Biogenesis of Secretory Vesicles

HSIAO-PING HSU MOORE, LELIO ORCI, AND GEORGE F. OSTER

I. Introduction
II. Structure and Function of Secretory Organelles
   A. Types of Secretory Vesicles
   B. Molecular Composition of Secretory Vesicles
III. Formation of Secretory Vesicles
   A. Molecular Sorting of Proteins: General Principles
   B. Sorting of Secretory Proteins in Storage Cells
   C. Budding from the Golgi: Mechanical Considerations
   D. Recycling of Membrane Components
IV. Conclusion and Perspectives
   References

I. INTRODUCTION

Most cells secrete continuously. The process of excreting substances into the extracellular milieu is accomplished by packaging materials into membranous vesicles and transporting them to the cell surface. There they fuse with the plasma membrane and dump their contents to the outside world. Historically, only specialized secretory cells, such as endocrine, exocrine, or neuronal cells, were classified as "secretory," for only in these types of cells could secretory vesicles be unequivocally identified. We realize now that all cells are equipped to secrete, but they have different types of secretory vesicles. The "professional" secretory cells store their products in morphologically distinguishable vesicles, which accumulate in the cytoplasm. Other cells do not present obviously distinguishable secretory vesicles, despite the fact that biochemical assays reveal that they are secreting quite prodigiously. For example, Fig. 1 shows two kinds of secretory cells. The insulin cell contains numerous
dense-core vesicles, while the plasma cell—which secretes quite vigorously—shows few discernible cytoplasmic vesicles. This is because the lifetime of a vesicle may be only a few minutes, so that not many vesicles accumulate within the cell. Thus we can distinguish between two types of secretion mechanism: (i) vesicles secreted as soon as they are created, the "constitutive vesicles," and (ii) "storage vesicles" which accumulate in the cytoplasm before release.

As we shall discuss, the two types of vesicles differ not only in their secretion properties but also in their mechanism of biogenesis. Thus it is important to bear in mind that the classic definition of "secretory granules" refers to only the storage-type secretory vesicles present in professional secretory cells. For discussion of this particular type of secretory vesicles, readers may consult several reviews (Palade, 1975; Winkler, 1977; Holtzman, 1977; Hand and Oliver, 1981; Orci, 1982; Steiner, 1984; Farquhar and Palade, 1981; Whittaker, 1986). In this chapter we shall devote our attention to the distinctions between the different pathways of protein secretion, and we shall discuss the biogenesis of both constitutive and storage vesicles.

An important advance in our understanding of the assembly of secretory granules came from the observation that a single cell can sometimes manufacture more than one type of vesicle. For example, recent experiments have shown that endocrine cells possess both constitutive and storage vesicles, and that each type of vesicle contains different sets of proteins (Gumbiner and Kelly, 1982; Moore et al., 1983b). Moreover, epithelial cells manufacture at least two different types of constitutive vesicles: some are routed to the apical surface, and others find their way to the basolateral surface. These vesicles provide the vehicles for transporting different proteins to distinct domains on the cell surface (for a review, see Simons and Fuller, 1985).

The realization that vesicles in the same cell may have different fates and contain different proteins raises an important question: How do proteins find their way to the appropriate vesicle? This question is stimulating much research on how secretory vesicles are formed and how the cell

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Fig. 1. Differences in pathways of protein secretion among higher eukaryotes: examples of storage secretory cells (A) and constitutive secretory cells (B). Both cells secrete large amounts of protein products, but only the storage cells contain morphologically identifiable secretory granules. (A) Classic endocrine secretory cell from the pancreas. The cell stores insulin and releases it when the plasma glucose level increases. The morphological hallmark of this type of secretory cell is the numerous dense-core secretory granules (sg) in the cytoplasm. (B) Plasma cell which constitutively synthesizes and exports immunoglobulins. In these cells, there is no accumulation of secretory vesicles, although the compartments of protein synthesis and membrane insertion (the rough endoplasmic reticulum, RER) and of protein processing and sorting (the Golgi complex, G) are conspicuous.
sorts and routes its proteins to the right vesicle. In this chapter we shall emphasize some recent progress in understanding the protein-sorting problem. However, we shall not attempt to cover sorting into all types of secretory vesicles; rather we shall focus mostly on a particular model system—the adrenocorticotropic hormone (ACTH)-secreting pituitary cell line (AtT-20)—under the assumption that the principles governing these cells can be applied to sorting and secretory activities in other neural and endocrine cells. For sorting in polarized epithelial cells, readers are referred to several outstanding recent reviews (Simons and Fuller, 1985; Rodriguez-Boulan et al., 1985; Matlin, 1986). In Section II we discuss the molecular structure and function of secretory organelles. Section III discusses how the components of the vesicles are sorted and assembled. Finally, we shall present our view on the most productive avenues of inquiry for future research.

II. STRUCTURE AND FUNCTION OF SECRETORY ORGANELLES

Proteins are passengers on a transport pathway destined for the cell surface. The Golgi plays the role of the train station, with stationmaster proteins that help direct the passenger proteins to the correct departure sites. The transport machines—the secretory vesicles—must also contain specific molecules that enable it to propel itself to, and fuse with, the plasma membrane. Thus the secretory vesicles must contain at least two varieties of molecules: the passenger proteins themselves as well as transport and recognition machinery. Since the mid-1970s vesicles from a number of systems have been isolated and their composition characterized. While the studies have greatly clarified the nature of the passenger proteins, our knowledge of the transport and recognition proteins remains quite sketchy. This is because good assay systems for these proteins have not yet been developed. Therefore, in this section we shall try to formulate a conceptual framework within which one can incorporate future knowledge about the various molecular components found in secretory vesicles. To appreciate the roles of the transport and recognition machinery we need to first discuss the life cycle of a typical secretory vesicle.

A. Types of Secretory Vesicles

1. Life Cycle of Secretory Vesicles

In most cases, the amount of membrane inserted into the cell surface is far greater than that required for cell growth. Therefore, much of the membrane material comprising the secretory vesicles must be recycled continuously. Figure 2 shows a schematic view of the life cycle of a secretory vesicle. For descriptive purposes, we can break down this cycle into the following steps: (1) assembly in the Golgi region; (2) transport to the cell periphery; (3) anchoring to the release site at the cell surface; (4) fusion with the plasma membrane and release of contents; and (5) retrieval of membrane components by endocytosis and recycling of membrane components back to newly formed secretory vesicles.

2. Classification of Secretory Vesicles

While all vesicles share this general life cycle, there are many types of vesicles, and their components differ according to their specific roles in the cell. One can classify secretory vesicles in several ways. The most obvious classification is by their physical appearance, which reflects differing contents. Many nerve terminals contain two types of secretory vesicles with distinct morphologies. Those vesicles containing small neurotransmitter molecules are typically translucent, while those containing peptide hormones have electron-dense cores (Fig. 3a). Another difference between vesicle types is their destination. Exocrine cells discharge the vesicle contents vectorially from their apical surfaces to the inner or outer surfaces of the organism, while endocrine cells secrete into the blood. Many epithelial cells can direct vesicles to either their apical or

Fig. 2. Life cycle of a secretory vesicle. (1) Proteins destined for secretion accumulate in the trans Golgi where they are packaged into secretory vesicles. (2) The vesicles are transported to the cell periphery. Storage vesicles remain in the cortex (3) until an external signal triggers membrane fusion and exocytosis (4). Constitutive vesicles do not require any external signal to fuse with the plasma membrane. Membrane proteins destined for recycling are reinternalized by endocytosis (5). They are then routed back to the Golgi for incorporation into newly formed secretory vesicles. EN, Endosome.
this classification is not exclusive. For example, a constitutive vesicle may be secreted in either a vectorial or a nonvectorial fashion. A vesicle can have more than one of these constituents and so possess more than one of the above characteristics.

B. Molecular Composition of Secretory Vesicles

1. Transported Proteins and Their Processing Enzymes

As discussed earlier, secretory vesicles contain two types of molecules: passenger proteins and transport machinery. In storage cells, the transported protein consists mainly of a specialized secretory product. For example, the storage vesicles of the pancreatic β cell contain insulin, and the somatotrophs in the pituitary contain growth hormone. In contrast, constitutive vesicles normally contain a variety of soluble and membrane proteins destined for the cell surface. In hepatocytes, for example, transferrin, albumin, and the glycoprotein of vesicular stomatitis virus (VSV) G are found in the same vesicles (Strouss et al., 1983). Similarly, yeast mutants defective in exocytosis accumulate in the same vesicle both the plasma membrane ATPase and the secreted enzyme, acid phosphatase (Holcomb et al., 1987).

Many of the peptide hormones undergo proteolytic processing at pairs of basic residues which, in insulin cells, takes place in the newly formed storage vesicles (Orci et al., 1985a). Therefore, the processing enzymes must accompany their substrate protein into these vesicles. Indeed, several enzymes associated with this processing have been found in secretory vesicles of storage cells (for review, see Loh et al., 1984). Two important questions are, Do enzymes capable of cleaving proteins at pairs of basic residues also reside in the constitutive vesicles, and if so, are they identical to those enzymes which process peptide hormones? This type of proteolytic processing can indeed take place somewhere in the constitutive pathway. For example, the constitutively exported viral protein, gp70, of murine leukemia virus is processed from its precursor, gp90, in AtT-20 cells (Gumbiner and Kelly, 1982). Moreover, yeast cells process the mating hormone α-factor, at dibasic residues in the secretory pathway, which is presumed constitutive (Julius et al., 1984a). At present, however, the exact location of processing in the constitutive pathway is still unknown. Moreover, the enzymes in the constitutive pathway do not appear to be identical to those found in the storage pathway. The α-factor processing enzyme, Kex2, has a different pH optimum than the pH found in the coated granules where proinsulin is processed (Julius et al., 1984b; Orci et al., 1986a).
Finally, it is worth noting that secretory vesicles containing large proteins typically acquire their cargo at the Golgi, while vesicles containing smaller molecules, such as neurotransmitters, may ingest them directly from the cytoplasm. In some cases it appears that biosynthesis of neurotransmitters continues in the vesicles, since biosynthetic enzymes are found there, e.g., dopamine β-hydroxylase (dopamine β-monooxygenase) in chromaffin granules.

2. Proton Pumps

In addition to the passenger proteins and their processing enzymes, secretory vesicles possess the molecular equipment required to perform the other functions suggested by Fig. 2. Important components of storage vesicles are proton pumps, which generate an acidic environment inside the granules (Fig. 4). These serve at least two functions. In vesicles containing dopamine or acetylcholine, the proton pumps provide the protonotive force for the uptake of neurotransmitters from the cytoplasm. Indeed, proton pumps and the transporter which performs this uptake reside in the vesicle membrane (Parsons et al., 1986; Henry et al., 1986; Johnson 1986; Kirshner et al., 1986; Apps and Percy, 1986). In vesicles containing peptide hormones the proton pump appears to provide a pH environment conducive to enzymatic processing. Lowering the internal pH may also play a role in sorting of peptide hormones, as we shall discuss below.

3. Transport Machinery

Once the secretory vesicle has formed, it must be transported to the cell surface. This could be accomplished either by random diffusion or via active transport along cytoskeletal elements. In cells where vesicles must travel long distances there is good evidence that they are actively transported. In neurons, vesicles move along microtubules, driven by the mechanochemical molecule kinesin (Vale et al., 1985). In pancreatic B cells disruption of microtubules also slows the intracellular transport of newly synthesized proinsulin and its conversion to insulin (Malaise-Lagae et al., 1979). However, microtubule-dependent transport has not been demonstrated in cells where vesicles do not travel far to reach the cell surface. In yeast cells, for example, disruption of the gene encoding for microtubules does not affect secretion rate nor the polarized membrane growth that accompanies budding. Instead, it appears that actin filaments play a role in transporting vesicles to the cell surface: disruption of actin structural genes slows the rate of secretion, leading to accumulation of vesicles in the cytoplasm (Novick and Botstein, 1985). In fibroblasts, following disruption of the microtubules, the overall rate of secretion is also unaf

Fig. 4. Storage granules of endocrine cells are characteristically acidic. The acidic compartments inside a cell can be identified by uptake of a weak base, 3-(2,4-dinitroanilino)-3-amino-N-methylidpropylamine (DAMP) (Anderson et al., 1984). DAMP contains a dimtrophenol moiety which can be labeled with antibodies against this hapten and visualized by protein A–gold techniques. DAMP immunostaining of an insulin (A) and an A1T-20 cell (B) are shown. In both cell types, gold particles are distinctly concentrated on the core of secretory granules (sg). In the insulin cell the core can appear as an elongated crystallloid, or a round mass, while in the A1T-20 cell all cores are roundish.

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4. Targeting

To date, no specific molecules which target secretory vesicles to the plasma membrane have been identified. However, there is suggestive evidence in several cell types that such molecules do exist. In neurons, for example, there are specific "zones of release, near which vesicles attach to the plasma membrane. It is possible that this involves interactions involving components of the cortical cytoskeleton, such as spectrin. In this regard, it is important to note that synapsis-1, a major protein found in brain synaptic vesicles, is homologous to the band 4.1 protein in red blood cells, which links spectrin and actin (Huttner et al., 1983; Llinas et al., 1985; Goldenring et al., 1986; Baines and Bennett, 1985). In chromaffin cells, there is also evidence that the subplasmalemmal microfilament network is modified during exocytosis (Marie-France et al., 1986).

5. Fusion and Regulation of Release

In storage cells, exocytosis of the secretory vesicle is often triggered by an external signal, such as membrane depolarization, binding of a hormone, or a neurotransmitter. Is there a common feature shared by these stimuli? Most triggered exocytosis is accompanied by a rise in intracellular calcium. Raising of intracellular calcium concentrations can be brought about in several ways (Fig. 5). In neuromuscular junctions, the membrane depolarization accompanying action potentials triggers a rise in internal calcium by opening voltage-dependent calcium channels (Fig. 5a). In other situations, intracellular calcium is controlled by different second messengers. For example, in corticotrophs, binding of corticotropin-releasing factor triggers the influx of extracellular calcium by first increasing the level of intracellular cAMP (Luini et al., 1985; Reisine et al., 1985; Fig. 5b). In many cell types the rise of intracellular calcium results from the release of internal stores, rather than influx from the surroundings. In these cases, binding of ligands triggers the hydrolysis of phosphatidylinositol to inositol trisphosphate (IP$_3$) and diacylglycerol (DG). IP$_3$ is the proximal trigger releasing calcium from membranous stores (Streb et al., 1983; Fig. 5c). Recent evidence suggests that IP$_4$ may also play a role in regulating the influx of external calcium (Houslay, 1987).

How does a rise in intracellular calcium promote exocytosis? There are two physical barriers that prevent fusion of lipid bilayers: the electrostatic repulsion between the negatively charged surfaces and the hydration layer that prevents the layers from coming in contact (Lucy and Akhong, 1986). In vitro experiments on fusion of lipid bilayers indicate that divalent cations are particularly effective in shielding the electrostatic forces, and an osmotic pressure difference across the membrane compartments is effective in removing the hydration barrier. However, as we shall discuss below, while calcium is implicated in regulating secretion in storage cells, the levels of calcium involved are not nearly as high as those required in vitro bilayer fusion. It is more likely, therefore, that calcium is a regulatory agent in controlling the activity of specific fusagens which overcome the electrostatic and hydration barriers via their hydrophobic structure. The best example of a protein-mediated membrane fusion is that produced by viral surface glycoproteins, such as the hemagglutinin of influenza virus (White et al., 1983). The activity of these fusagens is triggered by a drop in pH, which exposes a hydrophobic fusion peptide. It is possible that the fusion of vesicles with the plasma membrane in storage cells is mediated by a protein which responds to calcium in a similar fashion.

The fact that calcium appears to trigger exocytosis has stimulated the
search for regulatory intermediates. Several candidates are currently under investigation. Synexin is a protein that promotes aggregation of chromaffin vesicles in the presence of calcium (Scott et al., 1985). Calmodulin is a common intermediate in calcium-regulated responses; therefore, it is natural to seek a role for it in regulating exocytosis. Indeed, both calmodulin and calmodulin binding sites have been detected on synaptic vesicles isolated from electric fish (Hooper and Kelly, 1984a,b). Also, injection of calmodulin antibodies into chromaffin cells diminishes amine release (Trifaro, 1986).

Despite its apparent ubiquity, exocytosis is not always accompanied by a rise in intracellular calcium. Rink et al. (1983) found that diacylglycerol (DG) and phorbol esters—both activators of protein kinase C (PKC)—when applied to platelet cells can increase the release rate of serotonin without an associated rise in intracellular calcium. Several examples of such heightened secretion in storage cells, unaccompanied by elevated cytosolic calcium, have since been observed. It is interesting to note that constitutive secretion also does not appear to require elevated calcium levels (Tartakoff and Vassalli, 1978). It will be important to determine whether these types of calcium-independent exocytosis employ a different mechanism from calcium-dependent secretion. It is possible that they use the same mechanism, albeit with modified calcium sensitivity. In this regard, it is interesting to note that PKC can reduce the activation threshold for calcium dependence on exocytosis (Knight and Scrutton, 1984).

It is apparent from this abbreviated discussion that, aside from the passenger proteins and their processing enzymes, much work lies ahead before we fully understand the molecular components of secretory vesicles.

III. FORMATION OF SECRETORY VESICLES

The composition of the secretory vesicles is, as we have seen, quite complex. In addition to the peptide hormones, these vesicles must contain molecules for hormone processing, targeting, transport of the vesicle, fusion with the plasma membrane, and recycling of the membrane components. How are these components brought together during their biosynthesis? Unfortunately, most of the components of the secretory vesicles have not been explicitly isolated, and their mechanisms of action are only vaguely understood. Thus our understanding of the assembly of secretory vesicles has derived mostly from the study of the only component that has been well characterized: the peptide hormone itself. For the purposes of discussion, we shall divide the formation of the secretory vesicles into three steps: (a) sorting, assembly, and packaging of its constituents in the Golgi region; (b) budding of the vesicles; and (c) transport of the vesicle to the cell surface and the subsequent recycling of the membrane components. This breakdown may not correspond to the actual sequence of events. For example, sorting and budding may not be strictly sequential events (see below, and Fig. 8).

A. Molecular Sorting of Proteins: General Principles

1. Why Is Sorting Necessary?

Most proteins are manufactured by cytoplasmic ribosomes. Although many proteins remain in the cytoplasm, many others are inserted into membrane organelles, such as the rough endoplasmic reticulum (RER), nucleus, and mitochondria. Therefore, there must be subsequent sorting events to direct the different proteins to their appropriate target organelles.

2. Sorting of Proteins Is a Hierarchical Procedure

Sorting occurs in multiple steps, much like a zip code system, as shown schematically in Fig. 6 and 7. From the ribosomes the proteins are routed to four major destinations: the cytoplasm, nucleus, mitochondria, and endoplasmic reticulum (ER) (Fig. 6). Proteins that possess a specific “signal peptide” are inserted into the ER membrane and will then be routed to the secretory pathway. Similarly, proteins equipped with mitochondrial- or nuclear-specific tags are routed to the various mitochondrial or nuclear compartments (for reviews, see Schatz and Butow, 1983; Hurt and Loon, 1986; Dingle and Laskey, 1986). Proteins with no organelle-specific tag remain, by default, in the cytoplasm. (Here we are using the term “tag” to refer to either a particular primary sequence or to a post-translational modification.)

Within each of these pathways further sorting takes place (Fig. 7). For example, the ER is the entry port for at least five classes of proteins: those retained by the ER itself, and those destined for the Golgi, lysosomes, secretory vesicles, and plasma membrane. Similarly, mitochondria contain four subcompartments: the outer membrane, the intermembrane space, the inner membrane, and the matrix. Proteins must be further sorted into these subcompartments. These subsequent sorting processes work on the same principle as the primary sorting event at the ribosomes: there is a “default” destination that will be followed unless secondary molecular tags intervene. In the secretory pathway, there are two types of secondary molecular tags that affect the destination of a particular protein.
Fig. 6. Sorting of secretory proteins is a hierarchical process. Proteins assembled on the ribosomes (RB) are targeted to four major cellular compartments, the nucleus, mitochondria, the endoplasmic reticulum (ER), or the cytosol, according to their targeting sequences. We have indicated the targeting sequence by small circle with enclosed letters: N, nucleus; M, mitochondria; S, secretory pathway. Proteins with no signal sequence remain in the cytosol. It should be noted that the targeting signal for the secretory pathway and the mitochondria usually resides at the amino terminus of the polypeptide, whereas the targeting for the nucleus is usually not restricted to the amino terminus. After routing to their major compartments, many proteins are further routed to subcompartments. For example, mitochondrial proteins may be inserted into the outer membrane (OM), the intermembrane space (IMS), the inner membrane (IM), or the luminal matrix (MTX). Proteins in the secretory pathway may be retained in the ER, be shunted to the Golgi or lysosomes (LY), or pass to the cell surface.

(cf. Fig. 7A). A "retention tag" will cause a protein to be retained by an organelle (e.g., the ER or Golgi). A "branching tag" will cause the protein to be diverted from the secretory pathway to another compartment, such as the lysosomes. Those proteins that are neither retained nor diverted (e.g., plasma membrane and secretory proteins) will default to the cell surface via the "constitutive" secretory pathway, discussed below. A similar scheme operates to sort proteins to the various mitochondrial compartments (Fig. 7B). Proteins without retention tags for the outer membrane or the intermembrane space end up in the matrix (Hurt and Loon, 1986).

Fig. 7. The second stage of sorting is accomplished by retention and/or branching tags. (A) Sorting in the secretory pathway. All proteins with the signal sequence (S) are inserted into the lumen of the rough endoplasmic reticulum. A subset of these contains retention tags which are captured by the ER (Δ), or the Golgi (Ο). Another subset contains branching signals. Those containing the lysosomal tag (†) are diverted to lysosomes, and, in storage cells, those containing localization signals for storage vesicles (Δ) are diverted into secretory granules. In all cells, those proteins not retained or diverted into the above compartments are, by default, transported to the cell surface along the constitutive pathway. At the cell surface, soluble proteins are discharged into the extracellular environment. Membrane proteins containing an addition signal (Δ) are collected into coated pits for recycling, whereas those without such signals remain on the cell surface. We should emphasize that, for illustrative purposes, we have indicated a signal sequence on all proteins along the entire secretory pathway; however, most of them are removed at the rough endoplasmic reticulum. Note that most of the retention and branching tags have not been explicitly identified; therefore, their locations on the polypeptide have been drawn arbitrarily. (B) Sorting in the mitochondrial compartment. All proteins with the mitochondrial targeting sequence are captured by binding sites on the mitochondrial membrane. Some are retained on the outer membrane (Ο), while others containing additional cleavage signals (denoted by small arrows) are retained in the intermembrane space. Those not retained by the intervening compartments reach the luminal matrix. In contrast to the case of sorting within the secretory pathway, all of the mitochondrial targeting signals are found at the amino terminus.
3. Sorting Is Mediated by Sorting Domains and Receptors

How is the zip code system decoded by the cell’s machinery? The retention and diversion tags we have referred to are “sorting domains” (Blobel, 1980); the cell appears to use specific receptors to recognize and bind these domains. The two best characterized receptors are (a) the signal recognition particle and its receptor that route proteins to the secretory pathway (Walter and Blobel, 1981; Walter et al., 1981; Gilmore et al., 1982a,b; Meyer and Dobberstein, 1980) and (b) the mannose 6-phosphate receptor that targets lysosomal hydrolases to the lysosomes (for reviews, see Sly and Fischer, 1982; Kornfeld and Kornfeld, 1984; Farquhar, 1985).

B. Sorting of Secretory Proteins in Storage Cells

1. Are Secretory Proteins Sorted?

As we have discussed above, not all proteins require positive sorting. In the secretory pathway, it seems that the cell surface is the “default” destination for proteins not retained by or diverted to other organelles. That is, many proteins end up on the plasma membrane without any apparent signal for the cell surface—other than the signal peptide that routed them to the secretory pathway back at the ribosomes. For example, globin, a cytoplasmic protein, can be fused to the signal peptide of β-lactamase and threaded into the lumen of the ER (Lingappa et al., 1984). On cleavage of the signal peptide, it is efficiently secreted along the constitutive pathway (D. Quinn and H.-P. H. Moore, unpublished). Moreover, Weiland et al. (1987) recently demonstrated that a derivative of the tripeptide, Asn-Tyr-Thr, can be introduced into a number of constitutive cells, where it is glycosylated and transported to the cell surface with kinetics faster than any known secretory protein. Finally, yeast mutants defective in vacuole biogenesis shunt their vacuolar proteins into the secretory pathway (Rothman and Stevens, 1986).

These experiments demonstrate that a protein equipped only with the signal sequence will eventually reach the cell surface by the “default” constitutive pathway. Indeed, in many cell types secretory and plasma membrane proteins do not appear to be sorted and thus reach the surface by the same pathway. For example, in hepatocytes the plasma membrane glycoprotein G and the secretory proteins albumin and transferrin are found in the same secretory vesicles. Moreover, yeast mutants defective in secretion are also defective in export of plasma membrane proteins, supporting the view that they are transported via the same pathway (for review, see Schekman, 1985). Thus it appears that constitutive cells do not sort their secretory proteins. However, recent experiments indicate that storage cells do sort their secretory and plasma membrane proteins. This is necessary because these cells contain more than one secretory pathway.

2. Storage Pathway Coexisting with the Constitutive Pathway

As discussed earlier, professional secretory cells have a specialized secretory pathway. Does this imply that the constitutive pathway is discarded? Tartakoff and Vassalli (1978) noticed that secretory cells could be classified into two categories: those which could respond quickly to secretagogues and those which did not respond. He called the fast responding cells “regulated secretory cells,” since their secretion could be regulated by an external signal. This nomenclature was widely adopted, and appeared to define two distinct cell types. Moreover, it became generally accepted that each of these cell types possessed only one or the other secretory pathway. It has recently become clear, however, that the two pathways could coexist in the same cell. This makes sense, for the evolution of a specialized function does not preclude the necessity of serving the cell’s previous secretory tasks, such as supplying the cell surface with new membrane proteins.

3. Experimental Evidence Supporting the Coexistence of the Two Pathways

Gumbiner and Kelly (1982) examined the secretory pathways in the pituitary cell line AtT -20. They used antibodies against an endogenous viral membrane protein, gp70, to follow the secretory pathway of a plasma membrane protein and compared it with the pathway taken by the hormone ACTH. They concluded that there are at least two distinct routes to the cell surface in this cell. While ACTH is packaged into storage secretory vesicles and its secretion is accelerated by secretagogues, the viral protein is externalized by a different pathway. This protein is transported to the cell surface as soon as it is synthesized, i.e., without first being stored in secretory vesicles. Furthermore, its externalization is constitutive and not influenced by secretagogues. Does the constitutive pathway also provide a general route for secreting nonviral proteins made by AtT -20 cells? Moore et al. (1983b) examined the pathways taken by all endogenous secretory products after labeling the cells with [35S]methionine. A number of radiolabeled molecules were found in the tissue culture medium, and they all fell into one of two classes: those externalized by the constitutive pathway and those by the regulated pathway. Thus, in addition to gp70 and ACTH, other products in the secretory
apparatus are also sorted into these two pathways. The presence of these same two secretory pathways has also been found in other endocrine and neuronal cell lines. These include the growth hormone-producing pituitary tumor GH3 cells (H.-P. H. Moore, unpublished), the pheochromocytoma-derived PC-12 cells (Schweitzer and Kelly, 1985), and the pancreatic insulin-producing HIT cell line (Moore et al., 1983a).

4. Are Proteins Selectively Sorted into the Two Pathways?

The coexistence of these two secretory routes is important, for it raises the question of whether proteins are selectively sorted into the two pathways. In principle, one could imagine accomplishing fast response by simply shunting, without discrimination, some of the constitutively secreted material into the storage pathway. Experimental evidence indicates, however, that it does not work this way: proteins are not randomly distributed between the two pathways. Experimentally, it is not easy to demonstrate this, for there are few endogenously manufactured proteins that can be quantitated accurately. However, it is possible to transfect foreign DNA coding for known markers into A1T-20 cells, and their fate can be followed using specific antibodies. When these cells are transfected with human growth hormone (HGH)—a protein that is normally stored in secretory vesicles of somatotrophs—the foreign hormone is packaged efficiently into the ACTH vesicles. In contrast, a soluble form of a viral membrane protein (the VSV G protein with its membrane anchor truncated, VSV TG) is exported via the constitutive pathway by the same cell (Moore and Kelly, 1985).

5. What Kinds of Proteins Are Sorted?

The unequal distribution of proteins between the two pathways implies that at least one class of proteins is actively sorted. Which one? To address this question Moore and Kelly (1986) fused a constitutively secreted protein (TG) with a storage protein (HGH) and observed how the hybrid molecule was sorted by the cell. If the constitutive protein contained a sorting domain, then some fraction of the fusion product should be diverted to the constitutive pathway. Conversely, if the storage fragment contained the sorting domain, the hybrid protein should be directed to storage vesicles. It turned out that the viral protein TG was diverted from its normal route to the storage vesicles. These results strongly support the view that proteins are actively sorted into storage vesicles; that is, it appears that the storage pathway is the recipient of sorted proteins. Figure 8 summarizes the current view: the constitutive pathway is a "bulk flow" route to the cell surface, whereas the storage pathway is a shunt which receives selectively sorted proteins destined for storage vesicles to be used for controlled, rapid exocytosis.

6. Where Does Sorting Take Place?

Conceivably, segregation of proteins into the two pathways could take place in the RER, where different proteins are inserted into separate domains of the ER cisternae. Alternatively, they could be sorted at a later stage of transport, i.e., within a given Golgi cisterna or in some post-Golgi station. The intracellular sorting compartment has now been identified via immunoelectron microscopy (Orci et al., 1987). When transfected A1T-20 cells producing rat proinsulin were infected with the influenza virus, the constitutive marker hemagglutinin was found to be mixed together with the regulated protein proinsulin in all Golgi cisternae. Segregation appears to take place at the time the two proteins exit the Golgi: hemagglutinin was packaged into clear vesicles (i.e., with no electron-dense contents), whereas proinsulin became concentrated in the dense core of budding granules (Fig. 9 and 10). Thus, the two pathways appear to diverge at the trans-most Golgi cisternae, which is acid phosphatase positive.
Fig. 9. Influenza virus hemagglutinin and proinsulin are segregated into different secretory vesicles in AtT-20 cells. Consecutive thick serial sections of an insulin-transfected, influenza virus-infected AtT-20 cell following immunofluorescent staining of insulin (A) and influenza virus hemagglutinin (B) are shown. Insulin immunofluorescence appears mostly as a spotty perinuclear (Golgi region) staining; influenza virus hemagglutinin antibody also elicits a perinuclear reaction but in addition an intense cell surface staining.

Fig. 10. Immunocytochemical localization of influenza virus hemagglutinin (A and B) and insulin (C) in insulin-transfected, influenza virus-infected AtT-20 cells. (A) In the Golgi area (G) situated in the cytocenter (attested by the presence of a centriole, C), gold particles revealing influenza virus hemagglutinin are found in the Golgi cisternae and in clear vesicles (v), but they are absent from core-containing secretory granules (sg). (B) At the cell periphery, the gold particles decorate the cell surface, which is thrown into numerous microvilli (several particles are indicated by circles), but are absent from the numerous secretory granules beneath. (C) In the thin serial section consecutive to that shown in (B) and stained with anti-insulin antibodies, gold particles appear virtually restricted to the dense-core secretory granules.
7. What Is the Molecular Mechanism of Sorting?

The mechanisms the cell uses to divert proteins into the storage pathway remain obscure. However, some clues may be gleaned from examining related sorting systems, such as that which localizes the lysosomal hydrolases. In fibroblasts, sorting of lysosomal enzymes to the lysosomes is accomplished by the mannose 6-phosphate receptor, which binds to a common recognition marker on lysosomal hydrolases and diverts them to lysosomes. The hybrid protein experiment discussed above showed that storage proteins are positively sorted into secretory vesicles. This suggests that storage proteins may have a specialized sorting domain which is recognized by a specialized receptor. If this hypothesis is correct, what is the nature of the sorting domain and its receptor?

8. Molecular Structure of the Sorting Domain

Most peptide hormones are synthesized as a larger precursor consisting of a pre- or signal sequence followed by the prohormone sequence. During transit through the cell, the preprohormones undergo two sets of proteolytic processing events to yield the final mature hormones. The first cleavage event takes place in the RER where the presequence is removed by the signal peptidase as the nascent polypeptides are translocated through the membrane of the RER. A later processing step then cleaves the prohormones to separate the prosequences from the mature products. It is appealing to hypothesize that targeting signals for granular localization may reside on one of these cleavable sequences.

A clear advantage of using such sequences for address information is that, once a protein is correctly localized in the target organelle, removal of these signals could render the process irreversible. Recent results obtained from in vitro mutagenesis of storage proteins, however, do not support such a sorting function for the pre- or propeptide of hormones. Two lines of evidence suggest that the prepeptide is not involved in sorting into storage granules. First, identification of the trans-Golgi compartment as the sorting cite rules out the possible involvement of this sequence, since it is removed much earlier in the RER. Second, as mentioned earlier, the entire human growth hormone with its presequence is able to divert the VSV G fragment into storage granules. The presequence alone, however, is insufficient in rerouting the constitutive protein (L. Lehmicke and H.-P. H. Moore, unpublished results). Thus, the targeting signal does not reside on this hormone’s presequence. Moreover, the propeptide, or C peptide, of insulin can be either completely deleted or replaced with a much shorter sequence from the insulin-like growth factor without affecting its granule localization (S. Powell and H.-P. H. Moore, unpublished results). Taken together, these analyses suggest that the information responsible for granular localization is contained within the mature peptide hormone sequences, rather than in the pre- or prosequence.

9. Cellular Sorting Receptors

How does the cell decode the sorting information on mature hormone sequences? A likely mechanism is that the sequence is recognized by cellular carriers, or receptors, which concentrate and localize the hormones within selective regions of the Golgi. These receptors would then serve as a molecular filter: they would bind only to proteins destined for packaging into storage granules and leave those destined for the constitutive pathway untouched. There is preliminary evidence for such receptor proteins. Using affinity chromatography, K.-N. Chung, P. Walter, and H.-P. H. Moore (unpublished results) have isolated two proteins with molecular weights of 15K and 24K–29K from detergent-solubilized Golgi fractions of dog pancreas. These proteins selectively bind to storage proteins such as prolactin but do not show significant affinity for constitutive proteins. Interestingly, the pH dependence of ligand binding is similar to that of the mannose 6-phosphate receptor: binding occurs at neutral pH but is abolished at acidic pH. Since the internal pH of the storage granules and their precursor compartments are acidic (Orci et al., 1986a) (cf. Fig. 4), the dissociation of ligands from receptors at reduced pH may facilitate the unloading of transported proteins at the target compartments, much like the unloading of hydrolases in prelysosomal compartments (cf. Moore et al., 1983c).

10. Does Clathrin Play a Role in Intracellular Protein Sorting?

If sorting of storage proteins is indeed mediated by receptor proteins situated in the Golgi compartments, how then are the receptor proteins segregated to the storage granules? An intriguing observation came from studies of the intracellular distribution of clathrin. Orci et al. (1985b) and Tooze and Tooze (1986) found that, in peptide hormone-secreting cells, a significant fraction of the clathrin occurs in the Golgi areas. Electron microscopic examination of AT-20 cells revealed that the forming secretory granules are conspicuously coated with clathrin (Fig. 11). These clathrin molecules—and perhaps other associated proteins—may serve to aggregate receptors carrying ligands for granular localization, much like the recruitment of receptors by coated pits during endocytosis. Since transport via constitutive vesicles does not appear to require positive
11. Is There a Ubiquitous Sorting Mechanism in Storage Secretory Cells?

There are many types of storage cells. Endocrine cells typically secrete predominantly one hormone type: pancreatic β cells secrete insulin, somatotrophs in pituitary tissue secrete growth hormone, and corticotrophs secrete ACTH. Is it possible that each cell type has evolved a unique sorting mechanism, that is, a unique receptor to sort its hormone? In principle, there are two possibilities: (a) there is a common "zip code" that is recognized by a universal receptor and (b) each protein has a unique receptor. Transfection experiments have demonstrated that a number of endocrine and exocrine polypeptides can be packaged in the storage vesicles of heterologous cells, which seems to support the universal receptor model (Moore et al., 1983a; Burgess et al., 1985; Schweitzer and Kelly, 1985; Fig. 12). Examination of the molecular structure of these proteins, however, fails to reveal any common sequence. This may indicate that the recognition site resides in the 3-dimensional structure. This suspicion has several precedents, for example, in the lysosomal enzymes, where there appears to be a universal recognition system with no obvious consensus sequence. More work is required to identify the sorting domain and receptors before we can resolve this issue.

C. Budding from the Golgi: Mechanical Considerations

1. How Do Vesicles Bud from the Golgi?

Aside from the machinery to concentrate proteins in a particular region of the Golgi, there is another problem the cell must solve, namely, how to physically separate the condensed proteins from the Golgi into a secretory vesicle. While, much is known about the process of vesicle fusion (Wilschut and Hockstra, 1984), vesicle budding—both from the Golgi and from the plasma membrane—is still quite mysterious. Although probably requiring ATP, receptor-mediated endocytosis continues in the presence of cytoskeletal inhibitors; moreover, despite its suspected role, endocytosis appears to proceed independently of clathrin in yeast cells (Payne and Schekman, 1985).

The fundamental mechanical requirement for budding a vesicle or a tube from the Golgi is the following: somehow a bending moment must be generated across the lipid bilayer. This is a mechanical problem that can be solved in a number of ways; in Cheng et al. (1987) we discuss the various physical mechanisms for generating this force. Here we shall summarize some of our investigations into the mechanics of budding that may shed light on this crucial process (cf. also Israelachvili et al., 1980; Tartakoff, 1987; Cevc and Marsh, 1987).
2. The Mechanical Equilibrium of the Lipid Bilayer as a Balance between Interfacial Tension and Surface Pressure

Associated with each leaflet of a lipid bilayer is a "surface stress," which can be characterized in two equivalent ways: (1) the force per unit length (say, dyne/cm) tending to pull the surface apart* or (2) the energy per unit area (erg/cm² = dyne/cm) stored in the surface. The surface stress in a lipid bilayer is the result of an imbalance between opposing forces, the surface pressure and the interfacial tension, both of which act within each leaf of the bilayer. The net surface stress in each lipid layer (cf. Fig. 13a) can be written as

\[
\text{Surface stress} = \text{surface pressure} - \text{interfacial tension} \quad (1)
\]

The surface pressure arises from several contributions, including steric repulsions between the lipid head groups and hydrocarbon tails, as well as from electrostatic and hydration forces between the head groups (Israelachvili et al., 1980). These forces are counted as positive because they tend to expand the membrane surface area.

An alternative view of the surface pressure is to treat it as a "surface osmotic pressure" (Defay and Prigogine 1966; Davies and Rideal, 1961; Evans and Skalak, 1980). Since water can interpenetrate into the surface phase among the hydrophilic head groups, this region can be viewed as a nearly 2-dimensional solution. That is, the activity of the water is lower in the interfacial region; water molecules tend to diffuse into this region, thus increasing the local osmotic pressure.†

The interfacial (surface) tension arises from the hydrophobic interactions between the lipid tails and the aqueous solvent (Tanford, 1980). It is counted as is negative because it tends to shrink the surface area of each leaflet. In a planar membrane patch at mechanical equilibrium, these surface forces just balance, and the net surface stress vanishes.

* Imagine cutting a small slit in the surface and measuring the force tending to make the slit "gape."
† It is customary to include in this surface osmotic pressure the hydration forces and the electrostatic repulsions between the polar head groups (since the counterions induced by the fixed charges also must be counted as osmotically active particles; Hill, 1960).
3. Changing the Balance of Surface Forces Will Cause the Membrane to Bend

Within each leaflet, the net surface pressure and the interfacial tension need not act in the same plane (cf. Fig. 13a). This will create a bending moment within each leaflet. Only if conditions are identical in each leaflet will these moments cancel. In general, anything that disturbs the balance of forces in one face of the membrane will cause an imbalance of forces between the two lipid layers. If, for example, the interfacial tension in one face decreases with respect to the other face, then a bending moment $\mathbf{M}$ will be generated across the membrane (Fig. 13b):

$$\text{Bending moment} = \text{difference in surface stress} \times \text{membrane thickness}$$

This bending moment will cause the membrane to buckle; any mechanism that creates such a bending moment will suffice to create a membrane invagination. Whether this invagination leads to vesicle formation depends on a number of other factors, especially the capacity of apposed lipid layers to fuse (Wilschut and Hoekstra, 1984).

There are a number of plausible molecular mechanisms for perturbing the balance of forces in a bilayer (Israelachvili et al., 1980). However, some conclusions can be drawn from a mechanical analysis of the buckling of lipid bilayers that do not depend on the molecular mechanisms that creates the bending moment (Cheng et al., 1987):

1. For membrane systems where the ratio of membrane thickness to the membrane radius of curvature is small, buckling tends to create a vesicle. In the absence of surface convolutions, this situation would apply at the cell surface where the curvature of the plasma membrane is relatively small. However, when the ratio of the membrane radius of curvature to the thickness is not so large, as in the Golgi and ER, the membrane tends to extrude into tubes, rather than to form vesicles. For a given bending moment, when membrane is supplied to the site of buckling, tubes are favored over vesicles. This suggests that membrane flow to the trans Golgi will favor tubule formation. These conclusions are in accordance with the morphology of the trans-Golgi network; Griffiths and Simons, 1986. However, recent studies indicate that membrane tube extrusion may be an active process associated with cytoskeletal elements.

2. Curiously, a constant membrane moment alone will not generate a spherical vesicle; rather a mushroom-shaped protuberance results. In order to form spherical vesicles an internal force normal to the membrane is also required. This suggests that the protein contents of the vesicles play some mechanical role in producing spherical vesicles, perhaps by generating an internal pressure.
These conclusions do not depend on the mechanism for generating the membrane bending moment, but are simply geometric properties of any deforming elastic surface.* In order to say more about how proteins might exit the trans Golgi, one must inspect specific molecular models for generating the membrane bending moment.

Finally, surface tension considerations suggest that tubular membrane structures are not stable without some form of support (Israelachvili et al., 1975). This might explain why tubular membrane organelles, such as the tubular ER (and perhaps the trans-Golgi network and tubular lysosomal structures), appear to be colinear with cytoskeletal elements, and disappear when the cell is treated by agents which disrupt the cytoskeleton (Terasaki et al., 1986). In this connection it is interesting to note that heliozoan axopodia, which are tubular organelles, vesiculate when microtubules are disrupted (Schliwa, 1976).

4. How Might the Membrane Bending Moment Be Generated?

A possible clue to the mechanism of membrane budding is the observation that a molecular coat appears to form on the cytoplasmic face of constitutive Golgi vesicles (Orci et al., 1986b). This suggests the following scenario for vesicle and tube formation from the Golgi (Cheng et al., 1987). If a molecular species dissolves in the interfacial environment of one leaflet, the attractive forces may be reduced, and/or the surface osmotic pressure may increase. This will cause the leaflet to expand, and the membrane will buckle, as shown in Fig. 13b. As the curvature of the vesicle increases, the circumferential tensions created by the membrane curvature will pinch the neck of the protrusion until the lipid bilayers contact. This “surfactant theory” is only one of a number of theoretical possibilities (cf. Cheng et al., 1987; Israelachvili et al., 1976, 1980).

Any model for vesicle formation must address the issue of how the vesicle actually pinches off once the membrane surfaces contact. This is a complicated process involving the details of the transient structure of lipids in the bilayer (Wilschut and Hoekstra, 1984; Nagle, 1980; Gruner et al., 1985). As we mentioned in Section II.2.B, there is evidence that osmotic forces play an important role in membrane fusion (Lucy and Ahkong, 1986; Fisher and Parker, 1984; Horn, 1984). A macromolecular fusagen could surmount both the hydration layer barrier and electrostatic repulsion. Therefore, isolation of a vesicle-specific fusagen would be an important step in elucidating the mechanism of vesicle budding from the Golgi.

5. Intracellular Membrane Morphology May Be Controlled by Local Ionic Conditions

The above discussion of membrane forces suggests some further speculation about the role of physical factors in the functioning of the ER–Golgi system. Membranous organelles assume a variety of geometric shapes, including spherical vesicles, flattened sheets, fenestrated cisternae, and tubular reticulum. A clue to the function of the Golgi–ER system may be found in the differences in their respective shapes. Since the geometry of a membrane system is controlled by the balance of surface forces in the bilayers, these diverse geometries must reflect differences in local surface stress. These differences, in turn, can arise from differences in membrane composition and from local changes in ionic conditions:

1. It is known that different organelles are characterized by distinct molecular constituents, e.g., reticuloplasmins in the ER (Koch, 1987). Different compositions almost certainly imply different surface stresses, especially owing to differing molecular interactions between polar head groups (Shinada and Friberg, 1986) and different packing constraints (Israelachvili et al., 1980; Mitchell and Ninham, 1981). Thus one can expect that organelle shapes will correlate with their compositions. (Indeed, it may well be that the separation mechanism of differenct membrane components defines the organelle geometry.)

2. Solutions of mixed surfactants, when subjected to varying ionic environments, exhibit a sequence of phases that is very suggestive of biological structures (Longley and McIntosh, 1983; Tabony et al., 1987). Thus one candidate for controlling the surface stress of intracellular membranes is the local ionic and/or pH conditions.

As one adds salt to a micelle solution, the electrostatic repulsions between micelles is progressively screened, allowing them to contact and fuse. Increasing ionic strength also screens repulsions between polar head groups. According to Eqs. (1) and (2), this will cause a decrease in the local curvature, favoring the emergence of a lamellar phase. Sufficiently high ionic strength can even locally reverse the curvature from the micelle phase. Since the interfacial tension acts to minimize the total surface area, the global geometry of the membranous system is a complex compromise between the components of the surface stress. One solution to this problem is the formation of an interpenetrating minimal surface that has many

* Recall that a membrane bilayer is a “surface liquid,” i.e., with very low resistance to shear forces. The above conclusions are not altered by this property, however.

† The calculations discussed above are not contradictory to these conclusions. For the tubes generated by membrane bending moments alone are not indefinitely extruded.
of the features of the fenestrated cisternae and tubular networks characteristic of the Golgi stacks and the ER (Tabony et al., 1987). This similarity suggests that organelle geometry may be a reflection of local ionic and/or pH conditions that regulate membrane surface stress. It is worth noting that since a fluid membrane must flow down a gradient in surface stress, such gradients could play a role in transporting membrane-bound constituents within interconnected compartments.

D. Recycling of Membrane Components

Following exocytosis of the transported proteins, much of the transport equipment, including many membrane proteins, is probably recycled for subsequent reuse. This notion is supported by the observation that the turnover rate of the membrane proteins in secretory vesicles is much slower than that of the soluble contents. This retrieval process must be selective, since the composition of secretory vesicle membranes is quite different from that of the plasma membrane. Farquhar (1978) explored the route of recycled membrane by following the fate of cationized ferritin. When added to the extracellular fluid, cationized ferritin associates with the membrane, is internalized, and subsequently shows up in the Golgi and in newly formed secretory vesicles, rather than in its usual site, the lysosomes. In insulin cells, it has been shown that the externally applied tracer is found in the medial Golgi cisternae (Orci et al., 1986; see Fig. 14). These experiments clearly demonstrate that the membrane of secretory granules is recycled, but they do not address the mechanism of recycling.

Before we can understand the mechanism of recycling, we must first understand the route followed by recycled membrane molecules. An important—and unresolved—question in this regard is whether membrane components first disperse over the plasma membrane before retrieval, or whether they are retrieved as a group immediately following exocytosis. Two experiments suggest that dispersal may precede recycling. First, Heuser and Reese (1981) examined a time sequence of sections of neuromuscular junctions that had been rapidly frozen and freeze-fractured just following stimulation. They found that intramembranous particles appeared to disperse away from the site of exocytosis before retrieval. Second, Pfeffer and Kelly (1985) isolated coated vesicles from bovine brain, under the assumption that these were the vehicles of recycling. They found that two vesicle membrane components—defined by monoclonal antibodies—tended to segregate into different vesicles. Their results suggest that membrane components are recycled independently after dispersing over the plasma membrane.

Fig. 14. Examples of an extracellular tracer (horseradish peroxidase, HRP) taken up by endocytosis in the insulin cell (A). The tracer can be found in the Golgi stack (G), where cisternae situated in an intermediate (medial) position are preferentially labeled (Orci et al., 1986), as well as in secretory granules (sg) (B). Arrowhead in (A) indicates the lack of HRP labeling of a trans-condensing cisterna. Pulse-chase labeling with [3H]leucine coupled to the cytochemical demonstration of HRP showed that HRP-labeled granules are not those which are newly synthesized during the pulse-chase experiment (Sawano et al., 1986).
If membrane components are indeed dispersed before recycling, where do the dispersed components reassociate? This issue could be resolved by labeling two membrane markers and directly observing their distribution on the cell surface, and their subsequent route following endocytosis. Patzak and Winkler (1986) have made some progress in this direction by following a single vesicle antigen in chromaffin cells. If substances are indeed dispersed before retrieval, then the question arises of how the cell selectively recognizes these different components. This raises once again the specter of specific recognition molecules for each component. If true, this would open up an exciting avenue for further research.

IV. CONCLUSION AND PERSPECTIVES

It is clear that the molecular mechanisms underlying assembly of secretory vesicles are still largely terra incognita. The major point that emerges from studies of the secretory process is that there are many types of secretory vesicles, each with a characteristic ontogeny. Table 1 summarizes the major distinctions we have drawn between the constitutive and storage vesicles. It is likely that these, and perhaps additional, differences also exist between other vesicle types, such as those shuttling between apical and basolateral cell surfaces. We close with a list of issues that promise resolution in the foreseeable future.

1. What are the molecules that direct the sorting of storage proteins, and how are they routed to the correct locations?
2. How is the budding of vesicles from the Golgi accomplished? Are the molecular components involved in budding the same in constitutive and storage vesicles?
3. What is the function, if any, of clathrin in the assembly of storage vesicles?
4. How does intracompartmental pH influence the sorting and secretion process?
5. What is the molecular machinery for transporting and targeting vesicles between the different stations of the secretory pathways?
6. What are the mediators of membrane recognition and fusions?
7. After exocytosis, membrane proteins must be collected and rerouted to new secretory vesicles. Are the signals that guide recycling of these proteins the same as those previously used to target them to the secretory vesicles? That is, are addressing signals employed repetitively, or does each stage have its unique routing machinery?

### TABLE 1

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12. Biogenesis of Secretory Vesicles

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