

# INTRACELLULAR ASPECTS OF THE PROCESS OF PROTEIN SECRETION

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by

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## A SHORT HISTORY OF THE WORK

In the early 1950's, during the near avalanche of discoveries, rediscoveries, and redefinitions of subcellular components made possible by electron microscopy, those prospecting in this newly opened field were faced with the problem of what to do with their newly acquired wealth. It could be increased by extending the inquiry on the horizontal to many other cell types prepared by many other techniques; it could be extended in further depth, instrumental resolution permitting ("ultra" was the preferred prefix of the period); or it could be used as a guide to monitor cell fractionation procedures of the type previously developed by Claude (1). The last alternative seemed particularly attractive since the small dimensions of many of the newly discovered structures suggested that they were relatively simple macromolecular assemblies. At their level, structure - as traditionally envisaged by the microscopist - was bound to merge into biochemistry, and biochemistry of mass-isolated subcellular components appeared to be the best way to get at the function of some of the newly discovered structures. The example provided by the work on isolated mitochondria was recent and still shining (2, 3).

At the time the structures of interest were the "small particulate component of the cytoplasm" (4) soon to become in succession "ribonucleoprotein particles" (5) and "ribosomes" (6), and the endoplasmic reticulum (ER) originally discovered by Porter, Claude and Fullam (7) and then studied by Porter (8) and by Porter and myself (9-11). Philip Siekevitz joined me in 1955 and together we started a long series of integrated morphological and biochemical studies on the pancreas of the guinea pig using primarily a combination of electron microscopy and cell fractionation procedures.

The choice of the pancreatic exocrine cell, a very efficient protein producer, as the object for our studies reflected in part our training, and in part our environment. I was coming from a medical school where I had acquired an interest in "microscopical anatomy" and "physiological chemistry" and great respect for the work of Claude Bernard, Rudolf Heidenhain and Charles Garnier. Philip Siekevitz was coming from a graduate school with a Ph.D. in Biochemistry and had recently worked out one of the first *in vitro* systems for protein synthesis (12). Our environment was the Rockefeller Institute for Medical Research where a substantial amount of work had been carried out on the isolation, crystallization and characterization of pancreatic secretory proteins (cf. 13). But perhaps the most important factor in this selection was the appeal of the amazing organiza-

tion of the pancreatic acinar cell whose cytoplasm is packed with stacked endoplasmic reticulum cisternae studded with ribosomes. Its pictures had for me the effect of the song of a mermaid: irresistible and half transparent. Its meaning seemed to be buried only under a few years of work, and reasonable working hypotheses were already suggested by the structural organization itself.

The general aim of the project was to define the role played by the ribosomes, endoplasmic reticulum and other subcellular components in the synthesis and subsequent processing of the proteins produced for export by the exocrine cells of the gland. The approach worked rather well for a while (14, 15), but after a few years we ran into the common limitations of the cell fractionation procedures then in use: imperfect separation, incomplete recovery, and incomplete representation of subcellular components in the fractionation scheme. To resume the advance of the inquiry, Lucien Caro and I shifted to radioautography adapted to electron microscopy and obtained, in experiments carried out *in vivo*, a reasonable approximation of the route and timetable followed by newly synthesized, radioactive proteins from their site of synthesis to their site of discharge from the cell (16). Radioautography has, however, its own limitations connected primarily with its low resolution, so that in subsequent experiments uncertain radioautographic findings had to be checked by going back to cell fractionation procedures—this time with an advised mind. The experimental protocols were also changed to obtain better time resolution of the events under study, the major changes being the use of an *in vitro* subcellular system (17) and the adaptation by James Jamieson of an *in vitro* slice system (18) which later on evolved into a lobule system (19: 20).

#### ANALYSIS OF THE SECRETORY PROCESS IN THE PANCREATIC EXOCRINE CELL

Out of his combination of complementary techniques came a coherent representation of the secretory process, a “model” which has stood well the test of time. The current trend is to move from the subcellular to the molecular level in the analysis of the model, which means that its subcellular stage has been widely enough accepted.

The analysis of the secretory process of the pancreatic exocrine cell has not been the only research line pursued in our laboratory; membrane biogenesis, intercellular junctions and structural aspects of capillary permeability are other examples. But the corresponding bodies of information are either less fully developed or still under scrutiny by us and by others; besides none of them has affected the general thinking in our field to the same extent as the story of the secretory process. With these considerations in mind, I believe that this unique and solemn occasion would be put to good use if I were to depart from the apparent tradition, which favors a summary of past or current work, and assess instead the available evidence on the secretory process, pointing out its strengths as well as its weaknesses, and trying to figure out what can be done in the future to advance our knowledge still further.

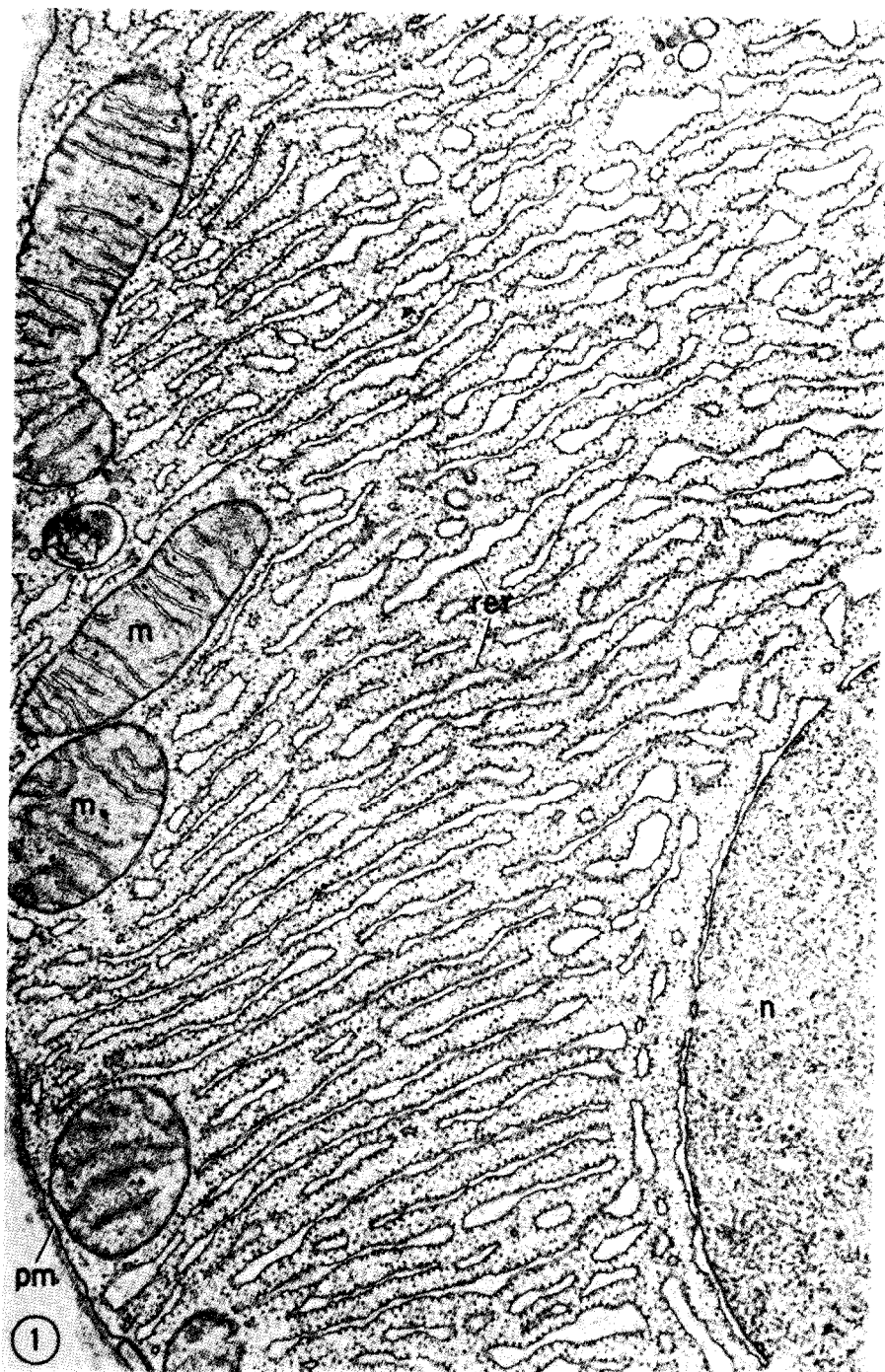


Fig. 1. Pancreatic exocrine cell. The basal region of the cell between the nucleus (n) and the plasmalemma (pm) is occupied by numerous cisternae of the rough endoplasmic reticulum (rer) and a few mitochondria (m).  
x 12,000

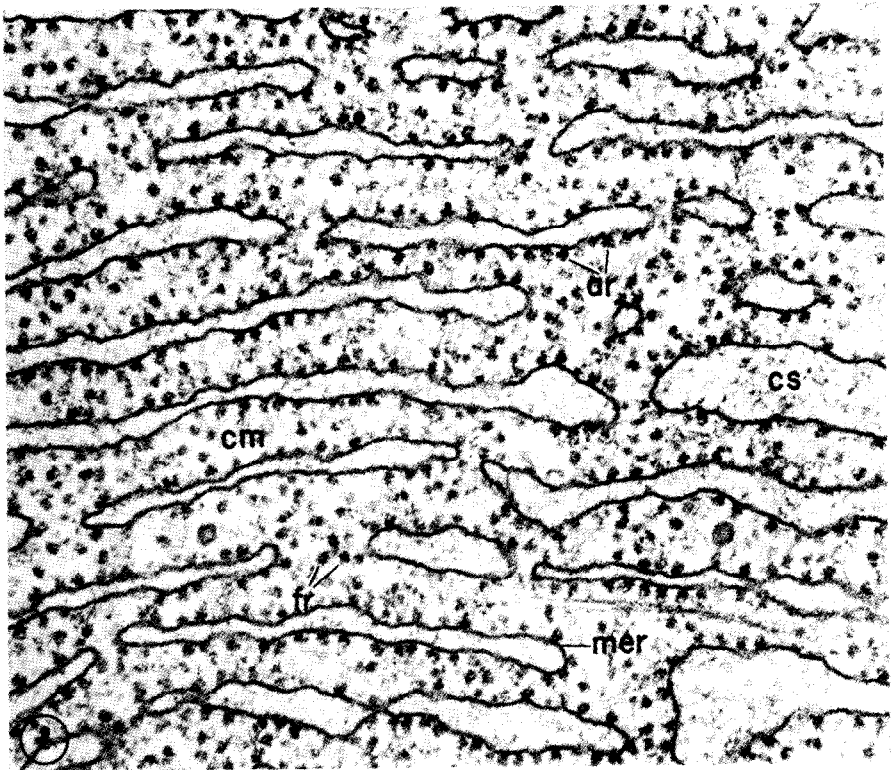


Fig. 2. Pancreatic exocrine cell. Array of cisternae of the rough surfaced endoplasmic reticulum.

cs, cisternal space; cm, cytoplasmic matrix (cell sol); fr, free ribosomes; ar, attached ribosomes; mer, membrane of the endoplasmic reticulum.

x 50,000

Our analysis recognizes in the secretory process<sup>1</sup> of the pancreatic exocrine cell 6 successive steps or operations of which the object is the secretory proteins. These steps are: 1) *synthesis*, 2) *segregation*, 3) *intracellular transport*, 4) *concentration*, 5) *intracellular storage*, and 6) *discharge*. Each of them will be considered in some detail in what follows.

#### 1. SYNTHESIS

Proteins for export are synthesized on polysomes attached to the membrane of the rough endoplasmic reticulum (Figs. 1-2). The first clear indication that this is the case came from early work carried out with Philip Siekevitz. After a short *in vivo* exposure to [<sup>14</sup>C]leucine, radioactive chymotrypsinogen appeared preferentially associated with attached polysomes isolated from the guinea pig pancreas (21) (Table I). The products of free polysomes were not investigated, but by analogy with the situation studied by others in the liver (22, 23) these polysomes probably synthesize proteins for intracellular

<sup>1</sup>For convenience, the term "secretory process" will be used in the rest of the text as a shorthand for "the process of protein secretion".

Table I. Specific radioactivity of chymotrypsinogen isolated from attached and free polysomes\*\* after in vivo labeling with [<sup>14</sup>C]leucine.

Fraction	Time after [ <sup>14</sup> C]leucine	
	1 min	3 min
Attached polysomes	22,100*	10,000*
Free polysomes	2,800	3,000

\* cpm/mg chymotrypsinogen (estimated from enzyme activity).

\*\* Guinea pig pancreas, from P. Siekevitz and G. E. Palade, *J. Biophys. Biochem. Cytol.* 7(1960)631.

use. Yet in all these cases, the results are - to some extent - ambiguous, since - as isolated - both polysome classes carry newly synthesized proteins irrespective of the latter's final destination. The differences are not qualitative as would be expected for strict specialization; they are definitely large, but only quantitative.

This finding could have a trivial explanation: e.g., leakage of newly synthesized proteins from cell compartments ruptured during tissue homogenization, followed by relocation by adsorption on the "wrong" class of polysomes. Available data indicate that artifactual relocation definitely occurs under these circumstances (24), but so far there is no reliable information concerning its extent. Alternatively the dual location may have functional significance since the position of the polysomes at the time of the initiation of translation is still unknown. Initiation in the free condition followed by enough elongation to expose either enzymic active sites or antigenic determinants before attachment seems unlikely but may occur, in principle. And the special sequence detected at the N-terminal of IgG light chains synthesized on detached polysomes (25) may function as a signal for attachment (cf. 26). To understand the situation, we need more information than we have at present on the relationship between free and attached ribosomes, on the position of polysomes at the time of initiation, and on the duration of polysomes attachment to the ER membrane.

Another aspect that should be considered at this point is the existence of two subclasses of attached polysomes: one synthesizing proteins for export and the other involved in the production of ER membrane proteins coupled with their insertion in this membrane (27). Much less is known about this second subclass, except that in its case the same uncertainties apply as to the location of the polysomes at the time of initiation. By analogy with a rather different system (chloroplast polysomes attached to thylakoid membranes during the synthesis of certain membrane proteins (28)), this type of attachment may be essentially transient, perhaps limited to a single round of translation for each site of attachment. It is generally assumed that all the soluble factors necessary for protein synthesis are present in molecular dispersion or in the form of soluble complexes in the cell sol or cytoplasmic matrix, but very few actual data are available in the case of the pancreatic exocrine cell - although this

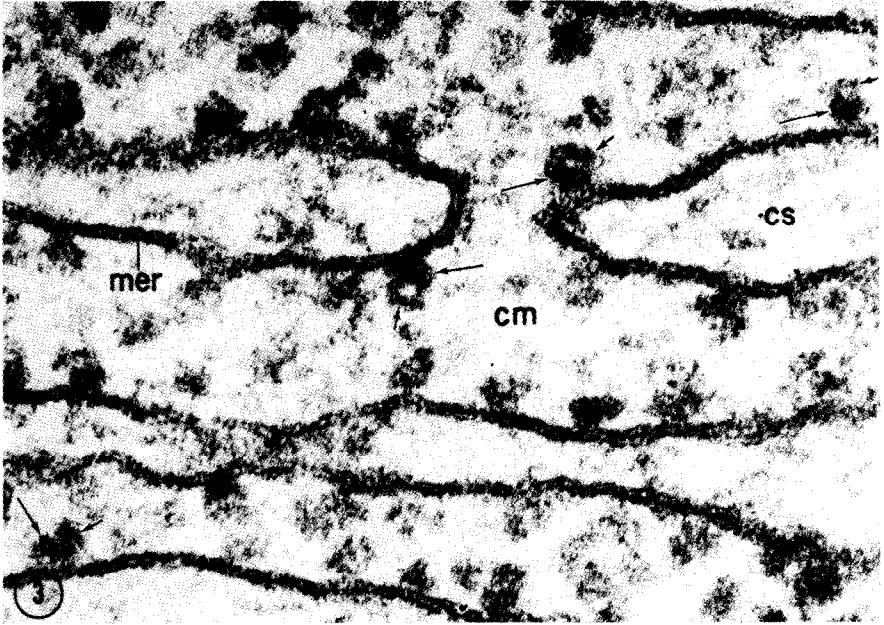


Fig. 3. Pancreatic exocrine cell. High magnification of a cytoplasmic region occupied by cisternal elements of the rough surfaced endoplasmic reticulum.

mer, membrane of the endoplasmic reticulum, cs, cisternal space, cm, cytoplasmic matrix (cell sol). The short arrows point to small subunits and the long arrows to large subunits of attached ribosomes.

X 275,000

cell is potentially a rich source of aminoacyl-t RNA synthetases, tRNAs and mRNAs. The presence of an active RNase among the secretory proteins produced by the cell has discouraged work along such lines, but this whole field may be open by using tissue taken from species known to have a very low pancreatic RNase content. Pancreatic proteolytic zymogens do not appear to constitute a problem, since their activation is either nil or controllable during cell fractionation.

## 2. SEGREGATION

The newly synthesized secretory proteins are segregated in the cisternal space of the rough endoplasmic reticulum. The first evidence that this is the case came from work carried out by Redman *et al* (17) on pigeon pancreatic microsomes synthesizing *in vitro* [<sup>14</sup>C] amylase. This radioactive secretory protein, initially associated with attached polysomes, preferentially appeared after ~ 3 min in the microsomal cavities. Experiments bearing on segregation were further refined in our laboratory by Redman and Sabatini (29) and Blobel and Sabatini (30). Their results indicate that the growing polypeptide chain is extruded through the microsomal membrane into the microsomal cavity which is the *in vitro* equivalent of the cisternal space of the rough endoplasmic reticulum. Upon natural or experimentally induced termination, the

## Segregation

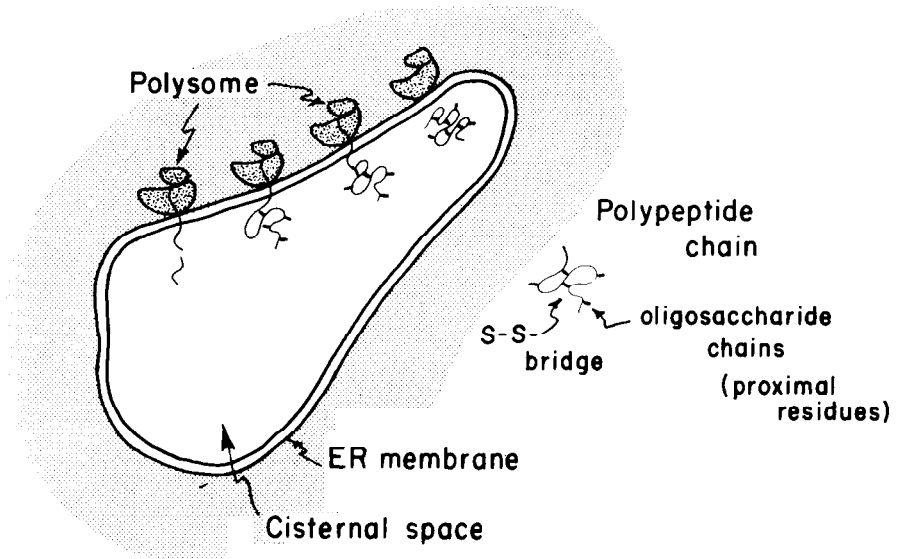


Fig. 4. Diagram of the segregation step.

newly synthesized chain separates with the microsomal vesicles and does not appear in the incubation medium, which topologically is the *in vitro* equivalent of the cell sol. Since it had already been established by Sabatini *et al* (3 1) that the ribosomes are attached to the ER membrane by their large subunits i.e., the bearers of nascent chains) (Fig. 3), it was concluded that segregation is the result of a vectorial transport of the newly synthesized polypeptide from the large ribosomal subunit through the ER membrane to the cisternal space.

This conclusion provides a satisfactory explanation for the basic structural features of the endoplasmic reticulum: a cavitory cell organ of complicated geometry which endows it with a large surface. All these features make sense if we assume that one of the main functions of the system is the trapping of proteins produced for export. With the exception of  $\text{Ca}^{2+}$  accumulation in the sarcoplasmic reticulum, i.e., the equivalent cell organ of muscle fibers, no other recognized function of the endoplasmic reticulum (e.g., phosphatide- and triacylglycerol synthesis, mixed function oxygenation, fatty acid desaturation) requires compellingly and directly a cavitory organ, at least according to our current knowledge. In detail, however, the forces and reactions involved in the trapping operation remain unknown. The interaction of the large ribosomal subunit with the ER membrane is understood only in very general terms (30), and precise information bearing on specific molecules involved in attachment is still lacking. Segregation appears to be an irreversible step: the nascent polypeptide is extruded in the cisternal space and, once inside, it can no longer get out (Fig. 4).

The membrane of isolated microsomes was found to be highly permeable to

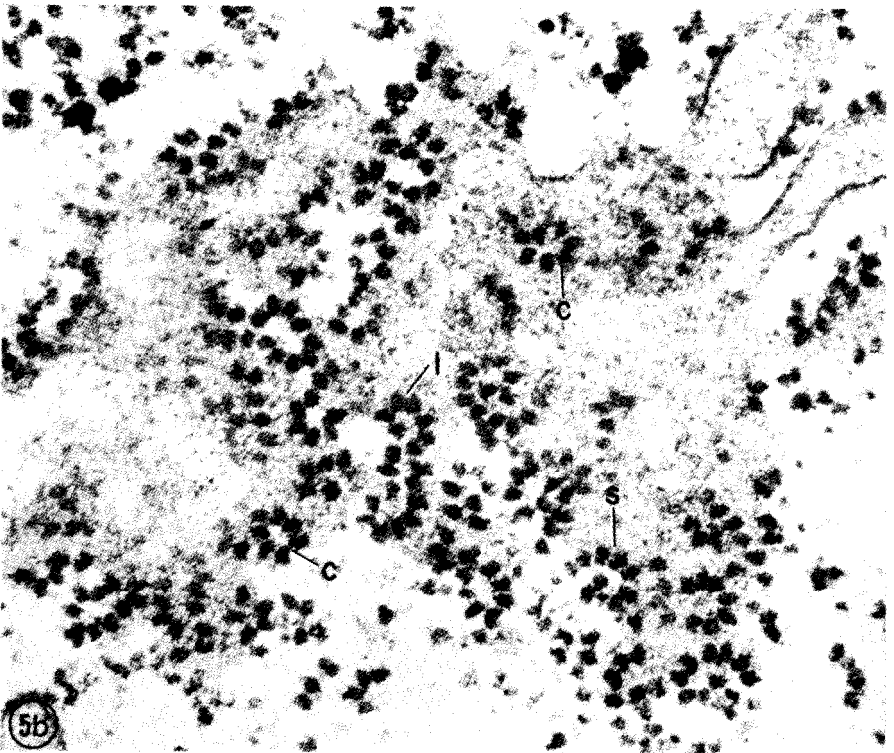
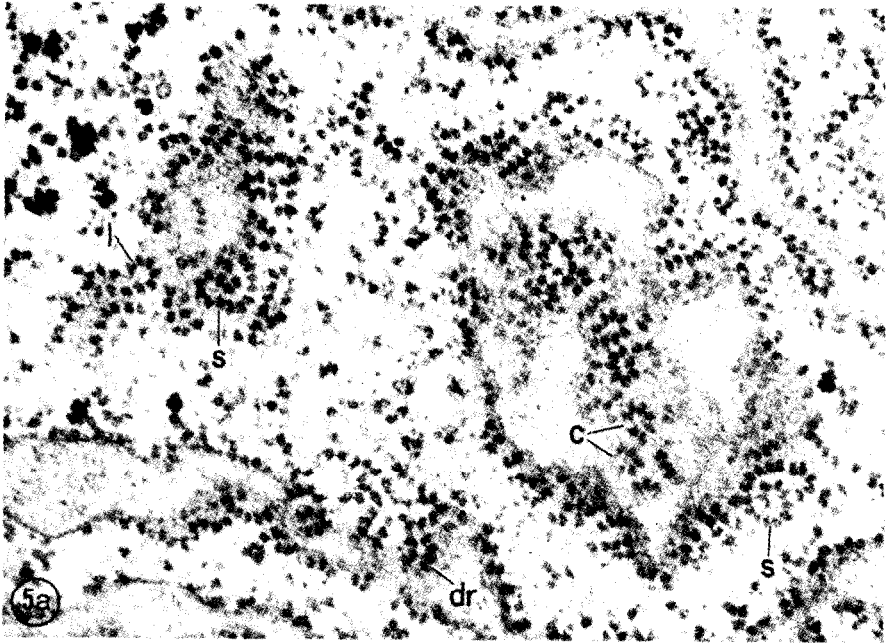


Fig. 5. Rat hepatocyte. The attached ribosomes (polysomes) form spirals (s), loops (l), circles (c) and double rows (dr) on the surface of the endoplasmic reticulum membrane.

a: x 55,000

b: x 90,000



molecules of  $\sim 10\text{\AA}$  diameter (32). Assuming that the same applies for the ER membrane *in situ*, it is reasonable to postulate that the imprisonment of the polypeptide is the consequence of its conversion into a globular protein too large ( $> 20\text{\AA}$  diameter) to permeate the membrane. This postulate is in keeping with a series of findings which show that enzymes associated with the ER membrane, or present in the cisternal space, are responsible for disulfide bridge formation (33), hydroxylation of proline and lysine residues (34), proximal glycosylation of polypeptide chains (35), and perhaps partial proteolysis (cf. 25). All these modifying operations are expected to affect directly or indirectly the tertiary structure of the secretory proteins which, once assumed, could render the proteins impermeant and their segregation irreversible (Fig. 4). Letting disulfide bridge formation aside, it would be of interest to know to what extent modifications of the type mentioned affect proteins produced for intracellular use. If the extent were nil or negligible, the differential modification of secretory proteins would provide an additional explanation for their segregation.

Available evidence either indicates or suggests that vectorial transport of secretory proteins to the cisternal space occurs in many other cell types (e.g., plasma cells (36), fibroblasts (37), granulocytes (38), parotid acinar cells (39) etc.) in addition to hepatocytes and pancreatic exocrine cells. Vectorial transport and its corollary-segregation-are most probably obligate functional features for all protein secreting cells, but further work is needed to check on the actual extent of their occurrence, as well as on possible exceptions (40).

Although the ER membrane is characterized by high fluidity (41), the polysomes attached to its cytoplasmic aspect maintain regular, characteristic patterns (Fig. 5) of rather constant geometry (4). One may wonder what prevents them from assuming a random coil conformation; or, in other words, how does the cell succeed in securing fixed attachment sites on a highly fluid membrane. This riddle must have an interesting answer.

### 3. INTRACELLULAR TRANSPORT

From the cisternal space of the rough endoplasmic reticulum, the secretory proteins are transported to the Golgi complex. In the case we have studied, i.e., the pancreatic exocrine cell of the guinea pig, the terminus of the transport operations is a set of large vacuoles on the trans side of the complex (16, 18) which, on account of their function (to be discussed later on), are called condensing vacuoles.

Intracellular transport was first recognized in radioautographic experiments carried out with Lucien Caro (16), but the details and requirements of this operation became evident only after James Jamieson and I shifted from intact animals to *in vitro* systems based on tissue slices (18). In such systems, short tissue exposure to radioactive amino acids ("labeling pulse") followed by effective removal of unincorporated label ("chase") became possible and, as a result, time-resolution in our experiments was considerably improved.



Fig. 6. Pancreatic exocrine cell. Golgi complex, partial view.

cv, condensing vacuoles; gc, Golgi cisternae; gv, Golgi vesicles; te, transitional elements; rer, rough endoplasmic reticulum.

x 26,000.

Results obtained in pulse-chase experiments showed that the pathway followed by the secretory proteins leads from the rough endoplasmic reticulum to the transitional elements of this system (Fig. 6), then to the small peripheral vesicles on the cis side of the Golgi complex (18) and finally, in about 30 min, to condensing vacuoles (42) (Table II, Fig. 7). An unexpected and intriguing finding was that intracellular transport requires energy (43) supplied (in the system investigated) by oxidative phosphorylation. In the absence of ATP synthesis, the secretory proteins remain in the rough endoplasmic reticulum, transport to condensing vacuoles being resumed upon resumption of ATP production. From these and other data, we concluded that the func-

# Intracellular Transport

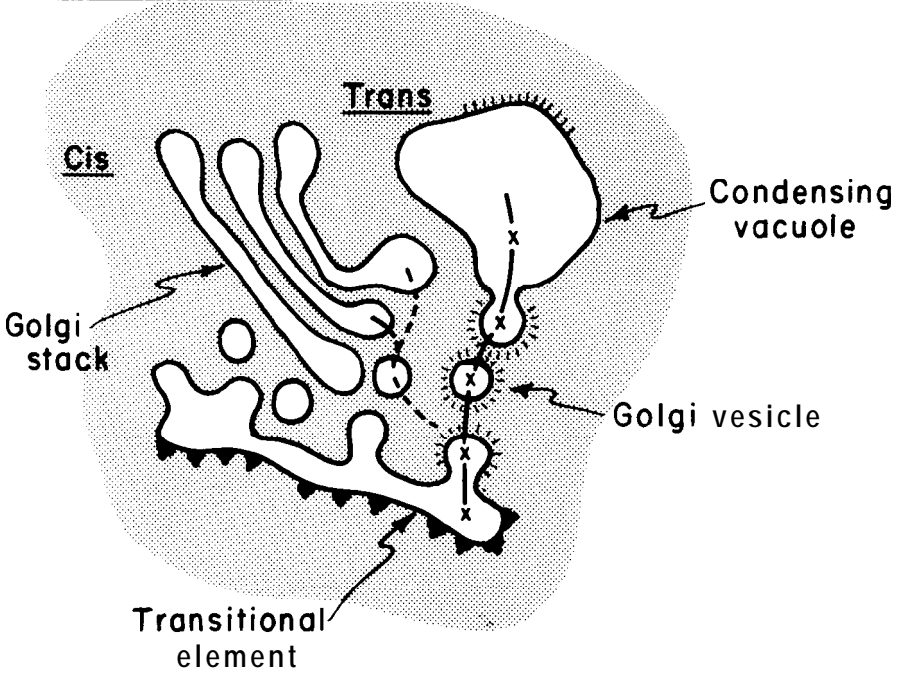


Fig. 7. Diagram of intracellular transport. X - - - X, pathway followed in the pancreatic exocrine cell of the guinea pig; ....., pathway followed in other glandular cells.

Table II. Guinea pig pancreas. Slices incubated *in vitro*\*

Cell Compartments	% radioautographic grains				
	Pulse 3 min	chase minutes			
		+7	+37	+117	
Rough endoplasmic reticulum	86.3	43.7	24.3	20.0	
Golgi complex	peripheral vesicles	2.7	43.0	14.9	3.6
	condensing vacuoles	1.0	3.8	48.5	7.5
Zymogen granules	3.0	4.6	11.3	58.6	
Acinar lumen	—	—	—	7.1	
Other compartments**	7.0	4.6	1.1	3.2	

\* Simplified from J. D. Jamieson and G. E. Palade, J. Cell Biol. 34(1967)597.

pulse: 200  $\mu$ Ci/ml L-[<sup>3</sup>H-4,5]leucine (40  $\mu$ M).

chase: <sup>1</sup>H-leucine (2mM).

\*\* Nuclei and mitochondria

For each compartment of the secretory pathway the maximal concentration figures are given in italics.

tional equivalent of a lock (or lock-gate) exists along the channels used for intracellular transport; that the lock is located at the level of the transitional elements of the endoplasmic reticulum, and that secretory proteins seem to flow vectorially to the Golgi complex, when the lock is opened.

The general pathway followed in intracellular transport appears to be the same in a variety of cell types (19, 44-48), but direct evidence on the pre-Golgi lock has been obtained only in the case of the exocrine pancreatic cell. Extension to other systems of the inquiry dealing primarily with the lock-gate is clearly needed. In addition, many aspects of the transport operation remain either unknown or unsettled. The geometry of the connections between the endoplasmic reticulum and the Golgi complex is still a matter of debate: according to some investigators (49, 50), the two compartments are permanently connected by continuous tubules; according to us (18), the connection is intermittent and is probably established by shuttling vesicles. The energy-requiring reactions are unknown, and equally unknown are the forces involved in transport and the means by which macromolecules are moved from the endoplasmic reticulum to the condensing vacuoles against an apparent concentration gradient.

We have uncovered an interesting process, but we are only at the very beginning of its analysis. Every one of the points mentioned above remains to be elucidated by further work.

#### 4. CONCENTRATION

The secretory proteins reach the condensing vacuoles in a dilute solution which is progressively concentrated at these sites to a level comparable to that eventually found in mature secretion granules. The exact concentration in each of the compartments involved in intracellular transport is unknown; but the increase in the density of the content in condensing vacuoles (as seen in electron micrographs), and the increase in number of radioautographic grains associated with the same vacuoles (42) (Fig. 8) suggest that the incoming solution is concentrated by a large factor. The final result of the concentration step is the conversion of the condensing vacuoles into mature secretion granules (16, 42), usually called zymogen granules in the case of the pancreatic exocrine cell.

Concentration is not dependent on a continuous supply of energy. *In situ*, neither condensing vacuoles nor zymogen granules swell when ATP production is blocked; and *in vitro*, isolated secretion granules are rather insensitive to the osmolality of the suspension medium at, or below, neutrality (51). They are instead highly sensitive to variations in pH and lyse promptly above pH 7.2 (52, 53). The findings rule out the hypothesis that concentration is achieved by ion pumps located in the membrane of the condensing vacuoles, and suggest that the cell uses for this step some other, energetically more economical mechanism. The synthesis of sulfate containing macromolecules in Golgi elements and their presence in secretion granules in murine, pancreatic acinar cells (54) as well as in other murine glandular cells (55) have been

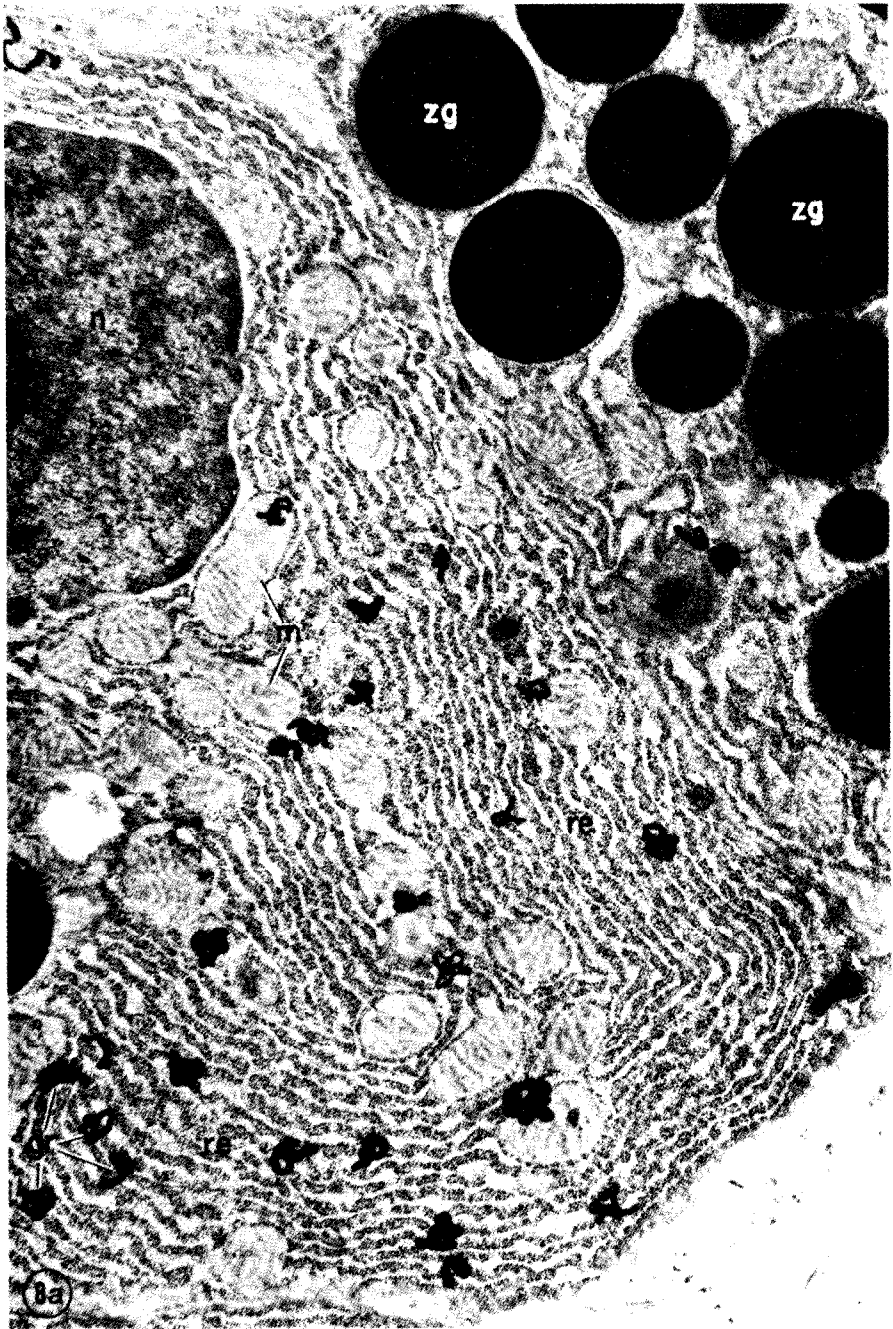


Fig. 8a. Pancreatic exocrine cell. (Guinea pig). Distribution of radioautographic grains in specimen fixed at the end of a 3 min. pulse with L-[ $^3\text{H}$ -4,5]leucine.

gr, radioautographic grains; n, nucleus; m, mitochondria; zg, zymogen granules; re, region of the cytoplasm occupied by the rough surfaced endoplasmic reticulum. At this time, ~ 85% of the grains are found associated with such regions.

x 12,000

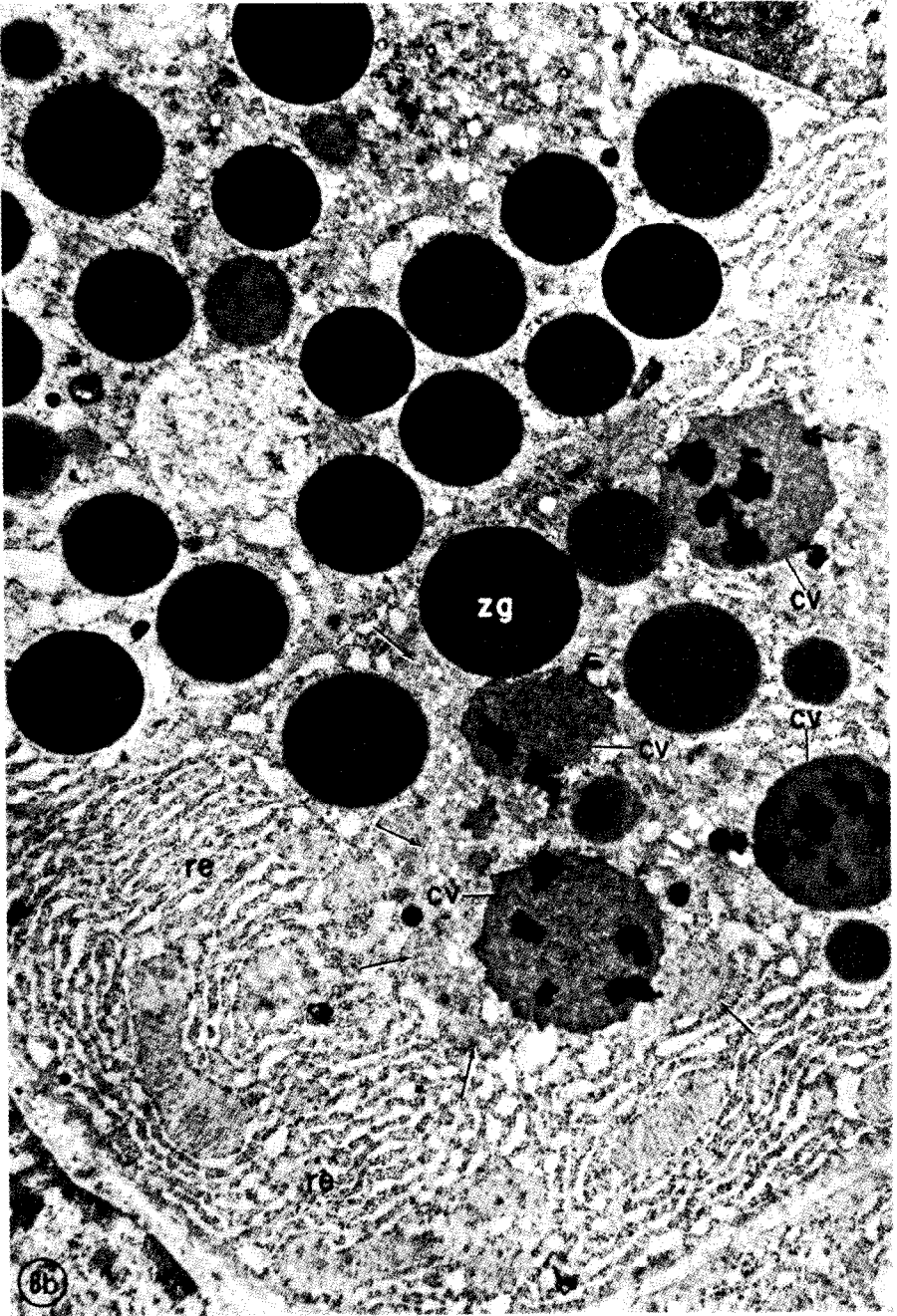


Fig. 8b. Pancreatic exocrine cell. (Guinea pig). Distribution of radioautographic grains at the end of a 37 min chase (after a 3 min pulse as in Fig. 8a).

*cv*, condensing vacuoles; *zg*, zymogen granules; *re*, region of the cytoplasm occupied by the rough surfaced endoplasmic reticulum. The periphery of the Golgi complex is marked by arrows. At this time, ~ 50 % of the radioautographic grains are associated with condensing vacuoles.

x 12,000

Figures 8a and 8b are taken from J. D. Jamieson and G. E. Palade, *J. Cell Biol.* 34, (1967) 597.

established by radioautography. Moreover, Tartakoff et al (56) have recently detected a sulfated polyanion ( $pI \simeq 3.4$ ), presumably a sulfated peptidoglycan, in the content of zymogen granules and in discharged secretion in the guinea pig pancreas. The formation of large aggregates by ionic interactions between this polyanion and secretory proteins, which are known to be predominantly cationic (56), could cause a reduction in osmotic activity within condensing vacuoles with concomitant outflow of water. In this case, energy would no longer be required past the synthesis of the polyanion and concentration would depend primarily on the stability of the postulated aggregates.

This hypothesis remains to be validated by the isolation and characterization of the sulfated polyanion, and especially by the demonstration of relevant aggregate formation under conditions likely to prevail *in vivo* within condensing vacuoles. The hypothesis is particularly attractive because it could explain not only concentration per se, but also intracellular transport against an apparent chemical gradient. Such a gradient may not exist, or may be reversed, if the secretory proteins of every new batch were to be aggregated and thereby osmotically inactivated upon their entry into condensing vacuoles.

In the pancreatic exocrine cell of the guinea pig concentration is effected in trans Golgi condensing vacuoles, but in the same cell of other species (rat, for instance) the step under discussion takes place in the last cisterna on the trans side of each Golgi stack. Finally in many other glandular cells (cf. 57) the same operation is carried out in the dilated rims of the last 2-3 trans Golgi cisternae (Fig. 7). Moreover, in guinea pig pancreatic lobules hyperstimulated *in vitro*, the usual condensing vacuoles are no longer present, and concentration of secretory proteins begins already in the Golgi cisternae, preferentially in those located on the trans side of the stacks (58). There are, therefore, variations according to species, cell type, and physiological conditions in the location of concentration sites within the Golgi complex, and it would be of interest to find out whether these variations reflect changes in the distribution of the sulfated polyanion (or other functionally equivalent compounds) within the complex.

Radioautographic findings (45-47, 59) and cell fractionation data (60) obtained on a variety of tissues indicate that terminal glycosylation of secretory proteins occurs in the Golgi complex. This operation is expected to affect only a fraction, not the totality, of the proteins produced for export.

In addition, the Golgi complex appears to be the site of partial proteolysis of proinsulin (61) and perhaps other secretory proteins. It is also the site of synthesis of polysaccharides in plant cells (cf. 62). The Golgi apparatus has, therefore, a multiplicity of functions in the processing of secretory products, but - with the exception of concentration - the location of the other activities among its elements is either uncertain or still unknown.

On the one hand, there is a rather extensive literature dealing with differences in cytochemical reactions within the same cisterna (63, 64) or among the cisternae of the same stack (65, 66) without any obvious functional correlation. On the other hand, we begin to have biochemical data on Golgi sub-

fractions, but so far they reveal no differences between Golgi cisternae and Golgi vacuoles (67).

Finally, at the level of the Golgi complex the secretory product is transferred from a high permeability membrane (i.e., the membrane of the endoplasmic reticulum), to a membrane whose lipid composition approaches that of the plasmalemma by its high content of cholesterol and sphingomyelin, and by the low degree of unsaturation of fatty acids in its phospholipids (68, 69)). Such a membrane is expected to have a low permeability, and therefore to be "exposable" without danger to the external medium at the time of discharge (see below).

In general, our knowledge of the functions of the complex is still rudimentary primarily because the isolation of Golgi fractions from tissue homogenates was achieved only recently (70-73) and is still limited to a few sources (liver, pancreas (68) and kidney (74)). The extent of compartmentation within the complex as well as the precise pathway followed by secretory products through it is still unknown. Finally, as a telling measure of our ignorance, it is worthwhile pointing out that we do not have any good idea about the functional meaning of the most prominent structural feature of the Golgi complex: the stacking of its cisternae.

##### 5. INTRACELLULAR STORAGE

Secretory proteins are temporarily stored within the cell in secretion granules which, as already mentioned, are condensing vacuoles that have reached the end of the concentration step. Their membrane comes, therefore, from the Golgi complex and their content is the product of attached polysomes, modified at subsequent steps as already described in the previous sections.

In the cases so far investigated, i.e., the exocrine pancreas of the cow (53, 75), rat (76), and guinea pig (56), and the parotid of the rabbit (77), the content of the secretion granules (more precisely, the extract obtained from reasonably homogeneous secretion granule fractions) and the physiologically discharged secretion contain the same proteins in the same relative amounts (Fig. 9). Since no other intracellular sources has been revealed or suggested by our evidence, we have concluded that the content of these granules is the sole precursor of the proteins found in the juice secreted by the gland.

In the case of glands which, like the exocrine pancreas, consist of an apparently homogeneous population of secretory cells which produce a complex mixture of secretory proteins, the question of specialization at the cellular or subcellular level was asked repeatedly and answered only in part. So far all the proteins looked for in the bovine pancreas (trypsinogen (78), chymotrypsinogen, DNase (79) and RNase (80)) were detected by immunocytochemical procedures in all the secretion granules of all cells examined. Each granule probably contains a sample of the mixture discharged by the gland, but it is hard to believe that all these microsamples are quantitatively strictly identical. Specialization at the cellular level is well established in a



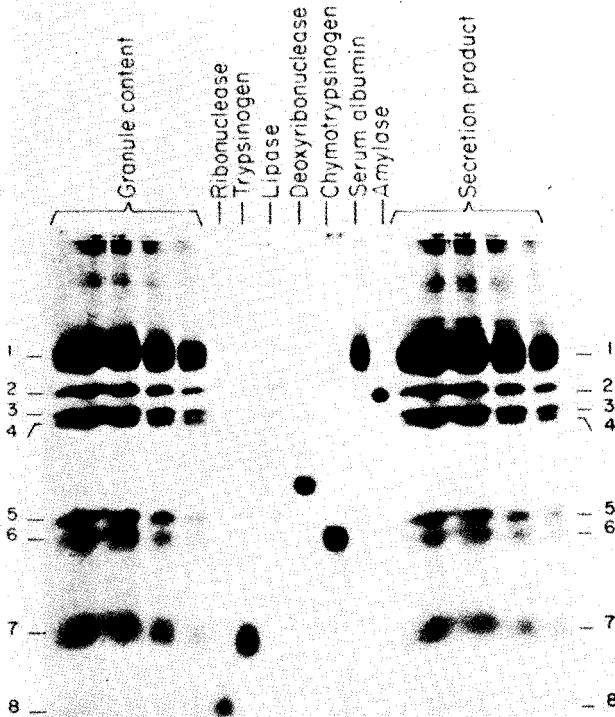


Fig. 9. Sodium dodecyl sulfate-olyacrylamide gel electropherograms of (left to right) zymogen granule content, standards, and secretion discharged by pancreatic lobules incubated and stimulated *in vitro*. Identification of bands: 1, unknown secretory protein and carrier bovine plasma albumin; 2, amylase; 3-4, procarboxypeptidases A and B and unknown secretory proteins; 5, unknown protein; 6, chymotrypsinogen; 7, trypsinogen; 8, ribonuclease.

From A. M. Tartakoff, L. J. Greene and G. E. Palade, *J. Biol. Chem.*, 249, (1974) 7420.

number of endocrine glands which are characterized by a morphologically heterogeneous cell population (cf. 57). Specialization at the subcellular level exists in polymorphonuclear neutrophil granulocytes (35). The formula used in the pancreas, i.e., intracellular storage of a complex mixture in apparently equivalent quanta, probably explains the lack of short term qualitative modulation of the secretory output (see (20, 81) for a more detailed discussion of this point). It can be assumed that this type of modulation is rendered unnecessary by the specialized nutritional habits of each species.

In the exocrine cells of the pancreas, secretion granules usually occupy the apical region between the Golgi complex and the acinar lumen. There are few microtubules in this region and few microfilaments, and there is no consistent pattern in their organization and distribution (except for the micro-

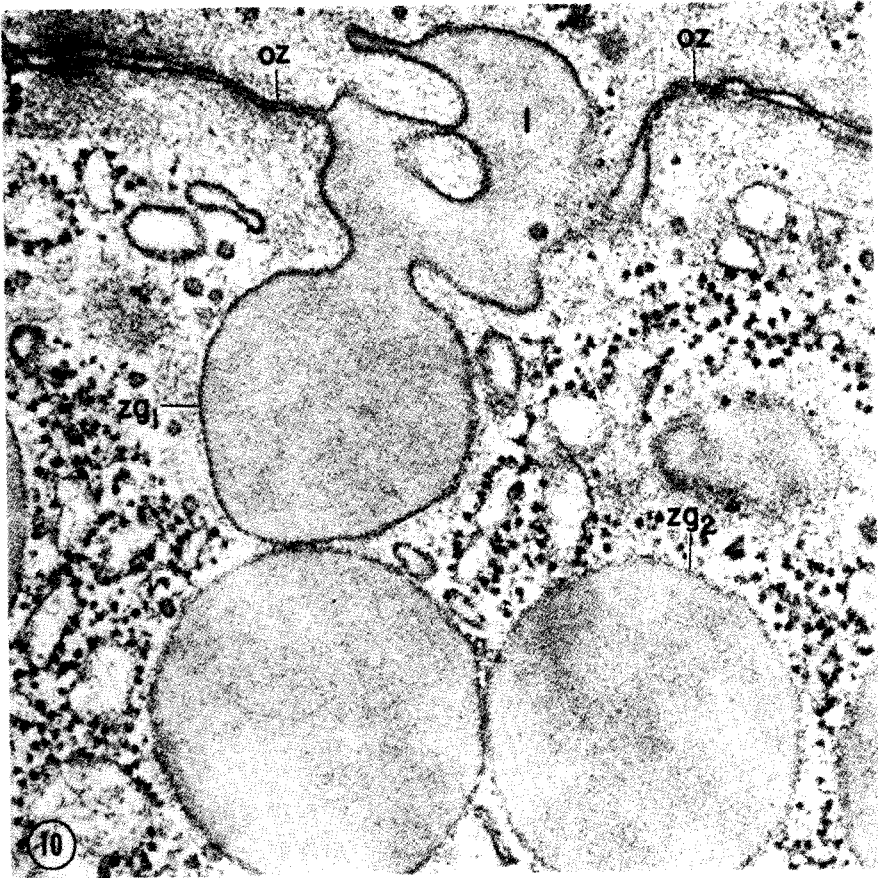


Fig. 10. Pancreatic enocrine cell. Apical region. *l*, lumen; *oz*, occluding zonules; *zg1*, discharging zymogen granule; *zg2*, zymogen granule still in storage.  
x 110,000

filaments associated with junctional elements and microvilli). In other cell types, it has been postulated that microtubules and microfilaments play a role in effecting secretory discharge (see below), as well as in directing or moving secretory granules to their sites of discharge. In pancreatic acinar cells, radioautographic findings show that newly formed, i.e., labeled, granules are distributed at random within the preexisting granule population (42), and biochemical data indicate that newly synthesized and preexisting proteins are discharged at random from the total zymogen granule population (20, 81). With the evidence at hand, these results can be ascribed to slow diffusion leading to thorough mixing of old and new granules within the apical region. In other cell types, the situation may be different on account of incomplete mixing within the granule population and uneven distribution of discharge sites (see below).

## 6. DISCHARGE

Relatively early in the investigation of the secretory process it was found that

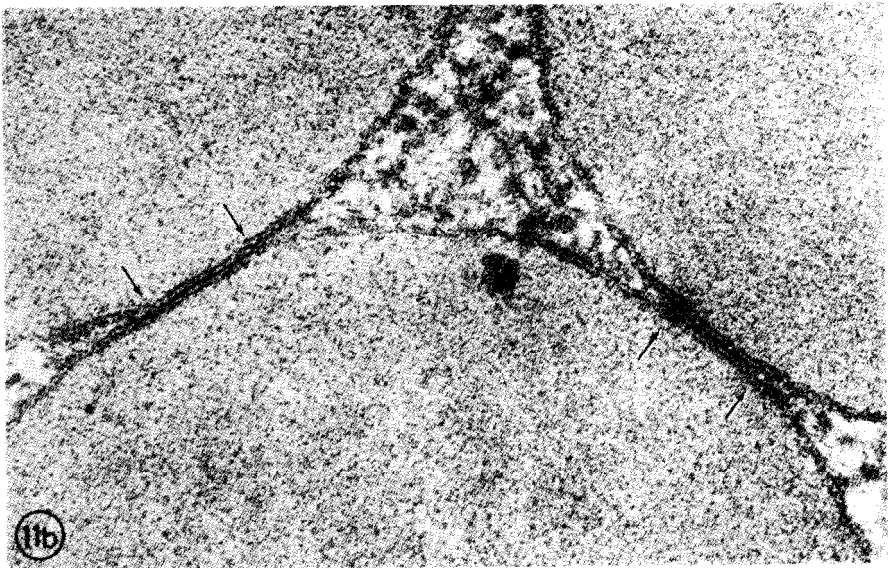
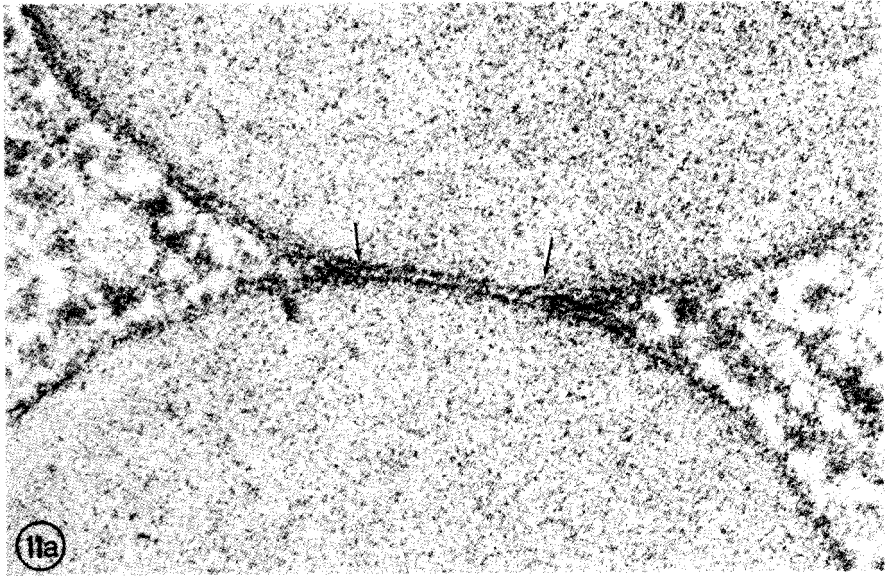


Fig. 11a, b. Pancreatic exocrine cells, Apical region. a. fusion of zymogen granule membranes followed by partial elimination of membrane layers (arrows), b. fusion of zymogen granule membranes (arrows);

a: x 220,000

b: x 160,000

secretion granules discharge their content into glandular lumina (Fig. 10) by a process originally called "membrane fusion" (82) and later on exocytosis (83). Morphological findings suggest that in preparation for discharge the membrane of the secretion granule fuses with the plasmalemma and that subsequent reorganization (i.e., progressive elimination of layers (Figs. 11, 12).

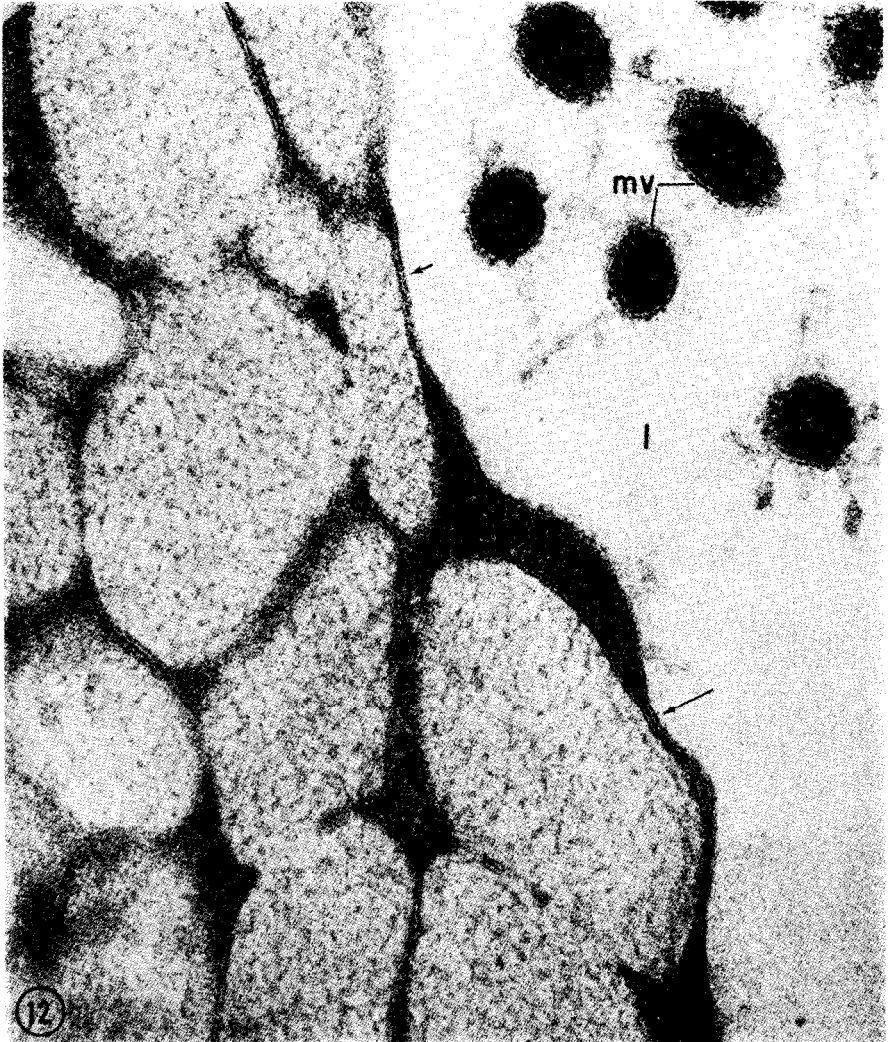


Fig. 12. Intestinal epithelium, Goblet cell. (Rat). Fusion of secretion granule membranes with the plasmalemma. Long arrows: simple fusion; short arrow: fusion with partial elimination of membrane layers.

*l*, lumen; *mv*, microvilli.

x 140.000

leads to fission of the fused membranes within the area of fusion. The final result is continuity established between the granule compartment and the extracellular medium (lumen), concomitantly with continuity of the granule membrane with the plasmalemma all around the orifice through which the granule content reaches the lumen (Fig. 13). This operation allows the discharge of the secretory product while insuring the maintenance of a continuous diffusion barrier between the cell sol and the extracellular medium. At the beginning, a few alternatives were considered, but by now exocytosis is recognized as a widely occurring, probably general mechanism for the dis-

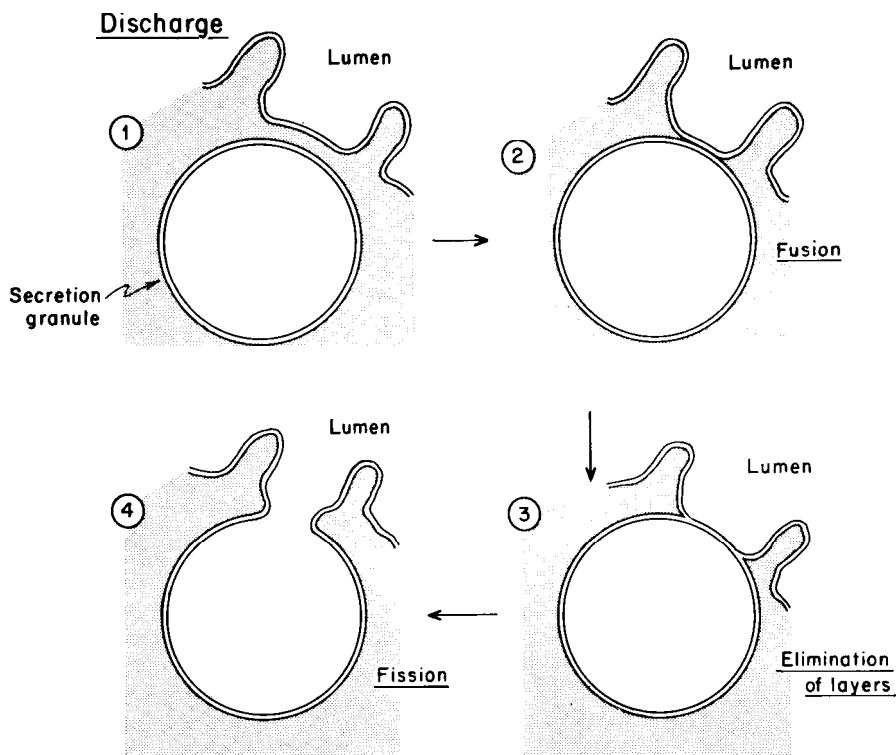


Fig. 13. Diagram of membrane interactions during secretory discharge.

charge of macromolecular secretory products.

The membrane fusion involved in secretory discharge has a high degree of specificity. The membrane of secretion granules fuses only with the plasmalemma, although there are at the time of this event and at comparable distances around the interacting pair many other types of cellular membranes. In the exocrine cells the specificity is even more stringent since ability to fuse is limited to the apical or luminal domain of the plasmalemma. The only permanently operative alternative is preliminary fusion of granule membrane to granule membrane leading eventually to discharge of two or more secretion granules in tandem (84). This type of specificity suggests the existence of complementary recognition sites in each interacting membrane which may be involved in binding preliminary to fusion. In some respects the postulated situation is reminiscent of the interaction between a hormone and its membrane receptor (85), except that in this case the events are intracellular and receptors as well as agonists are assumed to be membrane-bound.

Exocytosis has been extensively studied in a variety of secretory cells and by now its basic requirements for  $\text{Ca}^{2+}$  and energy are well established (86-88). Our own data demonstrated a stringent energy requirement for secretory discharge in the exocrine pancreatic cell and, hence, the existence of a second energy-dependent lock that controls the flow of secretory products from secretion granules in to the acinar lumina (58). Our data also showed that dis-

charge can proceed in the absence of continuous protein synthesis (58).

In certain glandular cells, pancreatic exocrine cells included, discharge is intermittent and well integrated with other activities of the organism. In such cases, the cell which without stimulation discharges at a slow, liminal rate, responds to stimulation by either hormones or neurotransmitters by a dramatic step-up in the rate of exocytosis. The stimulus-secretion coupling (87) often involves of cyclic nucleotide generating system (adenylate cyclase in most cases) and one or more protein kinases (89). But this coupling also involves a depolarization of the plasmalemma. In the case of the pancreatic exocrine cell stimulation definitely leads to membrane depolarization (90), while the activation of a cyclic nucleotide system is still uncertain (91 vs. 89). The final target of the protein kinases is unknown in secretory cells. A hypothesis advanced a few years ago ascribes this role to tubulin (92), but the evidence in case is open to question. Results obtained on other systems (93, 94) suggest that the target might be a membrane protein.

In recent years, a number of agents activating or inhibiting exocytosis have been described and among the latter colchicine and the vinca alkaloids have received considerably attention (95, 96), the general assumption being that their inhibitory effect implies the involvement of microtubules in exocytosis. At present the situation is rather confused and a reasonable interpretation of the numerous and in part contradictory data is hardly possible. A distinction should be made between agents affecting directly membrane fusion-fission, and agents affecting the superimposed regulatory systems which activate and inactivate the coupling between stimulation and secretion. Colchicine appears to affect the basic mechanism, rather than its controls, since it inhibits discharge in hepatocytes, (97, 99), i.e. in cells that appear to lack a stimulus-secretion coupling. In these cells the effect has been localized at discharge, the last step in the secretory process, all previous steps being unaffected (99). But the involvement of microtubules remains open to question since, at least in hepatocytes, the inhibitory effect is prompt and reaches its maximum long before the depolymerization of the microtubules becomes morphologically detectable. Hence, alternative targets should be considered, especially because colchicine binds to membranes (100) and inhibits a number of transport mechanisms in the plasmalemma (101).

As already mentioned, there is no elaborate organization involving microtubules and microfilaments in the apical region of the pancreatic exocrine cells. A rather modest fibrillar feltwork (terminal web) is found under the luminal plasmalemma, but there is no fibrillar lining on the cytoplasmic aspect of the membrane of the zymogen granules while still in storage. However, a fibrillar shell<sup>2</sup> often appears around discharging zymogen granules when their membrane is already in continuity with the plasmalemma. It is continuous with the terminal web, it may consist of contractile proteins (actin?

<sup>2</sup>A fibrillar feltwork exists also at the periphery of the Golgi complex in association with the transitional elements of the ER. Its function, and the function of fibrillar coats or layers occasionally found around Golgi vesicles and vacuoles are unknown.

myosin?), and it may promote the expulsion of the secretion granule content.

EFFECTS OF EXOCYTOSIS AND INTRACELLULAR TRANSPORT ON  
MEMBRANE DISTRIBUTION

The end result of exocytosis is - on the one hand - discharge of a secretory product, and - on the other hand - relocation of secretory granule membranes in the plasmalemma. Under normal steady state conditions, excess membrane must be removed from the receiving compartment (lumen) and membrane added to the donor compartment (secretion granules, or Golgi complex), since the distribution of membrane amounts among these compartments remains relatively constant with time.

The procedures used by the cell to recover and redistribute membrane after exocytosis are unknown. Morphological findings suggest coupled endocytosis and in a few cases, namely in nerve endings (102, 103) and in anterior pituitary cells (104, 105), recovery of organized membrane in the form of endocytic vesicles has convincingly been demonstrated with the help of cytochemical tracers. Moreover, in the case of pituitary cells the recovered membrane was eventually traced to trans Golgi vacuoles and cisternae (104, 105). But the exact nature of this membrane and its ultimate fate remain a matter of speculation.

In the case of discharge, the membranes of the secretory granules can be viewed as a set of individual vesicular containers which move forward from the Golgi complex to the surface during exocytosis and presumably back to the Golgi during coupled endocytosis. In the pancreas (106) as well as in the parotid (107), the rate of synthesis of the proteins of the granule membranes is generally slower than the rate of synthesis of the secretory proteins contained in the granules. Hence, reutilization or recycling of the membrane containers is possible, in principle, but so far it has not been proven.

Assuming that a similar shuttling system of membrane containers operates between the rough endoplasmic reticulum and the Golgi complex, recently obtained evidence indicates that there is no mixing among either the lipid (68, 69) or the protein (67, 108) components of the membranes of the two compartments in the pancreas (guinea pig) and in the liver (rat). These findings impose stringent limitations on membrane interactions since they suggest that lateral diffusion of components is prevented at the time the membranes of the two compartments establish continuity, and that incoming membrane is removed from the receiving compartment according to a non-random formula (67).

The situation may appear unexpectedly complicated, even confusing, but in fact it makes sense since the final result of the restrictions mentioned is the preservation of functional specificity for the membrane of each compartment. This specificity is implied in both the old concept of "marker enzyme," and the newer ideas on sequential modification of secretory proteins as they move along the secretory pathway. The most convincing example is that of the suc-

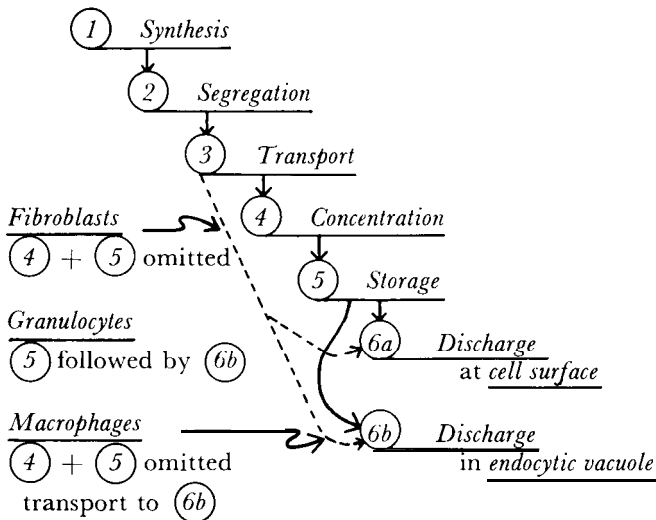
cessive glycosylation of glycoproteins (45-47, 60). The main difficulty is that we do not have at present any clear idea about the means used by the cell to carry through the various steps of the secretory process while imposing and maintaining the restrictions mentioned.

These are intriguing and challenging problems which stress the need for extending the inquiry from the processed product to the processing apparatus, especially to the membranes that outline the compartments which form the processing apparatus. Further understanding of the secretory process is now becoming dependent on adequate information on the chemistry of these membranes and on the reactions involved in their interactions.

VARIATIONS ON A COMMON THEME

The functional analysis of the pancreatic exocrine cell gave us a reasonably good representation of the steps generally involved in the secretory process. In addition, it helped us understand a series of special cases in other cell types which now appear to be recognizable variations on the theme already described. (Table III).

Table III. Secretory Process. Variations on a common theme.



Endocrine cells producing peptide or protein hormones follow the same sequence of operations but apparently discharge their secretory product along the entire plasmalemma (57), instead of discharging within restricted plasmalemma domains as exocrine cells do. In many secretory cells (e.g., fibroblasts, chondrocytes, plasma cells), the concentration step is omitted, secretion granules of usual appearance are absent, intracellular storage is reduced in duration or eliminated, and discharge seems to take place continuously. In such cells, the applicability of the last 3 steps of the general scheme was in doubt and the possibility of direct discharge from the cisternal space of the



endoplasmic reticulum was considered (109). But recently, equivalents of secretion granules were recognized in special fibroblasts, i.e., odontoblasts (110), as well as in ordinary fibroblasts after treatment with colchicine (111). Their secretory process now appears as a variation on the common theme with the variant step resulting from lack of extensive concentration in the Golgi complex. In plasma cells the equivalent of secretion granules is still not yet identified (47).

In polymorphonuclear neutrophil and eosinophil granulocytes, secretion granules are preferentially discharged into endocytic vacuoles (112, 113), discharge at the cell surface occurring only under special conditions (114). In eosinophils, the entire population of secretion granule consists of primary lysosomes, while in neutrophils the population includes "specific granules" in addition to primary lysosomes. In both cell types, all secretory proteins - irrespective of their nature - appear to be produced and processed according to the general scheme worked out for the pancreatic exocrine cell, except for the variant already mentioned at the discharge step.<sup>3</sup>

In macrophages, discharge of secretory proteins is also preferentially effected into endocytic vacuoles, but in addition the concentration step is apparently omitted. A dilute solution of acid hydrolases is carried probably by small vesicles (the local equivalent of primary lysosomes) from the Golgi complex to endocytic vacuoles. The latter are also able to fuse with secondary lysosomes which provide a second hand source of hydrolases (115). The variation on the common theme used by macrophages seems to be applied in all cells capable of autophagy and low efficiency heterophagy including cells specialized in protein production for export, like the hepatocytes, exocrine cells of the pancreas and cells of the anterior pituitary. A special problem arises in this case in connection with the separation of regular exportable proteins from lysosomal hydrolases. The separation seems to be reasonably efficient, though not perfect, since acid phosphatase activity has been repeatedly detected by histochemical procedures within regular secretion granules-mature and immature-and within trans Golgi cisternae (65, 116). In addition, it has been postulated that in a number of cell types lysosome formation takes place in a special compartment, called GERL (117), intercalated between the endoplasmic reticulum and trans Golgi elements. It is evident that all these cells are capable of handling concomitantly, and probably in the same production apparatus two "incompatible" lines of secretory proteins, but the means by which the products are separated or their inactivation prevented (in case of mixing) remain unknown. This riddle must also have an interesting answer.

Finally, another variation on the common theme has been found in glandular cells which produce protein or glycoprotein hormones, and are faced with an excess of stored product (116, 57). In this case the secretion granules are discharged directly into secondary lysosomes by simple membrane fusion. The process, called crinophagy was originally discovered in pituitary mammothrophs (116), but further work has shown that it probably occurs in all the cells of

<sup>3</sup>And except also for the fact that specific granules and primary lysosomes are formed on opposite sides of the Golgi complex of the neutrophil granulocytes (38).

the anterior pituitary (57) and probably in those of many other glands. The use of lysosomes for degrading excess secretory proteins stresses once more the need for understanding protective means against lysosomal hydrolases which must be at work along the entire secretory pathway beginning with the endoplasmic reticulum.

#### ON THE GENERALITY OF THE SECRETORY PROCESS

The evidence already discussed stresses the role played by the endoplasmic reticulum and the Golgi complex in the production and processing of secretory proteins. The stress put on secretion leads, however, to an apparent impasse. Since every eukaryotic cell possesses both an endoplasmic reticulum and a Golgi complex, it follows that all eukaryotic cells secrete proteins or that the organs of the secretory pathway have additional, perhaps more general and more important functions than secretion, which have been ignored or are still unknown.

This problem actually concerns fewer cell types than generally assumed since secretion of macromolecules has been recognized in recent years as an important activity in a wide variety of cells. Interestingly enough, all plant eukaryotes are secretory cells since they produce and discharge the polysaccharides and proteins of their cell walls (118). Among animal eukaryotes, male (119) and female (120, 121) gametes produce protein for extracellular use<sup>4</sup> and so do secretory nerve cells (122) including adrenergic (87) and presumably cholinergic (123) neurons. Smooth muscle cells have been recently recognized as producers of collagen, elastin and other proteins of the intracellular matrices (124), and the same probably applies for a variety of epithelia (including the vascular endothelium) in relation to the production of the corresponding basement membranes (125, 126).

For those animal cells for which a protein product for extracellular use has not been identified, an acceptable answer is provided by the production of lysosomal enzymes. As already mentioned, the production of these enzymes involves the same secretory apparatus (i.e., the endoplasmic reticulum and the Golgi complex) and the same sequence of steps (except for extracellular discharge) as in bona fide glandular or secretory cells. It appears, therefore, that - for the moment and with the evidence at hand - the problem can be solved in favor of the first alternative, i.e., all eukaryotic cells produce secretory proteins, the basic general secretory functions being the production of cell wall components in plant cells and the production of lysosomal enzymes in animal cells. To some extent, each type of basic production must be represented in the other kingdom. On top of these lowly but ubiquitous secretory activities, appears to be superimposed the production of highly specialized proteins exported by a variety of differentiated cell types. Our attention has been focused on the latter long enough to lose proper perspective and to

<sup>4</sup>In many species, female gametes produce vitellus proteins by using in part or in toto the secretory pathway (127).

assume (as we did until recently) that the secretion of proteins is a specialized function restricted to a few, highly differentiated, glandular cells.

Notwithstanding the conclusion reached in the preceding paragraph, the second alternative, i.e., the involvement of the secretory pathway in another general, but still unrecognized function, is not excluded. Among the non-secretory functions postulated for the endoplasmic reticulum and the Golgi complex is the production of cellular membranes, plasmalemma included (cf. 62). At present this postulate rests only on suggestive evidence, most of it morphological. This situation brings us back to the necessity of obtaining detailed and-if possible-comprehensive data on the chemistry and function of the different membranes of the secretory pathway and on their interactions. With this type of information, the second alternative could be put to test, and in the same time our understanding of the secretory process and of the basic organization of eukaryotic cells could be further advanced.

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