and nonparallel under another (*26, 35*).
37. Perhaps this is the case for the nonparallel effects seen with secretagogues that produce massive protein release by the pancreas (*16, 33*).

38. Supported by NIH grant AM16990.