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THE INTRACELLULAR TRANSPORT AND SORTING OF PRO-ACTH/ENDORPHIN

AND VIRAL MEMBRANE GLYCOPROTEINS IN PITUITARY CELLS

by

Barry Mark Gumbiner

DISSERTATION

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in the

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of the

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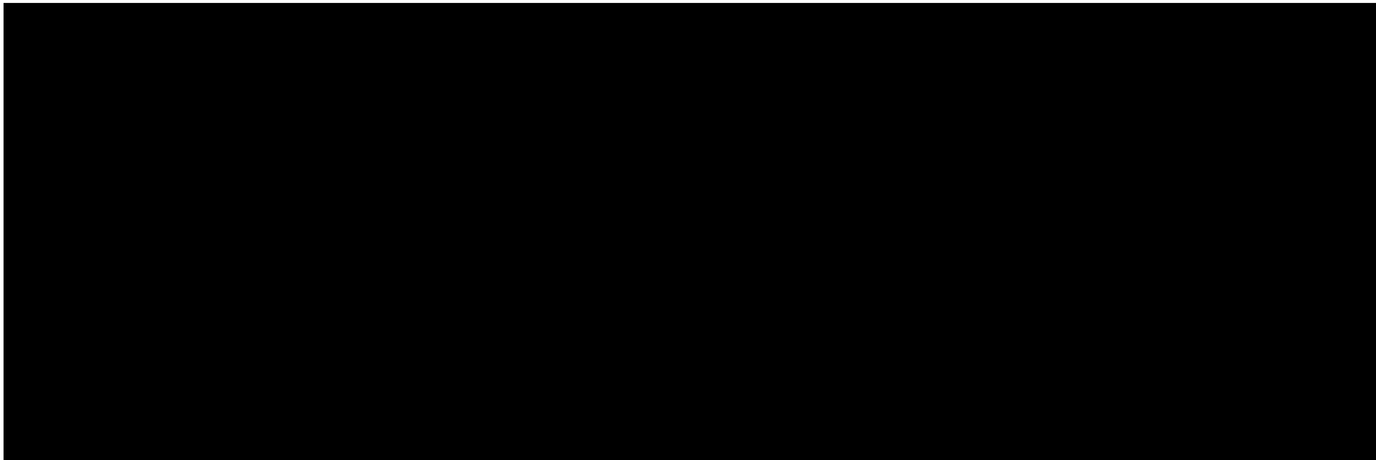

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THE INTRACELLULAR TRANSPORT AND SORTING OF PRO-ACTH/ENDORPHIN AND VIRAL MEMBRANE GLYCOPROTEINS IN PITUITARY CELLS

Dissertation Abstract

Barry Gumbiner

I have demonstrated that there are two distinct intracellular pathways for the transport of membrane and secretory glycoproteins to the surface of pituitary tumor cells. The pituitary cell line, AtT-20, secretes corticotropin (ACTH) and endophins after they are cleaved from a common polypeptide precursor. I have found that AtT-20 cells also produce a murine leukemia virus envelope glycoprotein and transport it to their surfaces. I have purified the ACTH and endorphin-containing secretory granules from AtT-20 cells and characterized their biophysical properties and polypeptide composition. The secretory granules contain only mature forms of the hormones and proteolytic processing of the precursor occurs at the time the hormones are packaged into secretory granules. I have shown by several criteria that the envelope glycoprotein does not utilize the hormone secretory granule for its transport to the cell surface. At the final step in the export of secretory and membrane glycoproteins, AtT-20 cells have two kinds of pathways. Mature ACTH and endorphins are stored in secretory granules so their release can be regulated by endocrinological secretagogues. The viral envelope glycoprotein is transported instead by a rapid constitutive pathway. Some of the ACTH precursor seems to be

secreted by a rapid constitutive pathway. Rapid secretion of the precursor may result from inefficient sorting in the Golgi apparatus.

To determine the intracellular location of the proteolytic conversion of the ACTH/endorphin precursor, the processing of the precursor and its packaging into secretory granules was examined in AtT-20 cells treated with the ionophore, nigericin. Carboxylic cation ionophores, including monensin and nigericin, block intracellular transport of plasma membrane and secretory proteins in the Golgi apparatus. Although proteolytic cleavage of the ACTH precursor was inhibited 75% by 10^{-6}M nigericin, most of the ACTH precursor that accumulated in treated cells had undergone terminal glycosylation. Incorporation of all hormone fragments derived from the precursor and several other presumptive secretory granule proteins into isolated secretory granules was also blocked. The results indicate that proteolysis of the ACTH precursor does not occur when transport from the Golgi to the secretory granule is blocked. Therefore, prohormone cleavage may occur in a compartment distal to the glycosyltransferase containing Golgi elements; either the secretory granule itself or a condensing vacuole.

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TABLE OF CONTENTS

PAGE

CHAPTER 1: The Intracellular Transport and Sorting of Lysosomal Hydrolases, Secretory Proteins and Membrane Glycoproteins (A Review) 1

I. Biosynthesis of Secretory and Membrane macromolecules at the rough endoplasmic reticulum 3

II. Transport of Proteins and Membranes from the RER to the Golgi Apparatus 6

III. Protein modification in the Golgi apparatus 13

IV. Functional compartmentalization of the Golgi apparatus 17

V. Route of protein transport through the Golgi apparatus 24

VI. Sorting of lysosomal hydrolases 29

VII. Biogenesis of secretory vesicles 36

VIII. The sorting of plasma membrane glycoproteins and secretory proteins 43

IX. Shuttle vesicles and membrane recycling 54

X. The biochemical basis of membrane protein sorting 58

CHAPTER 2: Secretory granules of an anterior pituitary cell line, AtT-20, contain only mature forms of adrenocorticotropic hormone and β-lipotropin 61

SUMMARY 62

INTRODUCTION 63

RESULTS

I. Secretory granule purification 65

II. Physical properties of the secretory granules 67

III. Secretory granule protein composition 68

IV. Comparison of proteolytic processing with packaging into secretory granules 70

PAGE

DISCUSSION 71

TABLES AND FIGURES 78

CHAPTER 3: Two Distinct Intracellular Pathways Transport Secretory and Membrane Glycoproteins to the Surface of Pituitary Tumor Cells 102

SUMMARY 103

INTRODUCTION 103

RESULTS

I. AtT-20s synthesize and process MuLV glycoproteins 105

II. Gp70 is transported to the exterior of AtT-20 cells 108

III. Kinetics of viral glycoprotein and ACTH release 110

IV. Viral envelope glycoproteins do not accumulate in ACTH secretory granules 111

V. Effect of ACTH secretagogues on the release of viral glycoproteins 112

DISCUSSION 113

FIGURES 120

CHAPTER 4: The Proteolytic Processing of Pro-ACTH/Endorphin and its Transport into Secretory Granules are Inhibited by Nigericin 142

SUMMARY 143

INTRODUCTION 144

I. Nigericin inhibits the processing of pro-ACTH/endorphin 146

II. Nigericin blocks the incorporation of newly synthesized proteins into secretory granules 148

III. General effects of nigericin on AtT-20 cells 150

DISCUSSION 151

FIGURES 156

PAGE

CHAPTER 5: Experimental Procedures 162

1. Cell Culture 163
2. Secretory Granule Purification 163
3. Radioimmunoassay 165
4. Labeling with 35S-Methionine 166
5. Cell Surface Iodination 166
6. Sample preparation and Immunoprecipitation 167
7. Antisera 168
8. Endoglycosidase-H digestion 169
9. Protease Digestion of Intact Cells 169
10. Ricin-Agarose Affinity Chromatography 170
11. SDS Gel Electrophoresis and Fluorography 170
12. Other Assays (protein and viscosity) 171
13. Materials 171

REFERENCES 173

ABBREVIATIONS

AChE	acetylcholine esterase
AChR	acetylcholine receptor
ACTH	adrenocorticotropic hormone
Asn	asparagine
8Br-cAMP	8-bromoadenosine 3':5'-cyclic monophosphate
cAMP	adenosine 3':5'-cyclic monophosphate
D_2O	deuterium oxide
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethylether)NN'-tetraacidic acid
EM	electron microscopy
Endo-H	endo-beta-N-acetylglycosaminidase H
GERL	acronym for Golgi-endoplasmic reticulum-lysosome (see text)
Glc	glucose
GlcNAc	N-acetylglucosamine
HRP	horse radish peroxidase
Ig	immunoglobulin
LPH	lipotropic hormone
MAN	mannose
Mulv	murine leukemia virus
PAGE	polyacrylamide gel electrophoresis
RER	rough endoplasmic reticulum
RIA	radioimmunoassay
SDS	sodium dodecyl sulfate

SER	smooth endoplasmic reticulum
SFV	Semliki Forest virus
TCA	trichloroacetic acid
TPPase	thiamine pyrophosphatase
UDP	uridine diphosphate
VLDL	very low density lipoprotein
VSV	vesicular stomatitis virus

CHAPTER 1

THE INTRACELLULAR TRANSPORT AND SORTING OF LYSOSOMAL HYDROLASES, SECRETORY PROTEINS AND MEMBRANE GLYCOPROTEINS (A REVIEW)

INTRODUCTION

The route of secretory protein transport has been generally established as rough endoplasmic reticulum (RER) to Golgi to the secretory granule which undergoes exocytosis (Palade, 1975; Morre et al., 1979; Farquhar & Palade, 1981). Lysosomal hydrolases have been envisaged to follow a similar route in which the lysosome can be considered analogous to a secretory vesicle which fuses with digestive vacuoles rather than the plasma membrane (Palade, 1975; Bainton, 1981; Novikoff, 1976). Although embedded in the lipid bilayer, plasma membrane glycoproteins are transported by the same or similar organelles as secretory proteins (Strous & Lodish, 1980; Lodish et al., 1981; Gumbiner & Kelly, 1982). These proteins are all cotranslationally segregated into the cisternal space of the RER; either partially or entirely translocated to the noncytoplasmic side of the membrane (Katz et al., 1977; Rothman & Lodish, 1977; Erickson & Blobel, 1979; Blobel, 1980; Lodish et al., 1981; Braell & Lodish, 1982; Sabatini et al., 1982). During their subsequent distribution to appropriate destinations within the cell, they are commonly processed, by glycosylation, proteolysis and other covalent modifications (Strous & Lodish, 1980; Lodish et al., 1981; Farquhar & Palade, 1981; Green et al., 1981). These biochemical events not only serve as signposts for the pathways of protein transport, but may also determine the route and sorting of classes of membrane and secretory proteins.

The purpose of this review is to consider how lysosomal enzymes, secretory proteins and transmembrane glycoproteins are

processed, sorted and transported to their ultimate destinations after synthesis at the RER. To this end I will discuss the major functions and properties of the organelles involved in their biosynthesis and transport. It is important to remember that our understanding of the role of these organelles is necessarily a composite picture taken from many cell types. Each of their functions are frequently best studied in a certain kind of cell. However, depending on their needs cells may vary considerably in the way they effect intracellular transport. When particularly relevant I will discuss potential differences between cell types with regard to the major theories of intracellular transport. Proteins that are posttranslationally inserted directly into membranes from the cytoplasm, bypassing the RER, will not be considered here (see Goldman & Blobel, 1978; Lodish et al., 1981; Gasser et al., 1982; Sabatini et al., 1982).

I. Biosynthesis of Secretory and Membrane Macromolecules at the Rough Endoplasmic Reticulum

The predominant function of the RER is the de novo biosynthesis of membrane and secretory macromolecules and their translocation across the membrane phospholipid bilayer. Secretory, transmembrane and lysosomal proteins are synthesized by polysomes firmly attached to the membrane and are transported through the lipid bilayer cotranslationally (Blobel & Dobberstein, 1975; Rothman & Lodish, 1977; Erickson & Blobel, 1979; Scheele et al., 1980). An amino terminal sequence of 25-30 amino acids or "signal peptide", is frequently cleaved off during translocation (Blobel, 1980; Sabatini et al., 1982), but not always (Palmiter et al., 1978;

Schechter et al., 1979; Braell & Lodish, 1982). A protein required for cotranslational translocation has been isolated from dog pancreas rough microsomes, a membrane fraction enriched in RER (Walter & Blobel, 1980). In the absence of membranes, this 'signal recognition protein' inhibits translation of mRNA for secretory but not cytoplasmic proteins, probably by binding to the nascent signal peptide and ribosomes (Walter et al., 1981; Walter & Blobel, 1981). Translation is continued only in the presence of rough microsomal membranes (Walter & Blobel, 1981). Walter & Blobel proposed that the function of the signal recognition protein is to ensure that secretory proteins are synthesized bound to the RER and not in the cytoplasm.

The RER is also the site for the synthesis and attachment of oligosaccharides linked to asparagine (Asn) residues of many extracytoplasmic proteins (reviewed by Hubbard & Ivatt, 1981). An oligosaccharide with the structure $(GlcNAC)_2$-$(MAN)_9(Glc)_3$ is transferred to Asn residues of the polypeptide (GlcNAc-N-acetylglucosamine, MAN-mannose, Glc-glucose) (Turco et al., 1977; Pless & Lennarz, 1977; Robbins et al., 1977). Glycosylation occurs either during or immediately after translation (Kiely et al., 1976; Rothman & Lodish, 1977; Roberts et al., 1978). The oligosaccharide donor is the lipid linked carrier, dolichol-pyrophosphate-oligosaccharide, and all of the enzymes for its biosynthesis are found in rough microsomes (Turco et al., 1977; Czichi & Lennarz, 1977; Hubbard & Ivatt, 1981). Very shortly after oligosaccharide transfer to protein, the three glucose residues are removed (Hubbard & Robbins, 1979). The glucosidase is enriched in rough and smooth

microsomal membrane fractions from rat liver which are distinct from the Golgi membranes that carry out terminal glycosylation of complex carbohydrates (Grinna & Robbins, 1979). This protein linked mannose rich oligosaccharide serves as a common precursor to both high mannose and complex type oligosaccharide chains which arise through extensive modifications during transport through the Golgi (Robbins et al., 1977).

The bulk of phospholipid biosynthesis occurs in RER and in smooth ER in cells where it is present (e.g. liver) (McMurray & Magee, 1972). Phospholipid biosynthetic enzymes are localized at the cytoplasmic surface of rough microsomes (Ballas et al., 1981). It is not yet understood how phospholipids are transported to other cell membranes. They may go by the RER to Golgi to plasma membrane route in association with proteins (Palade, 1975; Morre et al., 1979). It has also been proposed that transport is mediated by phospholipid exchange proteins (Wirtz, 1974; Morre et al., 1979). Many studies have established that the lipid composition of cellular membranes changes systematically from the RER to the plasma membrane (Keenan & Morre, 1970; Orci et al., 1981). The significance of this distribution is unclear.

The RER probably also catalyzes the oxidation of sulfhydryls to produce disulfide bonds in proteins (Fuchs et al., 1967; Vassalli et al., 1971; Tartakoff & Vassalli, 1979) and the hydroxylation of proline residues in procollagen (Olsen et al., 1973). It may also be the site for the assembly of multimeric secretory and membrane proteins such as H-2 antigens and immunoglobulin molecules (Tartakoff & Vassalli, 1979).

II. Transport of Proteins and Membranes from the RER to the Golgi Apparatus

The Golgi apparatus is a structurally complicated and diverse organelle or set of organelles which packages secretory proteins and carries out a wide variety of protein modifications. The review of the Golgi apparatus by Farquhar & Palade (1981) contains many excellent electron micrographs of the Golgi in several cell types. (Also, see schematic at end of this chapter.) In electron microscope (EM) thin sections the Golgi appears as a set of 3 to 6 stacked flattened cisternae (also called saccules or plates) surrounded by numerous smooth surfaced and coated vesicles which frequently appear to either bud or fuse with the cisternae. Although numerous stacks of cisternae are often apparent in thin sections, the available evidence indicates that the Golgi is a single continuous structure in animal cells (Rambourg et al., 1974; Novikoff et al., 1971). The stack exhibits a structural polarity with a convex "cis" face usually facing the transitional elements of the RER and a concave trans face which often seems to be associated with the formation of secretory granules. Traffic of both membrane and secretory products is often envisaged as entering the cis face, passing sequentially through the stack and leaving via secretory vesicles from the trans face (Morre et al., 1979; Rothman, 1981). The route of protein transport through the Golgi will be discussed after considering the biochemical and structural features of the Golgi stack.

Export of newly synthesized proteins from the RER is a poorly understood process. Different kinds of transport vehicles have

been proposed to carry proteins to the Golgi apparatus. Although these mechanisms have been presented as hypothetical alternatives, they could also represent a range of transport modes utilized by cell types which have different requirements. Depending on the nature of the export process, it may or may not be possible to sort proteins at the RER.

RER to Golgi transport may be mediated by vesicles. The most common observation in EM thin sections is a preponderance of small smooth surfaced vesicles, called peripheral Golgi vesicles or transitional vesicles, clustered between transitional elements of the RER (see below) and the Golgi apparatus (Jamieson & Palade, 1967; Tartakoff & Vassalli, 1977; Farquhar & Palade, 1981). In the exocrine pancreas secretory proteins in transit from the RER to the condensing vacuole were found associated with these vesicles by EM autoradiography (Jamieson & Palade, 1967). Similar observations have been made for the transport of secretory proteins from RER to Golgi in other cells such as the pancreatic β (insulin) cells, pituitary mammatrophs and plasma cells (Howell et al., 1969; Farquhar, 1971; Salpeter & Farquhar, 1981; Tartakoff & Vassalli, 1977). In cell fractionation studies of the exocrine pancreas, pulse radiolabeled secretory protein was found in the smooth microsome fraction between the times it was present in the rough microsomes and in the zymogen granule-condensing vacuole fraction (Jamieson & Palade, 1967). The proposed transport vesicles, however, have not yet been purified to homogeneity from the smooth microsomes. Coated vesicles also have been proposed to transport secretory proteins because they have been observed associated with

transitional elements of the ER and with Golgi membranes (Nichols et al., 1971; Franke et al., 1976; Morre et al., 1979; Farquhar & Palade, 1981).

Smooth ER tubules have also been implicated in RER to Golgi transport. In the hepatocyte very low density lipoprotein (VLDL) particles destined for secretion first appear at RER-smooth ER (SER) junctions, and accumulate in Golgi saccules (Claude, 1970). Claude (1970) has provided morphological evidence showing that SER tubules coalesce to form a fenestrated Golgi plate on the cis face of the Golgi stack which eventually matures to become a solid Golgi cisterna. Intact Golgi stacks isolated from rat liver have numerous tubules associated with the saccules (Morre et al., 1971) and the cis saccule appears to be made of anastomosing tubules (Bergeron et al., 1982). Cell fractionation studies of rat liver also indicate that newly synthesized serum proteins en route to the Golgi pass through the SER which, like the peripheral Golgi vesicles, is recovered in the smooth microsome fraction (Peters et al., 1971).

Are tubules rather than vesicles always involved in transport or are they a special feature of hepatocytes? The SER is highly elaborated in hepatocytes due to its role in lipid synthesis and chemical detoxification (Morre et al., 1979), but it is not so extensive in many cell types. High voltage electron microscopic studies of thick sections demonstrated that the cis Golgi saccules of neurons, leydig and serotoli cells also are made up of anastomosing tubules (Rambourg et al., 1974). The distinction between tubules and vesicles may not be easy to make in single EM thin

sections, since tubules appear as vesicles in transverse section. Also fragmentation of membranes during tissue homogenization could produce the small vesicles found in the smooth microsome fraction.

Unfortunately, temperature sensitive genetic lesions in the secretory pathway of yeast failed to reveal the nature of the RER to Golgi transport elements. Although the major secretory organelles, the RER, the Golgi and secretory vesicles accumulate in various mutants, no RER to Golgi transport vesicles or tubules were observed (Novick et al., 1981). It is possible that the appropriate mutation was not obtained in the collection of 188 mutants which includes 23 complementation groups. Alternatively, such organelles either do not exist per se or form Golgi elements independently of specific gene products. For example, direct transformation of ER membrane into Golgi saccules is possible.

Some investigators have proposed that secretory proteins pass directly from the ER to the Golgi through tubular connections between RER and Golgi membranes (Novikoff, 1976; Morre et al., 1979). If such continuities exist, they must either occur infrequently or intermittently because they are rarely observed (Palade, 1975; Farquhar & Palade, 1981). This hypothesis, in contrast to vesicular transport, does not require that excess membrane be degraded or recycled as secretory proteins are concentrated into secretory granules (see Section IX).

Even less is known about the transport of membrane proteins from RER to the Golgi. Rothman et al. (1980) have reported that the Vesicular Stomatitis Virus (VSV) G protein is transported by coated vesicles. However, the G protein is synthesized in large

quantities by virus infected cells, and contamination of the isolated coated vesicles by other membranes containing the G protein was not adequately ruled out.

Jamieson & Palade (1968) proposed that export of secretory proteins from the RER requires the formation of vesicles or a specific membrane budding event because it is energy dependent. Transport is blocked by metabolic poisons which deplete cells of ATP. The energy dependence of export has also been demonstrated in immunoglobulin (Ig) secreting plasma cells (Tartakoff & Vassalli, 1977) and yeast (Novick et al., 1981). When the exocrine pancreas is depleted of ATP secretory proteins accumulate in the transitional elements of the ER (Jamieson & Palade, 1968). Transitional elements are cisternae which have both ribosome studded and ribosome free surfaces, and they are usually located near the Golgi apparatus. EM autoradiography was not adequate to resolve whether secretory proteins accumulated in the transitional elements or in the peripheral Golgi vesicles, but the proteins accumulated in the rough microsomes rather than the smooth microsomes. Therefore it was concluded that energy is required to export proteins out of the transitional elements of the ER. Transitional elements have not yet been isolated from the rough microsome fraction.

Secretory proteins are transferred from their sites of synthesis on the RER to specialized exporting or 'transitional' elements even in the absence of metabolic energy expenditure (Jamieson & Palade, 1968). Are transitional elements a permanent subset of RER or do they arise from all portions of RER after sufficient synthesis of exportable protein? It is not certain whether proteins are

transported by flow through continuities in the ER network or by movement of entire ER cisternae toward the Golgi region.

During net membrane production by the ER phospholipids must be synthesized in addition to proteins. Are they made in specialized portions of ER such as the transition elements or the RER-SER junction? To what extent is phospholipid synthesis involved in the formation of vesicles and the export of membrane proteins or secretory proteins?

Export of proteins from the ER is commonly viewed as nonspecific and the sorting of proteins to different destinations is usually attributed to the Golgi apparatus (Farquhar & Palade, 1981; Rothman, 1981). If, however, it turns out that proteins can be sent to different portions of the Golgi or even bypass the Golgi entirely (see section V), then sorting must occur at the level of the ER. Transported proteins must be sorted away from indigenous ER proteins such as the enzymes involved in biosynthesis and translocation of glycoproteins. Such vectorial sorting could either be dual in nature or selective for classes of proteins.

Lingappa et al. (1978) showed that a membrane glycoprotein, the VSV G protein, and a secretory protein, prolactin, compete in vitro for the same rough microsomal translocation machinery. This finding does not rule out the potential for segregation of various proteins during synthesis in vivo at different portions of the RER. For example, in vitro reconstitution of cotranslational protein insertion into rough microsomes never indicated that mRNAs for secretory and cytoplasmic proteins might be distinguished by the translational apparatus (Blobel & Dobberstein, 1975; Scheele et

al., 1980). Also, Melcher (1980) has provided evidence that secretory proteins are initiated on cytoplasmic ribosomes. Thus, it appeared that the signal peptide alone is responsible for segregating secretory protein synthesis to membrane bound polysomes. However, mRNA injection into oocytes showed a differential capacity for and lack of competition between the translation of mRNAs for secretory and cytoplasmic proteins (Richter & Smith, 1981). This suggests that oocytes can distinguish between mRNAs for cytoplasmic and secretory proteins. Are similar distinctions of mRNAs made in vivo in order to segregate proteins into subregions of RER?

There is preliminary immunocytochemical evidence for the segregation of proteins in the RER. Immunoglobulin (Ig) in immature plasma cells and albumin in hepatocytes were found in restricted segments of the RER (Leduc et al., 1968; Yokota & Fahimi, 1981). Unfortunately no evidence was presented for the localization of different proteins to other regions of the RER. Even if translation of all extracytoplasmic proteins is intermixed along the RER, segregation could operate at the stage of export. Preliminary evidence was presented for selective export of albumin and VLDL particles in hepatocytes (Yokota & Fahimi, 1981). Immunocytochemically localized albumin was never found in the same transitional ER vesicles as VLDL particles. Curiously, they eventually reside in the same Golgi vacuoles. Much remains to be learned about the nature and selectivity of protein and phospholipid export from the RER.

III. Protein Modification in the Golgi Apparatus

Processing of N-asparagine linked oligosaccharides to form complex type glycoproteins and mature high mannose structures has been invariably attributed to the Golgi apparatus. The major biosynthetic pathway for the processing of complex type oligosaccharides of the VSV envelope glycoprotein and other glycoproteins has been elucidated (Tabas et al., 1978; Hubbard & Ivatt, 1981). Between 0 and 4 mannose residues can be trimmed from the $(GlcNAc)_2$-$(MAN)_9$ oligosaccharide precursor by the action of two α-mannosidases (Tabas & Kornfeld, 1979). This produces either mature high mannose glycoproteins or an intermediate for the synthesis of complex type glycoproteins which contains 5 mannose residues (Robbins et al., 1977; Godelaine et al., 1981). To initiate complex glycoprotein production GlcNaC is transferred from the sugar nucleotide, UDP-GlcNAc to one mannose residue and then two more mannose residues are rapidly removed by another mannosidase activity (Tabas & Kornfeld, 1978). Immediately thereafter more GlcNAc residues, galactose, fucose and sialic acid residues, are transferred from their respective sugar nucleotide donors (Robbins et al., 1977; Tabas et al., 1978; Bretz et al., 1980; Ivatt, 1981).

The generality of a Golgi location for terminal glycosylation was established early on by EM autoradiography. Tritiated fucose and galactose are incorporated initially at the Golgi in a wide variety of cells prior to their transport to the cell surface (Bennett et al., 1974). All of the glycosyltransferases which catalyze terminal sugar additions and the alpha-mannosidases are highly enriched in morphologically defined Golgi elements isolated

from rat liver (Tabas & Kornfeld, 1979; Bretz et al., 1980; Bergeron et al., 1982). Isolated Golgi elements for enzyme localization have been obtained almost exclusively from rat liver, presumably because of their light density resulting from a high content of VLDL's (Ehrenreich et al., 1973; Bergeron et al., 1973). Although some glycosyltransferase activity has been detected at the cell surface (Roth et al., 1971; Porter & Bernacki, 1975), the cell fractionation and autoradiographic data imply that most activity is localized to the Golgi.

The Golgi location of terminal glycosylation has been the major criterion to invoke the involvement of the Golgi apparatus in the transport of plasma membrane glycoproteins. A Golgi stopover during intracellular transport has been verified for the VSV G protein and the Semliki forest virus (SFV) envelope glycoprotein by immunocytochemistry (Bergmann et al., 1981; Green et al., 1981). Both of these glycoproteins were shown to transit the Golgi stack en route from the RER to the plasma membrane at the time of terminal oligosaccharide addition. Acetylcholine receptors and immunoglobulins have been detected in the Golgi stacks of muscle and plasma cells, respectively, but their kinetics of transport were not studied (Fambrough & Devreotes, 1978; Leduc et al., 1968; Ottosen et al., 1980). Golgi involvement in the transport of several membrane and secretory glycoproteins is also suggested by inhibition of their transport with the ionophore, monensin, which causes the Golgi to vacuolate (Tartakoff et al., 1978; Tartakoff, 1982; see Chapter 4). Thus it is reasonable to conclude that all N-ASN-linked complex glycoproteins, secretory or membrane bound,

are obligatorily transported through the Golgi apparatus for processing of their oligosaccharide side chains. There is no evidence that nonglycosylated membrane proteins pass through the Golgi apparatus, but nonglycosylated secretory proteins such as insulin and several pancreatic zymogens are concentrated into secretory granules in the Golgi complex (Howell et al., 1969; Jamieson & Palade, 1967).

Selective limited proteolysis of secretory and membrane proteins may occur either in the Golgi or in the secretory granule. Conversion of proinsulin to insulin and cleavage of the common precursor to adrenocorticotropic hormone (ACTH) and endorphins occurs near the time these proteins are packaged into secretory granules (Steiner, 1976; Gumbiner & Kelly, 1981; Glembotski, 1981; see Chapter 2). The proteases may be located in the membrane of forming and formed secretory granules. Conversion of proinsulin to insulin probably continues as the secretory granules mature in the cytoplasm and a proteolytic activity has been found in crude granule fractions from rat pancreas islets of Langerhans (Fletcher et al., 1981). An activity for the cleavage of ACTH/endorphin has also been found in a granule fraction obtained from the intermediate lobe of rat pituitary (Loh & Gainer, 1982). Processing of the common precursor to neurophysins and vasopressin in neurosecretory cells of the hypothalamus takes place during axonal transport (Gainer et al., 1977). The authors believe that filled secretory granules carry the hormones down the axon, but it is possible instead that axonal transport is mediated by the smooth axonal reticulum. Several viral membrane glycoproteins are also processed

proteolytically at a late stage of their maturation between the time of their terminal glycosylation and appearance at the cell surface (Witte et al., 1977; Ziemiecki et al., 1980; Green et al., 1981; Dickson et al., 1981). Uncleaved viral envelope protein precursors are normally not detectable on the surfaces of cells by iodination. However, the Semliki Forest virus envelope protein may be cleaved at the cell surface, since antisera to the protein inhibits proteolysis when it is added to intact cells (Ziemlecki et al., 1980). The processing enzymes seem to be similar for all prohormones and viral envelope proteins examined so far. Cleavage always occurs at a pair of basic amino acids (lysine or arginine) (Kemmler et al., 1971; Nakanishi et al., 1979; Porter et al., 1979; Garoff et al., 1980; Shinnick et al., 1981; Comb et al., 1982). Appropriate cleavages of proinsulin can be obtained in vitro using a combination of trypsin and carboxypeptidase B, and similar enzymes could act in vivo (Kemmler et al., 1971). The pancreatic islet and intermediate lobe granule converting activities appear, however, to be acid thiol-arginyl proteases, clearly differing from trypsin (Fletcher et al., 1981; Loh & Gainer, 1982). The actual Golgi or granule enzymes have never been isolated. There may be some additional proteolytic specificity. For example, the ACTH/endorphin precursor is processed differently in the anterior and intermediate lobes of the pituitary (Mains & Eipper, 1981b).

Several other processing activities have been attributed to the Golgi membranes. Viral and other glycoproteins acquire a fatty acid near the time of mannose trimming (Schmidt & Schlesinger, 1980). The site of fatty acylation on the polypeptide backbone has

not yet been determined, nor has the appropriate enzyme activity been demonstrated. The role of fatty acylation is not known yet, but the lack of fatty acid on a mutant VSV G protein which is not transported out of the Golgi is intriguing (Zilberstein et al., 1980). Sulfate incorporation into oligosaccharides also occurs in isolated rat liver Golgi (Katona, 1976) and has been localized to Golgi stacks in several cells by EM autoradiography (Young, 1973). The Golgi also seems to be responsible for glycosaminoglycan biosynthesis (Silbert & Freilich, 1980) and the addition of O-linked oligosaccharides to serine, threonine and tyrosine residues in animal cells (Hanover & Lennarz, 1981).

IV. Functional Compartmentalization of the Golgi Apparatus

Two major views concerning the functional significance of stacked Golgi cisternae have been proposed. The observation that the cis Golgi saccule appears "ER-like" while the trans saccules look more like the plasma membrane has been interpreted to indicate a simple progressive modification of cisternae across the stack (Brown & Willison, 1977; Morre et al., 1979). The cisternae are believed to be formed by coalescence of vesicles at the cis face and used up by formation of secretory vesicles at the trans face (see Membrane Flow Hypothesis, section VIII). In the alternate view the cisternae are believed to be distinct functional compartments, with secretory and membrane proteins being transferred between compartments (Farquhar & Palade, 1981; Rothman, 1981). In support of the latter view, there is cytochemical evidence for enzymological differences between the cisternae and preliminary biochemical evidence for Golgi subcompartments. The compartmental-

ization of functions in the Golgi has important implications for the route of membrane traffic and is considered below.

Terminal glycosylation may be carried out within a single compartment. When isolated rat liver Golgi fractions are subjected to a variety of conditions including mild detergent treatments, all the enzymes which catalyze terminal glycosylation of complex oligosaccharides copurify as if they are contained within the same vesicles (Bretz et al., 1980). This is consistent with the hypothesis that terminal glycosylation is carried out by multienzyme complexes (Roseman, 1970). Functional interactions between GlcNAC transferase and galactosyltransferase have been demonstrated in vitro, providing preliminary support for a multienzyme complex (Ivatt, 1981).

Do these enzymes reside in a distinct Golgi subcompartment? The evidence obtained so far by several approaches is conflictive. Subfractionation of rat liver Golgi has not yet revealed sublocalization of glycosyltranferases. Light and heavy subfractions of Golgi which are believed to be enriched in trans and cis elements, respectively can be isolated from rat liver (Ehrenreich et al., 1973; Bergeron et al., 1973). The specific activities of the terminal glycosyltransferases are slightly greater in the light fraction than the heavy fraction, but the total activities are similar in both fractions (Bretz et al., 1980). Also, both fractions incorporate ^{3}H-galactose and ^{3}H-sialic acid into endogenous glycoproteins (Banerjee et al., 1976; Bergeron et al., 1982). These results have been interpreted to mean that glycoproteins are glycosylated in all Golgi saccules as they are transported through

the stack. However, since there is considerable overlap between Golgi elements from the light and heavy subfractions it is difficult to assign any activity to any particular element. On the other hand, cytochemical staining for thiamine pyrophosphatase (TPPase), an enzyme believed to be a marker for membranes involved in terminal glycosylation, is usually restricted to the one or two most trans cisternae of the Golgi in many cells including hepatocytes (Novikoff, 1976; Novikoff et al., 1977; Broadwell & Oliver, 1981; Farquhar & Palade, 1981). TPPase is the same enzyme as nucleoside diphosphatase (Yamazaki & Hayaishi, 1968). Kuhn & White (1977) have provided evidence that it breaks down UDP generated by galactosylation in the lumen of Golgi vesicles. TPPase does copurify with galactosyltransferase when rat liver Golgi is partitioned in aqueous two phase polymer systems (Hino et al., 1978). The localization of TPPase is consistent with the restriction of terminal glycosylation predominantly to the trans saccules of the Golgi apparatus.

Rothman (1981) has proposed that mannose trimming enzymes and terminal glycosyl transferases reside in distinct early and late Golgi compartments which may represent cis and trans elements. Although alpha-mannosidases copurify with galactosyltransferase in the total Golgi fraction isolated from rat liver (Tabas & Kornfeld, 1979) their distributions in light and heavy subfractions have not been reported. On the other hand, fractionation of chinese hamster ovary cell membranes on sucrose density gradients showed that alpha-mannosidase activity peaked at a slightly greater density than galactosyltransferase activity (Dunphy et al., 1981). There

was, however, considerable overlap in the enzyme distributions. The authors claim that the enzymes reside in distinct compartments because they believe they can reconstitute transport between such compartments in vitro. For reconstitution, donor membranes were obtained from VSV-infected mutant cells which can remove mannose but cannot initiate terminal glycosylation (Fries & Rothman, 1980). Donor membranes can be shown to fuse with rat liver Golgi membranes during reconstitution because the G protein acquires terminal carbohydrates (Rothman & Fries, 1981). Fusion depends on a soluble factor and a continuous supply of ATP. The ability of donor membranes to transfer G protein in vitro occurs transiently as the intracellular transport of G protein proceeds in the donor cells (Fries & Rothman, 1981). The loss of transferability in vitro is believed to result from the transport of G protein in vivo to a new compartment which cannot act as a donor. The new compartment is thought to be one which catalyzes terminal glycosylation because the decline in transferability in vitro occurs with the same kinetics as the normal conversion of G protein in whole cells from a trimmed to a fully glycosylated state (Dunphy et al., 1981). It is possible however that the decline in transferability results from the transport of the G protein out of the Golgi entirely. The donor membranes have not yet been identified and it is possible that fusion occurs between identical Golgi elements. It is interesting in this regard that Paiement et al. (1982) have reported ATP dependent fusion between isolated Golgi membranes which contain galactosyltransferase. Rothman (1981) has proposed that the postulated early and late compartments containing alpha-mannosidases and

glycosyltransferases, respectively, represent cis and trans Golgi cisternae, consistent with the segregation of TPPase to trans Golgi. However, the morphological entities in their reconstitution are unknown. The inhibition of intracellular transport by monensin also has been attributed to an early Golgi to late Golgi step between mannose trimming and terminal glycosylation (Tartakoff & Vassalli, 1979; Tartakoff, 1982). I present evidence, however, that the block in transport occurs after terminal glycosylation (see Chapter 4). If α-mannosidases and glycosyltransferases are segregated into cis and trans Golgi cisternae, respectively (Rothman, 1981), the postulated cis to trans route for transport must occur, at least for complex type glycoproteins.

Concentration of secretory products into secretion granules may also be effected by a distinct Golgi compartment. Endocrine and neurosecretory cells usually form secretory granules in the transmost Golgi saccules (Howell et al., 1969; Farquhar, 1971; Broadwell & Oliver, 1981; Farquhar & Palade, 1981). In exocrine tissues such as pancreas and parotid, secretory granules are derived from a special organelle, the condensing vacuole, which generally lies near and may be associated with the trans Golgi saccule (Jamieson & Palade, 1967; Castle et al., 1972). The requirement for a morphologically distinct condensing vacuole may be due to the enormous storage capacity of exocrine cells. The hyperstimulated pancreatic acinar cell has no condensing vacuoles and forms smaller than normal zymogen granules in the trans Golgi cisterna (Jamieson & Palade, 1971b). Does condensation occur in a compartment distinct from the site of terminal glycosylation?

Secretory products are eventually segregated away from glycosyltransferases in some cells. The membranes of both chromaffin and zymogen granules are devoid of galactosyl transferase activity (Hortnagl, 1976; Ronzio, 1973). Secretory granules frequently are observed to arise from a cisterna, often called GERL, which stains histochemically for acid phosphatase (Novikoff, 1976; Hand & Oliver, 1977; Broadwell & Oliver, 1981). (GERL is an acronym for Golgi, endoplasmic reticulum, lysosome.) This compartment often has been distinguished from the TPPase staining trans Golgi saccule. In fact, the condensing vacuole of the exocrine pancreas cell sometimes reacts positively for acid phosphatase, but does not stain for TPPase (Novikoff et al., 1977). Novikoff (1976) claims that GERL is continuous with the ER and is an organelle distinct from the Golgi apparatus. However many investigators who acknowledge the existence of GERL as a special acid phosphatase positive cisterna consider it to be part of the trans Golgi and not continuous with the ER (Hand & Oliver, 1977; Broadwell & Oliver, 1981; Farquhar & Palade, 1981). Assuming that TPPase is a consistent and accurate marker for membranes containing terminal glycosyltransferases, these observations imply that condensation of secretory protein and terminal glycosylation occur in separate compartments. Segregation of TPPase and granule formation is not always maintained however. When vasopressin production and secretion by hypothalamic supraoptic neurons is stimulated by hyperosmotic stress, secretory granules appear to arise from all Golgi saccules and all of the saccules stain for TPPase (Broadwell & Oliver, 1981).

Other observations raise doubts about the involvement of GERL in the formation of secretory granules. Peroxidase cytochemistry and neurophysin immunocytochemistry of the exorbital lacrimal gland and hypothalamic supraoptic nucleus respectively, revealed these secretory products in the trans saccule of the Golgi stack, but not in GERL (Hand & Oliver, 1977; Broadwell et al., 1979). Yet, these authors observed secretory granules, identified solely by morphological criteria, forming in continuity with acid phosphatase positive GERL. They proposed that the transmost Golgi saccule may be converted to GERL during the process of secretory granule formation and that the secretory contents are extruded from the saccule into the granule as it collapses into GERL. The potential role of GERL or acid phosphatase in the condensation of secretory proteins remains to be clarified. Acid phosphatase could have a function in condensation as yet undescribed. Alternatively, acid phosphatase may represent the distribution of lysosomal hydrolases in the Golgi as originally proposed (see Section VI) and its association with secretory granules could indicate degradation of excess secretory protein or membrane. Degradation of secretory protein by fusion of secretory granules with lysosomes, called crinophagy, has been described in pituitary mammatrophs (Smith & Farquhar, 1966; Farquhar, 1971). The decrease in acid phosphatase staining of forming secretory granules in supraoptic neurons after stimulation could reflect a decrease in vasopressin degradation (Broadwell & Oliver, 1981).

Selective proteolysis of prohormones could also be localized to a specialized condensing compartment since it often occurs near

the time of packaging (Steiner, 1976; Gumbiner & Kelly, 1981; Chapter 2). For example, the ACTH/endorphin precursor is fully glycosylated before it is cleaved in AtT-20 cells (Phillips et al., 1981). Some of the glycosylated precursor is released from the cells before it is cleaved, apparently by a pathway not involving the secretory granules which release cleaved hormones (Gumbiner & Kelly, 1982; Chapter 3). Thus proteolysis seems to be associated mostly with the concentration step and unpackaged precursor is secreted by another route immediately after glycosylation. Does the secreted precursor bypass the condensing compartment entirely? Perhaps the proteases are highly concentrated in the secretory granule membrane or the membrane of the late Golgi compartment where granules form.

V. Route of Protein Transport Through the Golgi Apparatus

What is the route of membrane and secretory protein transport through the Golgi? There is good immunocytochemical evidence that secretory and membrane glycoproteins are present in all saccules of the Golgi stack. The best examples include IgG secretion by plasma cells and the transport of the envelope glycoproteins of VSV and SFV (Leduc et al., 1968; Ottosen et al., 1980; Bergmann et al., 1981; Green et al., 1981). Evidence for cis to trans movement through the Golgi has been obtained by immunocytochemical localization of a temperature sensitive mutant VSV G protein (Bergmann, 1981; Bergmann et al., 1981). The ER to Golgi transport of this mutant can be synchronized by a change from the nonpermissive to the permissive temperature. The G protein is initially added asymmetrically to one side of the Golgi stack. The cis or trans

sides could not be discerned.

A direct RER to condensing vacuole route of transport bypassing the flattened saccules of the Golgi stack has been proposed for zymogen proteins in the pancreas and for peroxidase in the lacrimal gland (Palade, 1975). No autoradiographic grains were found over the Golgi stack in pulse-chase studies of zymogen transport (Jamieson & Palade, 1967). The flattened Golgi saccules in lacrimal gland were negative for peroxidase cytochemistry (Herzog & Miller, 1972). It is possible that only accumulated secretory products could be detected in these experiments and that transient passage through the Golgi stack was overlooked. Amylase has been detected in Golgi saccules of the pancreas by immunocytochemistry (Geuze et al., 1979) but the lack of quantitation makes its significance difficult to determine. Although some peroxidase activity has been demonstrated in Golgi stacks of the lacrimal gland in more recent studies using different cytochemical procedures, the Golgi saccules contained much less reaction product than either the RER or the condensing vacuoles (Hand & Oliver, 1977). Peroxidase staining has been localized in all Golgi cisternae in polymorphonuclear leukocytes, however (Bainton & Farquhar, 1970). In my opinion, the involvement of the Golgi stacks in the transport of zymogens and peroxidase remains unresolved. Interestingly, most of the zymogens are not glycosylated (Ronzio, 1973; Lehrner & Malacinski, 1975). Is it possible that only glycoproteins must pass through the Golgi stack in order to be processed while nonglycosylated secretory proteins can be sent directly to a condensing compartment? There is evidence in yeast for a direct RER to plasma

membrane pathway which bypasses the Golgi. Yeast mutants that block the transport of most secretory and membrane proteins at the Golgi or secretory vesicle stage do not affect the appearance of galactose permease at the cell surface (R. Schekman, personal communication). ER mutants do inhibit its transport. Unfortunately, the biosynthesis and carbohydrate processing of galactose permease have not yet been studied. Perhaps the route of intracellular transport is determined to a large extent by the requirement for posttranslational processing.

What is the functional significance of the stacking of multiple Golgi cisternae? Rothman (1981) has proposed that all plasma membrane proteins must pass sequentially through the Golgi saccules in order to be efficiently purified away from the RER. Since plasma membrane proteins must be purified ten thousand fold from the RER, he hypothesized a multistep purification or "distillation" through the stacked cisternae. It is not yet known, however, to what extent membrane proteins can be segregated during a single vesicle budding event. If 100 fold sorting can occur, only two budding events would be required to achieve ten thousand fold purification.

The alternate membrane flow model, that the stacked Golgi saccules represent progressive temporal modifications of a single membrane cisterna en route to the cell surface, is difficult to reconcile with the concept of distinct enzymological compartments. Yet membrane transport in some cells seems to operate in this manner. A progressive cis to trans development of cisternae appears to take place during the production of scales in some algae (Brown

& Willison, 1977). Yeast cells normally appear in the EM to have little in the way of a secretory apparatus, presumably due to the very rapid rate of secretion compared to mammalian cells (Novick et al., 1980). However, Golgi-like stacks of membrane cisternae accumulate in mutants blocked in secretion and cell surface growth at a stage where the proteins are terminally glycosylated (Esmon et al., 1981; Novick et al., 1981). Therefore membrane in transit can form stacks when secretory vesicle formation is blocked or slowed relative to the rate of membrane production by the RER. It seems possible then, that a slower rate of glycosylation or vesicle formation in other types of cells could account for the stacking of membranes en route to the cell surface. The apparent difference between models which invoke distinct Golgi compartments versus models of progressive cisternal transformations may represent a range of transport modes exhibited by various cell types. The distinction only depends on the proportion of membrane en route to the cell surface relative to membrane that is indigenous to Golgi. As the complexity of membrane traffic increases in cell types which accommodate more pathways, such as storage pathways and pathways for membrane recycling, the Golgi may take on a more compartmentalized function in addition to its role in the unidirectional elaboration of surface membrane (see Section VIII).

Farquhar and Palade (1981) have proposed that most traffic of membrane and secretory products is restricted to the dilated peripheral rims of the Golgi cisternae. Concentrated secretory proteins are probably exported from the dilated rims. The rims are the sites of secretory granule formation and the destination of

membrane that has been recycled from the surface of secretory cells (Herzog & Farquhar, 1977; Farquhar, 1978). Also a plasma membrane marker, adenylate cyclase, has been localized to the cisternal rims of rat liver Golgi by enzyme cytochemistry (Cheng & Farquhar, 1976). Farquhar and Palade also conclude that incoming traffic from the RER fuses with the dilated cisternal rims because presumptive ER marker enzymes are enriched in peripheral Golgi elements isolated from rat liver (Ito & Palade, 1978). Although a peripheral route of transport through the cisternal rims may occur in some secretory cells such as the exocrine pancreas, it clearly is not the pathway taken by viral membrane glycoproteins. Both the VSV G protein and the SFV spike protein were found randomly distributed throughout all portions of every Golgi cisterna by immunocytochemistry (Bergmann et al., 1981; Green et al., 1981). It is possible that membrane traffic enters and leaves the Golgi at the rims, but at least some of it must mix with the flattened central portions of the saccules. It is not yet known where the export vesicles for nonconcentrated secretory proteins or membrane glycoproteins arise from the Golgi.

Presumably, membrane proteins en route to the cell surface must be sorted away from Golgi processing enzymes such as the glycosyltransferases. This appears to be the case for zymogen granule and chromaffin granule membranes (Ronzio, 1973; Hortnagl, 1976). (But some of the glycosyltransferases residing at the surface of some cells could result from inefficient sorting.) The properties of the glycosyltransferases suggest one model for their segregation from exported glycoprotein substrates. If the terminal

glycosyltransferases are all associated in a multienzyme complex as proposed by Ivatt (1981), they may cluster into distinct membrane domains. Glycoprotein substrates would interact with the enzyme complex until completion of glycosylation, and then they would be free to dissociate. Ito and Palade (1978) have suggested that glycosyltransferases are segregated into the flattened central portions of the Golgi cisternae from rat liver. Immunoadsorbed Golgi elements enriched in VLDL packed dilated cisternal rims were not enriched in glycosyltransferase activity. This result is consistent with the segregation of secretory product away from glycosyltransferases in the trans Golgi cisterna, but the intra Golgi location of glycosyltransferases needs to be more rigorously determined. Could similar intramembrane interactions between biochemically related enzymes or structural proteins account for the sorting of proteins that occurs in other organelles such as the RER or plasma membrane?

In summary, the rigorously documented functions of the Golgi apparatus are the processing of glycoconjugates and the proteolytic processing and concentration of secretory products. The commonly assumed role of the Golgi in the sorting of proteins which have different intracellular destinations will be discussed in the following sections.

VI. Sorting of Lysosomal Hydrolases

Lysosomes contain a variety of hydrolytic enzymes including proteases, glycosidases, nucleases, lipases etc. which act optimally at acid pH (deDuve & Wattiaux, 1966; Bainton, 1981). Segregation of lysosomal hydrolases away from many cell constituents is

an obvious requirement. The discovery of a lysosomal hydrolase recognition marker which mediates their translocation to lysosomes is the best example of an intracellular sorting event (Neufeld, 1981).

Unlike most other cellular organelles, lysosomes were discovered by biochemical procedures rather than microscopic observations. They were originally defined as sedimentable particles with membrane enclosed acid hydrolases (initially acid phosphatase) and are now known to comprise a morphologically heterogeneous set of vesicles (deDuve et al., 1955; deDuve & Wattiaux, 1966; Fawcett, 1966; Bainton, 1981). Since there is no unique morphological definition of a lysosome, their identification inside of cells frequently requires enzyme cytochemistry. Acid phosphatase activity copurifies with acid hydrolases and has been commonly used to identify lysosomes by electron microscopy. Acid phosphatase may not be a unique marker for lysosomes however, since it has been demonstrated in secretory organelles such as condensing vacuoles and secretory granules (Novikoff, 1976; Novikoff et al., 1977; Broadwell & Oliver, 1981). Acid phosphatases are also known secreted proteins of yeast and Dictyostelium, and could have a similar role in mammalian cells (Novick et al., 1980; Dimond et al., 1981). Aryl sulfatase activity has also been used as a cytochemical lysosomal marker (Nichols et al., 1971; Bainton, 1981).

Special kinds of lysosome are found in various tissues, including the dense bodies of liver or azurophile granules of polymorphonuclear leukocytes (Fawcett, 1966; Bainton & Farquhar, 1970). Morphological heterogeneity of lysosomes also occurs within a cell.

Different structures represent the various stages in the life at the lysosome (deDuve & Wattiaux, 1966; Friend & Farquhar, 1967; Bainton, 1981). Small (20-50 nm diameter) coated and uncoated Golgi associated vesicles and the larger dense bodies found in liver and polymorphonuclear leukocytes are considered primary lysosomes because they are thought to have not yet fused with vesicles containing material destined for digestion. Secondary lysosomes contain material being digested and include multivesicular bodies, phagolysosomes and autophagic vacuoles which may be involved in endocytosis, phagocytosis and degradation of whole organelles respectively (Geuze & Kramer, 1974; Muller et al., 1980; Bainton, 1981). Residual bodies are strongly osmiophilic structures thought to represent a late stage of digestion. Their ultimate fate is usually unknown, i.e. whether they accumulate intracellularly or whether the hydrolases can be reused after this stage of digestion. Dictyostelium cells egest the product of their digestive vacuoles by exocytosis (Dimond et al., 1981), but residual bodies such as lipofuscin pigment granules may accumulate in human tissues with age (Fawcett, 1966). Unlike secretory vesicles, mature lysosomes usually do not exocytose, at least not in fibroblasts. Only lysosomal enzyme precursors that have not yet been cleaved in the lysosome are secreted by fibroblasts and then only when their delivery to lysosomes is diverted by increasing lysosomal pH with weak bases or as a result of inherited disease (Hasilik & Neufeld, 1980a). Cell surface membrane must be internalized in order to fuse with lysosomes. Fusion with lysosomes and exocytosis of secretory vesicles presumably depend upon different

recognition features.

Lysosomal hydrolases contain N-ASN linked oligosaccharides (Tabas & Kornfeld, 1980) and one enzyme, cathepsin D, has been shown to be cotranslationally segregated at the RER (Erickson & Blobel, 1979). They are presumed to be transported to lysosomes by a route through the Golgi similar to secretory proteins. Novikoff has identified an acid phosphatase positive Golgi associated cisterna in continuity with the RER. He has called this organelle GERL and proposed that it gives rise to new lysosomes (Novikoff et al., 1971; Novikoff, 1976). Although many authors recognize GERL as a special acid phosphatase reactive cisternae at the trans side of the Golgi, its continuity with the RER has been questioned (Hand & Oliver, 1977; Broadwell & Oliver, 1981). Novikoff's generalization of GERL to include secretory granule formation confuses the role of GERL in lysosomal enzyme segregation (Novikoff, 1976; Novikoff et al., 1977). The functional meaning of GERL remains to be clarified. The actual route for the transport of hydrolases will undoubtedly be elucidated now that the proteins involved in their targeting have been identified.

The recognition marker for the targeting of lysosomal hydrolases was discovered by comparing enzymes secreted from normal fibroblasts and fibroblasts obtained from patients with inherited I cell disease. Although I cells secrete rather than store their own hydrolases they are fully capable of taking up and storing enzymes secreted from normal cells (Sly & Stahl, 1978; Neufeld, 1981). The high uptake forms of the enzymes have one or two 6-phosphomannosyl groups on their oligosaccharides (Hasilik & Neufeld, 1980b). The

hydrolases secreted by I cells do not have phosphorylated oligosaccharides because the I-cells do not express the required glycosyltransferase activity (see below) (Reitman & Kornfeld, 1981a).

Internalization of high uptake hydrolases depends on their binding to a cell surface receptor (Sly & Stahl, 1978; Rome et al., 1979; Neufeld, 1981). Mannose-6-phosphate blocks both uptake and binding by competing for the recognition marker (Kaplan et al., 1977; Sly & Stahl, 1978). Because there is a functional surface receptor and because normal fibroblasts secrete high uptake forms of the hydrolases, Neufeld et al. (1977) proposed a "secretion-recapture" route for normal delivery to lysosomes. The secretion-recapture model has been proved wrong subsequently because normal fibroblasts cultured in mannose-6-phosphate were not depleted of their stores of hydrolases (Sly & Stahl, 1978). Apparently normal fibroblast secrete newly synthesized hydrolases only when they are cultured in weak bases such as ammonia or chloroquine (Hasilik & Neufeld, 1980a,b; Gonzalez-Noriega et al., 1980). Ammonia can arise in cell culture media from deamination of glutamine.

An intracellular receptor was proposed to mediate the transport of newly synthesized enzymes to the lysosome (Fischer et al., 1980). In rat liver there is an intracellular receptor with the same hydrolase binding properties as the fibroblast surface receptor. At least 80% of the liver receptor is intracellular. Most of the receptors fractionated with rough microsomes and were occupied by endogenous lysosomal enzymes. Thus it was proposed that the receptor carries newly synthesized acid hydrolases from the RER to the lysosome. The function of the surface receptor is unclear. It

either constitutes a minor pathway for salvage or is a part of the receptor's normal life cycle (Neufeld, 1981).

Biosynthesis of the mannose-6-phosphate recognition marker appears to be the initial event which distinguishes the lysosomal hydrolases from other secretory glycoproteins. A glycosyltransferase first transfers N-acetylglucosamine phosphate from UDP-N-acetylglucosamine to the 6 position of the oligosaccharide mannose residues, forming a phosphate diester linkage (Reitman & Kornfeld, 1981a). The N-acetylglucosamine is then removed by the action of α-N-acetylglucosaminyl phosphodiesterase to unmask the mannose-6-phosphate recognition marker (Tabas & Kornfeld, 1980; Waheed et al., 1981a). A partially purified preparation of the N-acetylglucosaminyl phosphotransferase utilizes high mannose oligosaccharides of lysosomal enzymes as substrates 100 times more efficiently than high mannose oligosaccharides of nonlysosomal glycoproteins (Reitman & Kornfeld, 1981b). The acceptor activity of lysosomal enzymes is greatly reduced by heat denaturation. Reitman and Kornfeld therefore proposed that lysosomal hydrolases probably all have a common protein domain recognized by the phosphotransferase that is ultimately responsible for their sorting away from secretory glycoproteins.

Sorting of lysosomal enzymes from secretory glycoproteins probably occurs in the Golgi apparatus rather than the RER. Both the phosphotransferase and the diesterase activities fractionate with galactosyltransferase containing Golgi membranes from rat liver (Waheed et al., 1981b). Also, phosphorylation of β-glucuronidase in macrophages lags behind mannose addition in the RER by

15-30 min (Goldberg & Kornfeld, 1981). Unmasking of the recognition marker by the phosphodiesterase occurs with the same kinetics as the trimming of mannose residues attributable to the Golgi. If lysosomal enzymes are first distinguished from other glycoproteins by the phosphotransferase, there is no reason to expect that they are segregated prior to their transport through the Golgi. The claim that hydrolases first bind to the mannose-6-phosphate receptor in the RER (Fischer et al., 1980) is difficult to reconcile with the idea that they do not obtain the recognition marker until the Golgi. The discrepancy may result from problems with cell fractionation. For example, transport vesicles not derived from the RER that contain bound receptors (e.g. coated vesicles) could contaminate the rough microsome fraction.

Gonzalez-Noriega et al. (1980) have provided evidence that net transport of acid hydrolases from the Golgi or cell surface could result simply from its receptor binding properties. Binding occurs maximally at neutral pH, but dissociation is favored at the acidic pH found inside of lysosomes. Hydrolases could bind to the receptor at the surface or in the Golgi at neutral pH and dissociate from the receptor upon delivery to a low pH compartment.

In fibroblasts unbound receptors probably recycle to the surface after they have internalized hydrolases, since enzyme uptake exceeds receptor binding capacity when cell protein synthesis is blocked (Rome et al., 1979). If intracellular receptors function similarly, they would act as cycling shuttles between Golgi and lysosome. That intracellular receptors function this way is supported by the finding that chloroquine enhances lysosomal

enzyme secretion and inhibits lysosomal enzyme pinocytosis by impairing receptor recycling (Gonzalez-Noriega et al., 1980). Because choloroquine raises the pH of the lysosome, the enzymes might not dissociate from their receptors in the lysosome. Secretion of newly synthesized enzymes develops over time with chloroquine treatment even though lysosome pH increases immediately. The enzymes may be secreted because their synthesis continues after all the intracellular receptors become occupied.

Coated vesicles mediate endocytosis of receptor bound hydrolases (Willingham et al., 1981), and have been proposed to be involved in the transport of lysosomal enzymes from the Golgi (Friend & Farquhar, 1967). Are the small (20-50 nm) coated and uncoated acid phosphatase positive Golgi vesicles that are considered primary lysosomes the morphological equivalent of the intracellular shuttle vesicles? With which organelles do these vesicles fuse to unload their contents; with larger primary lysosomes, GERL or directly with secondary lysosomes such as phagolysosomes, multivesicular bodies, autophagic vacuoles or the recently described endosome or receptosome (Helenius et al., 1980; Willingham et al., 1981)? Probably all of these compartments have an acidic interior in which hydrolases could dissociate from their receptors and accumulate (Geisow et al., 1981; Maxfield & Tycko, 1981).

VII. Biogenesis of Secretory Vesicles

Exocytosis or fusion of preformed vesicles with plasma membrane is usually accepted as the event by which all secretory proteins and integral membrane glycoproteins are delivered to the surface of eucaryotic cells (Palade, 1975; Morre et al., 1979;

Farquhar & Palade, 1981; Lodish et al., 1981). Most information on the biogenesis and exocytosis of secretory vesicles has come from studies of dense core secretory granules of exocrine and endocrine cells and neurons. Because these storage vesicles accumulate within cells and exhibit unique and homogeneous physical properties, they can be identified by EM and isolated by standard physical techniques. To what extent these secretory granules represent all exocytotic vesicles is unclear.

Secretory granules often contain high concentrations of proteins or transmitters which are osmiophillic, resulting in their dense core appearance in EM. Analysis by high resolution EM autoradiography indicates that pituitary prolactins are concentrated in granules approximately 200 times greater than in the RER (Salpeter & Farquhar, 1981). Isolated granules have a very high specific activity of secretory product, for example, 50% hormone content for ACTH granules (Gumbiner & Kelly 1981; see Chapter 2) and greater than 96% secretory product for zymogen granules (Ronzio et al., 1978). Secretory proteins can be condensed in the granule in an osmotically inactive state. Insulin appears crystalline in beta granules (Grieder et al., 1969). Zymogen, parotid and prolactin granules all resist osmotic lysis (Palade, 1975; Castle et al., 1975; Giannattasio et al., 1975). In fact, the protein core of prolactin granules can be isolated in stable form after the membranes have been removed by mild detergent extraction (Giannattasio et al., 1975). Is the condensation of secretory product into a nonosmotic state characteristic of most protein containing dense core secretory granules?

Although the physical basis for condensation or concentration is not yet understood, several theories have been proposed. Any likely process must be reversible, since the secretory proteins must dissociate when exposed to the extracellular environment. pH dependent processes have frequently been invoked, since a low intragranular pH would be raised to neutrality upon exocytosis. There is considerable evidence that secretory granules, like lysosomes, have a low internal pH. pH's of 5.5 have been measured directly in chromaffin granules which contain catecholamines and in neurohypophyseal granules containing vasopressin and neurophysins (Scarpa & Johnson, 1976; Russell & Holz, 1981). Although pH gradients have not been measured in other granules, their condensed state requres low pH. Insulin granules are particularly unstable at neutral or high pH (Howell et al., 1969). Despite their resistance to osmotic lysis the contents of both zymogen granules and prolactin granules are readily solubilized by pH's slightly above neutrality (Giannattasio et al., 1975; Reggio & Dagorn, 1978). The transmembrane pH gradient of chromaffin and neurohypophyseal granules is probably generated by a proton pump that is dependent on ATP (Scarpa & Johnson, 1976; Russell & Holz, 1981).

Palade (1975) has argued against a role for ion pumps in the condensation process. Energy inhibitors do not block the conversion of condensing vacuoles to zymogen granules in the pancreas (Jamieson & Palade, 1971a). Whether this energy independent conversion represents concentration by the addition of more secretory protein or the condensation of protein already present in condensing vacuoles is unclear. It is interesting, in contrast, that

secretory vesicle formation in yeast does depend on expenditure of metabolic energy (Novick et al., 1981). Instead of ion pumping Reggio and Palade (1978) proposed that the polyanionic sulfated proteoglycans found in zymogen granules and pancreatic discharge form large aggregates with secretory proteins by ionic interactions. Sulfated proteoglycans or glycosaminoglycans have been found in other secretory granules including chromaffin, prolactin and histamine granules and cholinergic synaptic vesicles (Winkler, 1976; Geissler et al., 1977; Zanini et al., 1980; S. Carlson, personal communication). In most cases however the polyanions are present in proportions too small relative to content to account for the required binding capacity. Reggio and Dagorn (1978) were able to form precipitates of some zymogen granule proteins in vitro by adding larger quantities (5-8%) of chondroitin sulfate. They hypothesized that smaller amounts may precipitate secretory proteins in vivo by initiating crystallization. Interestingly, aggregate formation in vitro is pH dependent, fulfilling the requirement for reversibility. The formation of other ionic complexes such as zinc-insulin crystals (at low pH) could also be the basis for condensation (Steiner, 1976).

Binding proteins such as the neurophysins of the posterior pituitary and chromogranins in chromaffin granules have been proposed as intragranular carrier proteins for secretory products (Gainer et al., 1977; Winkler, 1976). Binding of neurophysin to vasopressin is maximal at pH 5.5 with dissociation favored at extracellular pH (Camier et al., 1973). It is noteworthy that neurophysins and the hormones that they bind, oxytocin or vasopres-

sin, are generated by limited proteolysis of a common precursor (Gainer et al., 1977). It seems however, that the proposed carrier function of neurophysins would be obviated, if, as proposed by Gainer et al. (1977), proteolysis of the common precursor occurs in already formed secretory granules. Is prohormone cleavage generally involved in hormone packaging? Both events often occur together in time. It has been suggested that proteolysis could provide a chemically irreversible change in the state of secretory products to allow their concentration under appropriate conditions (e.g., pH) (Steiner, 1976). Interestingly, proteolysis of proinsulin and proglucagon by an impure preparations of granules is maximal at low pH (Fletcher et al., 1981). Specific limited cleavage of the ACTH/endorphin precursor by secretory granules isolated from rat neurointermediate lobe also occurs maximally at pH 5.5 (Loh & Gainer, 1982). Acidification is again implicated in the processes of granule formation.

Could the process of condensation underlie the sorting of secretory granule proteins away from other proteins? If so, the process must be specific for granule proteins. Yet many proposed mechanisms invoke nonspecific processes such as ionic interactions. Alternatively, secretory proteins could be sorted prior to their delivery to a specialized condensation compartment of the Golgi (Section IV).

Exocytosis of secretory granules in exocrine, endocrine and neuronal cells is regulated physiologically by changes in plasma membrane potential or surface receptor occupancy. An increased intracellular level of Ca^{++} is believed to be the final signal that

stimulates exocytosis. Increased levels of Ca^{++} usually result from physiologically regulated influx of extracellular Ca^{++}, but Ca^{++} may also be released from intracellular stores (Rubin, 1974; Palade, 1975; Kelly et al., 1979). Cyclic nucleotides may also play a role in regulating exocytosis, either directly or indirectly by increasing intracellular Ca^{++} (Haymovits & Scheele, 1976; Schubart et al., 1980). Whether proteins at the cytoplasmic surface of secretory granules or plasma membrane catalyze Ca^{++} dependent memebrane fusion is not yet known for certain (Kelly et al., 1979). A protein called synexin has been proposed to mediate exocytosis of chromaffin granules (Creutz et al., 1978). Although a direct role for synexin in exocytosis has not been demonstrated, it does seem to facilitate Ca^{++} dependent fusion of artificial liposomes (Hong et al., 1982). Because of its role in Ca^{++} regulated processes, calmodulin has also been implicated in exocytosis. An inhibitor of calmodulin binding, trifluoperazine, blocks secretion in many cells (Schubart et al., 1980; Steinhardt & Alderton, 1982). Calmodulin binding sites on secretory granule membranes have been found (Bartlett & Scheele, 1981; J. Hooper, personal communication). Most interesting is the finding that antibodies to calmodulin block the Ca^{++} dependent exocytosis of cortical granules in a lysed preparation of sea urchin eggs (Steinhardt & Alderton, 1982). Another candidate is a 65 Kd membrane protein defined by a monoclonal antibody that is found at the cytoplasmic surface of synaptic vesicles (Matthew et al., 1981). This protein is found in tissues where exocytosis is regulated by Ca^{++}, but is absent from secretory tissues such as liver or muscle where exocytosis occurs

constitutively. The function of this protein is unknown. Exocytosis of some secretory vesicles occurs constitutively even in cells which have a regulated exocytotic pathway (Gumbiner & Kelly, 1982; Chapter 3). The function of a regulatory protein may be to confer Ca^{++} sensitivity on an otherwise constitutive fusion event.

Much less is known about secretory vesicles which constitutively deliver unconcentrated secretory proteins or membrane proteins to the cell surface. Presumably these vesicles do not accumulate to any great extent in cells. Because they are not osmiophillic by EM, it has been assumed that their secretory content is not concentrated, but it is possible that concentration could occur to a lesser extent. Although they are less distinctive than secretory granules morphologically, such vesicles in some cases have been identified. Numerous small clear vesicles have been observed near the plasma membrane of epithelial cells and plasma cells (Benett et al., 1974; Tartakoff & Vassalli, 1977) and in growing tips of neurons (Pfenninger & Maylie Pfenninger, 1981) and in buds of yeast (Novick et al., 1980). In only a few cases have the contents of such vesicles been identified, including acid phosphatase vesicles in yeast (Novick et al., 1980), immunoglobulin containing vesicles in plasma cells (Ottosen et al., 1980), VLDL secretory vacuoles in liver (Yokoto & Fahimi, 1981) and casein micelle containing vesicles in mammary epithelial cells (Franke et al., 1976). Coated vesicles have been reported to mediate Golgi to plasma membrane transport of the VSV G protein, but contamination of the isolated coated vesicle fractions was not ruled out (Rothman et al., 1980). Non-concentrating or constitutive secretory vesi-

cles have been isolated from only one source, i.e. yeast mutants blocked in exocytosis which accumulate large numbers of acid phosphatase containing vesicles (T. Etcheverry, personal communication). It will be interesting to compare these vesicles to the condensing Ca^{++} regulated secretory granules to see, for example, whether they also have ATP driven proton pumps or calmodulin binding sites.

VIII. The Sorting of Plasma Membrane Glycoproteins and Secretory Proteins

It has become apparent that both secretory proteins and plasma membrane glycoproteins are synthesized and transported by similar if not identical pathways. Both are cotranslationally segregated and glycosylated at the RER, undergo carbohydrate and proteolytic processing during transport through the Golgi and are delivered to the cell surface by exocytosis of carrier vesicles. Clearly, then it is possible that plasma membrane and secretory proteins are transported from the RER to the cell surface by the identical membranous structures, differing only by whether they remain associated with the plasma membrane phospholipid bilayer. However, the results that I present in Chapter 3 and the evidence discussed below demonstrate that newly synthesized membrane and secretory proteins are in some cases segregated from each other during their transport to the cell surface. The segregation of transport pathways appears not to depend upon a membrane versus secretory protein distinction, but rather depends on the functions of the pathways that are required by each particular cell.

Two major theories concerning the relationship between protein secretion and plasma membrane biogenesis have been proposed. According to the membrane flow hypothesis of Morre and colleagues (1979), membrane (proteins and lipid) is transported unidirectionally from its site of synthesis at the RER to the plasma membrane, carrying secretory proteins within its contents to be released into the extracellular space. To account for the retention of the compositional specificity of organelles such as the ER and the Golgi and for the modifications of glycoproteins en route to the cell surface, Morre et al. (1979) proposed a 'flow-differentiation' hypothesis. The implications of the flow hypothesis for traffic through the Golgi are discussed in section V. Palade (1975) on the other hand proposed that secretory proteins are transported between functionally and compositionally distinct membrane compartments, i.e. ER to Golgi and Golgi to plasma membrane, by special shuttle vesicles. The shuttling vesicles would retain their specificity after fusing with the recipient membrane and bud off selectively to return empty to the donor compartment for another round of transport. Plasma membrane biogenesis is usually believed in this theory to require a separate pathway (Rothman, 1981; Farquhar & Palade, 1981). The two theories make testable predictions about the composition and fate of the containers that transport secretory proteins. The shuttle concept predicts that secretory vesicles are different from the plasma membrane, make no permanent contribution to it after exocytosis and that their membrane is retrieved and reutilized for secretion. The flow hypothesis predicts that secretory vesicles are the same as or similar to the plasma membrane and

deliver components to it during secretion. When protein secretion occurs in great excess of the requirement for plasma membrane growth, excess membrane would be degraded in lysosomes rather than reutilized. We will see below that certain aspects of both theories describe membrane traffic, depending on the particular pathway or cell examined.

In several cells, protein secretion is constitutive and appears to be linked to plasma membrane biogenesis. For example, secretion of acetylcholinesterase (AChE) and externalization of the acetylcholine receptor (AChR) seem to be coupled in culture muscle cells. Their appearance at the cell surface occurs with identical kinetics and a variety of pharmacological agents inhibit their transport coordinately (Rotundo & Fambrough, 1980). Although it is possible that the two proteins are transported by similar but distinct pathways, their segregation does not seem to be necessary. Because both proteins are ultimately localized together at the synaptic cleft in vivo it might be efficient for the muscle cell to transport them together. It is not yet known whether their transport vesicles (believed by some to be clathrin coated; Porter-Jordan et al., 1981) also carry other membrane and secreted proteins. Nor is it known whether some components of the vesicle are reutilized for shuttling. Curiously, the AChR and AChE are transported to the muscle cell surface more slowly than most of the well studied plasma membrane and constitutively secreted glycoproteins (Rotundo & Fambrough, 1980), with a lag of 2 hr compared to 15-30 min for IgM, H-2 antigens, the ACTH precursor and the glycoproteins of SFV and VSV (Tartakoff & Vassalli, 1979; Croze &

Morre, 1981; Gumbiner & Kelly, 1982; Green et al., 1981; Rothman et al., 1980). Is this a characteristic of the muscle cells themselves or a property of a specific pathway such as the biogenesis of synaptic membrane?

Hepatoma cells probably transport membrane and secretory proteins together. The kinetics of transferrin secretion is much slower than the externalization of the VSV membrane glycoprotein, the difference being attributable to the RER to Golgi step (Strous & Lodish, 1980). However, the two glycoproteins have been localized to the same Golgi vesicles by immunocytochemistry (H. Lodish, personal communication). It is possible however that the viral glycoprotein is not a truly representative plasma membrane protein since, like secretory proteins, it is ultimately released from the cell (by viral budding). The reason for the slow transport of transferin is perplexing, since other glycoproteins are externalized by the hepatoma cells with the same kinetics as the VSV G protein.

A continual loss of plasma membrane proteins from the cell surface by budding occurs physiologically in the mammary epithelium. Milk fat globules are enveloped in the apical plasma membrane as they are extruded from the cell (Keenan et al., 1970; Franke et al., 1976; Morre et al., 1979). The cells also secrete casein by exocytosis of secretory vesicles at their apical surface (Franke et al., 1976). Compositional data suggest that the casein micelle containing secretory vesicles deliver the fat globule envelope components to the apical plasma membrane (Powell et al., 1977; Morre et al., 1979). Since the cells probably regenerate

their apical plasma membrane every 8 to 10 hr to form milk fat globules during lactation (Franke et al., 1976), there may be no need for them to recycle the casein secretory vesicle components. Cotransport of components involved in milk production is not surprising and in this case the notion of membrane flow for the apical surface is conceivable.

Enzyme secretion by yeast cells appears tightly coupled to cell surface growth. Growth of the yeast cell surface and cell wall occurs by enlargement of the bud prior to cell division. The incorporation of the secreted enzymes, acid phosphatase and invertase, into the cell wall is also restricted to the bud (Field & Schekman, 1980). Novick et al. (1980) have identified 23 genetic complementation groups in the yeast secretory pathway. In all of their conditional mutants enzyme secretion, cell surface growth and incorporation of plasma membrane sulfate permease activity are blocked at the restrictive temperature. Novick has also found that secretion and plasma membrane incorporation of all the major externalized ^{35}S-methionine proteins are blocked at the restrictive temperature in the mutants (R. Schekman, personal communication). In sum, these findings suggest that a single major pathway exists for enzyme secretion and plasma membrane protein incorporation during cell growth. Although rare in wild type yeast, the major organelles in the secretory pathway, ER, Golgi and secretory vesicles, accumulate in the various mutants (Novick et al., 1981). They disappear when cells are returned to permissive temperature. Thus, the organelles seem like kinetic intermediates in the secretory pathway. This apparently unidirectional coordinate transport

of secretory proteins and plasma membrane is certainly consistent with the membrane flow hypothesis. Of course Golgi related processing enzymes must be transiently associated with membrane in transit. Some minor plasma membrane proteins of yeast cells may be transported by an entirely different pathway. The incorporation of galactose permease activity into the cell surface is blocked only in the RER stage mutants, but not in mutants at the Golgi or secretory vesicle stage (R. Schekman, personal communication). Also, acid phosphatase containing secretory vesicles isolated from mutant yeast contain no appreciable levels of two plasma membrane enzymes, chitin synthetase and the vanadate sensitive ATPase, even though the enzymes continue to accumulate at the restrictive temperature (T. Etcheverry, personal communication). Thus minor transport pathways detectable by enzyme activity may be segregated from the major pathway that is responsible for cell growth. Is there some functional reason that surface expression of these enzymes should not be coupled to cell surface growth per se?

In contrast to the cells discussed above, secretion by endocrine, exocrine and neuronal cells is physiologically regulated at the level of exocytosis (see Chapter 3, section VII). The secretory products are usually stored at high concentrations within secretory vesicles or granules so that they can be released by exocytosis on demand. Massive stimulated exocytosis in pancreatic acinar cells and parotid acinar cells causes expansion of the luminal plasma membrane far in excess of the need for surface growth (Jamieson & Palade, 1971b). Since the luminal plasma membrane returns to its normal dimensions following exocytosis, the

excess membrane is retrieved and either reutilized or degraded. The major parotid and pancreatic zymogen granule membrane proteins are probably reutilized since they turnover at a slower rate than the secreted contents (Meldolesi, 1974; Wallach et al., 1975). Differential turnover rates were also demonstrated in chromaffin granules in which the membrane bound portion of dopamine β-hydroxylase was synthesized much more slowly than the soluble portion (Geissler et al., 1977; Winkler, 1977). There is also compelling evidence in pancreatic and parotid exocrine cells and pituitary prolactin cells that at least some components of the secretory granule membrane are retrieved and recycled through the Golgi apparatus to be reincorporated into secretory granules (see Section IX). The specific membrane components that recycle remain to be identified. Certainly, the membrane flow hypothesis does not adequately describe the secretory process for these cells in which exocytosis is not entirely coupled to net surface growth. The secretory membrane at least in part behaves like a shuttle that carries secretory proteins between the Golgi apparatus and the plasma membrane.

Because secretory granule membranes are reutilized many investigators have proposed that plasma membrane glycoproteins are transported to the cell surface by a pathway distinct from the regulated secretory pathway (Palade, 1975; Farquhar & Palade, 1981; Rothman, 1981). The differences between the protein compositions of secretory granule membranes and plasma membrane enriched fractions from exocrine pancreas, parotid and chromaffin cells has also been construed as support for this hypothesis (Ronzio et al., 1978;

Castle et al., 1975; Wilson & Kirsher, 1976; Winkler, 1977). Even if it is assumed that plasma membrane fractions were pure enough to make valid comparisons, these findings do not exclude the possibility that proteins are delivered to the plasma membrane as minor nonreutilized components of the granule membrane. Also, the observations that membranes at different domains of the cell usually differ in their protein composition (Farquhar & Palade, 1963; Rodriguez-Boulan & Sabatini, 1978) and that secretory granule exocytosis is restricted to the apical domain in exocrine cells (Palade, 1975; Herzog & Farquhar, 1977) has led to the conclusion that secretory granule membranes cannot serve as vehicles for transport of proteins to all domains of the cell (Farquhar & Palade, 1981). Of course, it is possible that granules do contribute membrane proteins to the apical domain and also that proteins destined ultimately for other domains are first delivered to the apical domain and subsequently sorted by endocytosis (see below). In this regard, it is interesting that the major zymogen granule membrane glycoprotein, GP2, has been localized by immunocytochemistry to both the basolateral and the apical surfaces of the exocrine pancreatic cell (Geuze et al., 1981).

In Chapter 3 (Gumbiner & Kelly, 1982) I have presented direct evidence that a newly synthesized plasma membrane glycoprotein is transported to the surface of pituitary cells by a pathway distinct from the physiologically regulated pathway for ACTH release. The envelope glycoprotein of an endogenous retrovirus does not use the ACTH/endorphin secretory granule, but instead is delivered to the cell surface by a rapid, constitutive pathway. The regulated

pathway for hormone release is maintained distinctly from the pathway for plasma membrane biogenesis even though the tumor cells grow rapidly doubling every 24 hr. Therefore, the need to independently regulate hormone secretion is probably the reason for segregating secretory and membrane proteins into different exocytotic vesicles. I cannot yet exclude the possibility that the secretory granule membrane makes a permanent contribution to the plasma membrane during exocytosis. It will be important to determine whether the granule membrane proteins are recycled. Segregation of the two pathways may occur in the Golgi apparatus. Both proteins are similarly glycosylated and a portion of the fully glycosylated ACTH precursor molecules is released by the constitutive pathway, presumably because it has escaped packaging into secretory granules. Perhaps the large demand for cell surface growth is responsible for the rapid constitutive secretion of the ACTH precursor, causing inefficient sorting in the Golgi apparatus.

For cells which regulate secretion at the level of exocytosis there is therefore a requirement for at least two distinct pathways to transport newly synthesized proteins to the cell surface. In the simplest view, such cells could have a single constitutive pathway for all plasma membrane glycoproteins and some secreted proteins. Secretory proteins destined for storage in regulatable secretory granules could be sorted away from the constitutive pathway in a manner analogous to the segregation of hydrolytic enzymes into lysosomes (see section VI). There may be situations, however where cells require more than one pathway for the transport of plasma membrane glycoproteins.

Many cells have two or more plasma membrane domains with differing protein compositions (Rodriguez-Boulan & Sabatini, 1978). Because the domains are often set off by tight junctions that prevent lateral diffusion of membrane proteins (Farquhar & Palade, 1963; Fawcett, 1966), segregation of membrane proteins may occur by vesicular transport through the cytoplasm. (Unless of course the proteins reach the membrane before the junctions form.) Sorting of this type is often attributed to the Golgi apparatus, but so far there is no supporting experimental evidence. Alternatively, newly synthesized membrane proteins could be first transported together to one domain of the plasma membrane and subsequently sorted by transcytosis. Proteins probably can be sorted at the plasma membrane by endocytosis in coated vesicles (Goldstein et al., 1979; Bretscher, 1980). Also transcytosis is capable of mediating net transport of membrane proteins in some cells. For example, Mostov et al. (1980) have shown that the receptor for the transcytosis of Ig across epithelial cells, secretory component, is made as an integral membrane protein. The secretory component is cleaved to become a soluble protein and eventually is released from the apical side of the cell. The authors proposed that the newly synthesized integral membrane form is delivered from the Golgi to the basolateral plasma membrane where it functions as the Ig receptor. Once bound to Ig, the receptor would be endocytosed in coated vesicles and cleaved to be released from the membrane during transport across the cell. Transcytosis could also be responsible for the segregation of membrane glycoproteins into the apical and basolateral plasma membrane domains of MDCK cells. When MDCK cells are

infected with VSV, virions bud from the basolateral surface, but when infected with influenza or SFV, virions bud from the apical surface (Rodriguez-Boulan & Sabatini, 1978). Selective budding is due to the segregation of the viral envelope glycoproteins into the respective domains (Rodriguez-Boulan & Pendergast, 1980). When the SFV envelope glycoprotein is exogenously introduced into the apical surface of MDCK cells by liposome fusion, it remains at the apical surface (A. Helenius, personal communication). However, exogenously introduced VSV G protein rapidly becomes internalized and transported to the basolateral surface of the cells. Although these results do not prove that transcytosis mediates the segregation of the G protein in vivo, they demonstrate that MDCK cells have the capacity to sort membrane proteins by transcytosis.

Rapid expansion of the cell surface during growth may require a different biogenetic pathway than membrane modifications or continual turnover of plasma membrane. Pfenninger and Maylie-Pfenninger (1981) have shown that new axonal membrane is added at the growing tip. Glycoproteins which bind ricin are selectively incorporated into the distal regions or filopodia of growth cones in sprouting cultured sympathetic neurons. The membrane composition is probably altered subsequently. In freeze fracture, newly added surface membrane is free of intramembrane particles, but eventually acquires intramembrane particles as growth continues distally (Pfenninger & Bunge, 1974). Protein addition could either occur by lateral diffusion from the axolemma, direct insertion of proteins from the cytoplasm or by the fusion of new vesicles. In contrast to the restricted localization of surface growth, newly

synthesized α-bungarotoxin receptors are inserted into the plasma membrane at the same rate at both the cell body and axons of sympathetic neurons (Carbonetto & Fambrough, 1979). Since the addition of new axonal surface area occurs in a different region than the insertion of some membrane proteins, separate intracellular pathways are implicated. It is possible that surface growth also constitutes a separate pathway from membrane maintenance in other growing cells such as rapidly dividing cells. Remember for example that yeast cells may incorporate some enzymes into the plasmalemma independently of surface growth. It may be important in general to distinguish between pathways for the addition of membrane surface area in growing cells and the turnover or modification of preexisting membranes in theories of membrane biogenesis.

IX. Shuttle Vesicles and Membrane Recycling

Shuttle vesicles which retain their molecular specificity could mediate the transport of proteins between many different compartments. How commonly is this recycling mechanism used for intracellular transport? The best evidence for membrane recycling has been obtained by studying the fate of exocytosed secretory granule membrane in exocrine, endocrine and neuronal cells. When the frog neuromuscular junction is stimulated to release acetylcholine, exogenous horseradish peroxidase (HRP) is endocytosed via coated vesicles (Heuser & Reese, 1973). Shortly thereafter it appears in synaptic vesicles. In this case, there is no need for the vesicle membrane to return to the Golgi to acquire new transmitter. Synaptic vesicles may arise directly by endocytosis. (An intermediate stopover with the axonal SER is possible however.) In

exocrine and endocrine cells it was necessary to demonstrate that retrieved secretory membrane recycles through the Golgi apparatus to acquire newly synthesized secretory proteins. Initial experiments showed that exogenous tracers such as HRP and ferritin were transported predominantly to lysosomes (Geuze & Kramer, 1974; Oliver & Hand, 1978). Tentatively, this led to the conclusion that retrieved secretory membrane was degraded. More recently Herzog and Farquhar (1977) were able to show that exogenous dextran was transported to the Golgi saccules and condensing vacuoles as well as the lysosomes of stimulated parotid and exocrine pancreas cells. Dextran was sent to lysosomes to a greater extent in dissociated cells compared to intact glands. The authors suggested that the pathway to lysosomes could arise from basolateral membrane surfaces which do not participate in exocytosis of secretory granules.

The destination of exogenous tracer depends upon which tracer is used. While native ferritin ends up exclusively in the lysosomes of prolactin secreting cells from the pituitary, cationized ferritin goes to both the Golgi and the lysosomes (Farquhar, 1978). This difference was attributed to the fact that cationized ferritin binds to membranes, and therefore traces the path of the membrane rather than contents. It is also possible that the binding of cationized ferritin alters the destination of endocytotic membrane. Cationized ferritin appears in lysosomes sooner than the Golgi in both plasma cells and thyroid follicle cells (Ottosen et al., 1980; Herzog & Miller, 1979). It has been suggested therefore that incoming vesicle traffic first fuses with lysosomes to unload unwanted exogenous soluble contents en route to the Golgi for

recycling. Two independent pathways from the cell surface cannot be excluded. By perfusing tracers into the pancreatic duct in vivo, Herzog & Reggio (1980) provided evidence for two independent pathways of endocytosis. Dextran appeared exclusively in Golgi saccules and condensing vacuoles in 5 min. HRP (which in this case is thought to bind to membranes) was found only in lysosomes and only after longer periods of time.

These tracer experiments demonstrate that a plasma membrane to Golgi apparatus pathway exists in regulated secretory cells, but specific components of the secretory granule membrane have never been shown to recycle. It is possible that some portion of membrane recycling may be unrelated to regulated exocytosis per se. Endocytosis and plasma membrane protein recycling is widespread even in cells without a regulated secretory pathway (Goldstein et al., 1979; Schneider et al., 1979; Muller et al., 1980). Also recycling to the Golgi occurs even in unstimulated pancreatic exocrine cells (Herzog & Reggio, 1980). The dependence of exogenous tracer recycling on acute exocytotic stimulation has not yet been published, although Farquhar (1978) did claim that recycling was less evident in mammotrophs from nonlactating female rats compared to lactating animals. It is important to determine which of the components of the granule membrane recycle, i.e. which proteins and what portion of the phospholipids. Some components may recycle but some could also stay in the plasma membrane or even be degraded within the lysosome. The molecules which recycle would presumably be permanent components of the shuttle vesicle. A freeze fracture study of exocytosis in the parotid suggested that

the granule membrane was retrieved from the plasma membrane intact (DeCamilli et al., 1976). Retrieval occurred by gross membrane invagination in this case rather than endocytosis. In any case, the fate of specific proteins cannot be determined by freeze fracture alone.

Secretory vesicle membrane may recycle in nonregulated secretory cells. Cationized ferritin is sent from the surface to the Golgi apparatus and lysosomes in immunoglobulin secreting plasma cells and myeloma cells (Ottosen et al., 1980). The tracer entered the secretory compartment since it was found in Golgi saccules that also contained immunoglobulins. It is not clear however to what extent the retrieved membrane is related to the exocytosed secretory vesicles. Nothing yet is known about the composition of the immunoglobulin secretory vesicles. Even if some vesicle components recycle it is possible that membrane glycoproteins are delivered to the plasma membrane via the secretory vesicle membrane. There is no reason to exclude the possibility that vesicles which transport integral membrane glycoproteins also operate as cyclical shuttles between the Golgi and the cell surface.

Palade (1975) proposed that the vesicles presumed to transport secretory proteins from the RER to the condensing vacuole or Golgi also operate by a shuttle mechanism. Secretory proteins are concentrated in the condensing vacuoles of the exocrine pancreas and parotid, but the membrane area of the vacuole does not increase. Thus, excess membrane must either be degraded or recycled to the ER for another round of transport. At present there is no evidence that membrane ever returns to the RER after it has left. Tracers

added extracellularly have never been found to reach the ER, even if they are soluble and therefore free to diffuse through any continuities (Herzog & Farquhar, 1977; Farquhar, 1978; Herzog & Miller, 1979; Herzog & Reggio, 1980; Ottosen et al., 1980). If there is vesicular traffic to the ER from the Golgi, the Golgi must act as a kind of one way valve, allowing ER to plasma membrane traffic but not the reverse. Lectin cytochemistry of intracellular membranes in the exocrine pancreas revealed high mannose but no complex forms of glycoproteins in the ER (Pinta da Silva et al., 1981). So far there is no good evidence that membrane proteins ever return to the ER once they have reached the compartment for terminal glycosylation. The findings of Elhammer et al. (1975) that glycoproteins in the liver go from the Golgi to the ER by transport through the cytoplasm probably result from the relocation of secretory proteins that occurs during tissue disruption (Scheele et al., 1978). Perhaps vesicles return to the ER from Golgi membranes which are proximal to the site of terminal glycosylation (see section IV). Alternatively, vesicular transport could be unidirectional and excess membrane could be degraded at the Golgi or condensing vacuole. If the localization of acid phosphatase in the condensing vacuole, Golgi or GERL is indicative of the presence of lysosomal hydrolases in the secretory compartment (see Section IV), there is a potential mechanism for membrane degradation. Much remains to be learned about transport between the ER and Golgi.

X. The Biochemical Basis of Membrane Protein Sorting

Presumably, some features of membrane and secretory proteins are recognized by the transport machinery in order to be sorted to

different destinations. Lysosomal hydrolases use a mannose-6-phosphate recognition marker to be sorted into lysosomes (Section VI). An analogous role for carbohydrate moieties on secretory and membrane glycoproteins has been ruled out in several cases. The intracellular transport of some glycoproteins is inhibited by preventing their glycosylation in the ER with tunicamycin (Leavitt et al., 1977; Hickman et al., 1977). Because the transport of some glycoproteins is unaffected, Gibson et al. (1980) concluded that this inhibition is probably an idiosyncratic property of each protein. Even proteins as similar as the G proteins of two strains of VSV differ in their susceptibility to inhibition of transport by tunicamycin (Gibson et al., 1979). Appropriate protein conformation rather than the carbohydrate moiety per se is probably required for efficient transport. Green et al. (1981b) have also shown using tunicamycin that glycosylation does not determine the segregation of viral envelope proteins into plasma membrane domains of MDCK cells. Thus, the evidence to date suggests that a structural feature of the protein itself is recognized for transport and sorting. Blobel (1980) has postulated a role for 'sorting sequences' of amino acids analogous to the signal sequence for cotranslational membrane insertion.

There are several mutant virus strains in which variant envelope glycoproteins are defective in their intracellular transport. One mutant of the VSV G protein that is blocked in transport from the Golgi is also defective in fatty acylation (Zilberstein et al., 1980). It is possible that fatty acylation or other modifications other than glycosylation could have a role in intracellular trans-

port. Nucleotide sequence data on some envelope protein variants reveal mutations in the noncytoplasmic portion of these proteins (A. Helenius, personal communication). Therefore a simple defect in recognition of the glycoproteins by clathrin or cytoskeletal proteins at the cytoplasmic membrane surface cannot account for the loss of transportability. Perhaps the membrane proteins need to be recognized by cellular membrane proteins involved in the transport process. By analogy to defective lysosomal hydrolases of I cells, the variant proteins might fail to recognize a receptor within a transport vesicle. Alternatively, mutant proteins might be abnormally retained by structures in the ER or Golgi, mimicking enzymes which are indigenous to those membranes. Of course, the mutant glycoproteins may only nonspecifically denature in the membrane. The normal envelope glycoproteins of VSV and SFV must be distinguished at the apical plasma membrane of MDCK cells since they can be segregated by transcytosis (Section VIII). The proteins must be distinguished either by selective retention in the apical plasma membrane or by selective endocytosis. Further analysis of mutants and the construction of variant proteins by gene splicing should provide much information on the features of proteins which are recognized for intracellular transport and sorting. The structures or receptors that are recognized by the transported proteins remain to be identified. There must also be recognition between intracellular membranes which fuse in order to deliver proteins to the appropriate compartments. The nature of these intermembrane recognition events is still obscure.

CHAPTER 2

SECRETORY GRANULES OF AN ANTERIOR PITUITARY CELL LINE, AtT-20, CONTAIN ONLY MATURE FORMS OF ADRENOCORTICOTROPIC HORMONE AND β-LIPOTROPIN

SUMMARY

The pituitary cell line, AtT-20, synthesizes the precursor to adrenocorticotropic hormone (ACTH) and β-endorphin and correctly glycosylates and cleaves the precursor to make the mature forms of the hormones before they are secreted. To use this cell line for the study of the intracellular transport, packaging and secretion of these hormones I have isolated and characterized the secretory granule from the cells. After appropriate homogenization, differential centrifugation and isopycnic sedimentation on a deuterium oxide-Ficoll gradient, a secretory granule preparation has been obtained which has a specific activity of ACTH/protein (90 μg/mg) that is 30-90 fold greater than whole cells. The granules have unique density characteristics and a sedimentation coefficient that is appropriate for spheres of 1000 Å radius. They contain all of the fragments of the initial ACTH/endorphin precursor, but almost undetectable amounts of the intact precursor. The fragments constitute about 50% of the protein in the secretory granule fraction and from density measurements we estimate that they are present in approximately 60,000 copies per vesicle. The cell line secretory granules appear, therefore, to be similar to mature secretory granules in normal differentiated tissues. ACTH first appears in the secretory granule between 30 and 45 min after it is synthesized. Cleavage of the precursor to mature ACTH occurs at about the same time in the whole cell. Therefore proteolysis of the prohormone to ACTH and to β-lipotropin is a metabolic event that can be correlated with the packaging of the hormone into a mature

secretory granule. Cleavage of β-lipotropin to β-endorphin occurs later, probably in the secretory granule.

INTRODUCTION

Morphological studies of highly differentiated secretory tissues such as the pancreas and parotid gland have delineated the intracellular route of transport of proteins destined for secretion (Palade, 1975). Secretory protein is synthesized at the rough endoplasmic reticulum transported to the Golgi apparatus or the condensing vacuole where it is packaged into mature secretory granules and then discharged from the cell by exocytosis. The molecular events underlying this process are poorly understood. It is not known how secretory proteins are segregated and packaged into secretory granules or how specific granule membrane components are acquired. The nature of the recognition event whereby appropriate membranes fuse during transport and secretion remains obscure. Although highly structured tissues like the pancreas are optimal for morphological studies, the advantages of cell lines for the study of biochemcal processes has long been appreciated. It is reasonable to expect that the molecular events involved in packaging and secretion of proteins could be optimally studied in a cell line which retains normal secretory functions.

I have chosen the mouse pituitary cell line AtT-20 as a model secretory system because the cells retain many important biochemical and physiological properties of pituitary corticotrophs. The biosynthetic pathway of the major secretory products, ACTH, β-lipo-

tropin (β-LPH) and β-endorphin, has been elucidated using AtT-20 cells (see Herbert, 1981 for review). These hormones are first synthesized as a common polypeptide precursor (Mains et al., 1977; Roberts & Herbert, 1977a,b) which is glycosylated and subsequently cleaved into the final hormone products (Roberts et al., 1978; Phillips et al., 1981). In other tissues the enzymes involved in post-translational processing of membrane and secretory proteins have been localized to specific organelles such as the rough endoplasmic reticulum of the Golgi apparatus (see Chapter 1). If normal transport and processing occur in AtT-20 cells, it should be possible to use ACTH processing activities to monitor transport between intracellular compartments or as biochemical markers for the corresponding organelles. AtT-20 cells retain additional characteristic physiological properties of corticotrophs. The rates of biosynthesis and secretion of ACTH and β-endorphin are reduced by glucocorticoids (Roberts et al., 1979; Herbert et al., 1978) and stimulated by corticotropin releasing factor (Herbert et al., 1978; Allen et al., 1978). The cells can be stimulated to secrete with either vasopressin (Johnson et al., 1980), potassium chloride depolarization (Simantov, 1978), norepinephrine (Mains & Eipper, 1981) or 8-bromo cyclic AMP (Chapter 3).

I have begun biochemical studies of the secretory processes in AtT-20 cells by isolating the final organelle in the secretory pathway, the secretory granule. Secretory vesicles have been purified close to homogeneity from many tissues including the parotid (Castle et al., 1975), the adrenal medulla (Winkler, 1976), and the elasmobranch electric organ (Carlson et al., 1978). Prepar-

ations of secretory granules from the anterior pituitary have been reported (Hymer, 1975), but due to the heterogeneity of cell types there is usually quite a bit of cross contamination between various hormone containing granules. This is not a problem when starting with a clonal cell line. In this chapter I describe a deuterium oxide (D_2O) density gradient procedure which is effective in producing a highly enriched population of ACTH containing secretory granules that has distinct physical properties. The large number of fully processed hormones in the vesicle supports the contention that mature secretory granules have been isolated from AtT-20 cells. Also proteolytic processing of the ACTH/endorphin precursor occurs simultaneously with the packaging of the hormones into the mature secretory granule.

RESULTS

I. Secretory Granule Purification

In seven secretory granule purifications, 33 to 88 fold increases in the specific activity of ACTH/protein over whole cells have been obtained. The average secretory granule specific activity was 74±11 μg ACTH/mg protein (mean ± S.D.). A summary of a typical purification resulting in a 33 fold increase in specific activity is given in Table 1. A variety of homogenization conditions were tried in order to maximize the fraction of cellular ACTH that could be recovered in a 10^6 g x min pellet after first removing nuclei and cell debris with a low speed centrifugation. Six strokes with a Dounce homogenizer in the appropriate buffer lysed

essentially all of the cells (determined by trypan blue exclusion), released less than 5% of the ACTH to a soluble fraction, and yielded 40% of the cellular ACTH in the high speed pellet. Conditions for the differential centrifugation were determined empirically to minimize the loss of ACTH during the low speed centrifugation step, but to increase the specific activity of the high speed pellet (P_2) (Fig. 1). From this experiment, 5 minutes at 9000 rpm was chosen for the low speed spin to remove large contaminants. In the purification shown (Table 1) 37% of the cellular ACTH was recovered in the P_2 resulting in a 4 fold increase in specific activity over the homogenate.

Several types of density gradients were tried to purify further the secretory granules. No purification was achieved on conventional equilibrium sucrose gradients because the ACTH and total protein cosedimented throughout the gradient. Isopycnic iso-osmotic centrifugation using a Ficoll density gradient gave a single ACTH peak with only a 2 fold increase in specific activity. Successful purification was achieved using a density gradient of D_2O containing Ficoll. When centrifuged in a gradient of a membrane permeable solute the density of a closed vesicle is determined less by the water it contains and more by the vesicle contents (Wagner et al., 1978). The successful purification with the D_2O gradient presumably results from the permeability of granules to D_2O and the high density of the granule contents.

The D_2O gradient separated the ACTH containing structures into two major peaks (Fig. 2). The appropriate amount of Ficoll throughout the D_2O gradient was critical to obtaining optimal separation

between the two peaks (see Chapter 5 & 2). The lighter peak equilibrated within the D_2O gradient and contained a large amount of contaminating protein. The denser secretory granule peak equilibrated in the Ficoll step gradient at the bottom and copurified with a very small peak of protein. Six percent of the cellular ACTH was recovered in the peak fractions which had a very high specific activity (89 μg ACTH/mg protein).

Velocity sedimentation of the P_2 on a sucrose gradient did purify ACTH containing membranes away somewhat from other contaminants (Fig. 3). However all of the organelles containing ACTH sedimented as a single broad peak. This procedure may be useful as an additional purification step for secretory granules; either preparatively or analytically to evaluate copurification of presumptive granule components.

II. Physical Properties of the Secretory Granules

Several physical properties of the secretory granules are listed in Table 2. The densities of the secretory granules determined in iso-osmotic Ficoll solutions were less in H_2O than D_2O as expected (Wagner et al., 1978). To determine the sedimentation coefficient, peak fractions from a D_2O/Ficoll gradient were pooled, diluted 5 fold with iso-osmotic D_2O buffer and centrifuged in a swinging bucket rotor at 18,000g average for varying lengths of time. The amount of ACTH in each pellet was measured by RIA and expressed as a fraction of the total pelletable ACTH (Fig. 4). The sedimentation coefficient was calculated for each time point using the following expression (Cotman et al., 1970).

$$S = \frac{1}{2\omega^2 t} \ln \left[\frac{R_m^2}{R_p^2} + (1-f_t)(1- \frac{R_m^2}{R_p^2}) \right]$$

where ω = the angular velocity in radians/sec; R_m = the distance of the solution meniscus from the center of rotation; R_p = the distance of the pellet from the center of rotation; f_t = fraction of the total pelletable ACTH in pellet at time t; t = time of centrifugation in seconds. The average sedimentation rate from five time points was 189±38 S. The secretory granules must be fairly homogeneous in size. The sedimentation rate for ACTH did not decrease systematically with increasing centrifugation time, in contrast to the sedimentation rate of membranes from side fractions of the D_2O gradient (Fig. 4). The ACTH containing membranes of the P_2 were heterogeneous in sedimentation rate (Fig. 1).

III. Secretory Granule Protein Composition

Several polypeptides copurify with secretory granules. Figure 5 shows SDS gels of D_2O gradient fractions after the cells had been labeled with ^{35}S-methionine. Bands that comigrate with ACTH, β-endorphin, β-LPH, glycosylated ACTH (13K), and amino terminal fragments of the ACTH/ endorphin precursor are highly enriched in secretory granule fractions. By quantitative densitometry of autoradiograms, these polypeptides represent 44%, 47% and 47% of the labeled protein in fractions 4, 5 and 6, respectively.

To confirm that the indicated bands on the gel are indeed ACTH and endorphin related peptides, D_2O gradient purified granules from ^{35}S-methionine labeled cells were subjected to immunoprecipitation

with ACTH antiserum and with β-endorphin antiserum (Fig. 6). In each case specificity was determined by including a control precipitation in the presence of excess cold hormone to displace the labeled hormone. ACTH and a smeared band of 13 kd corresponding to glycosylated ACTH were precipitated from the granules by anti-ACTH antiserum (lanes e and f). Bands at the molecular weight positions of β-endorphin and β-LPH were specifically precipitated by an anti-β-endorphin antiserum (lanes g and h). Comparison of the immunoprecipitates with the total secretory granule fraction (lane i) suggests that most if not all of the ACTH, endorphin and β-LPH size material is immunoprecipitated by the appropriate antiserum. The ACTH/endorphin precursor was only faintly detectable after a very long period of fluorography (more than 4 weeks). By comparison with shorter exposures, they constituted less than 1% of the labeled secretory granule ACTH or endorphin peptides. Precursor forms were readily immunoprecipitated from homogenates of whole cells (lanes a through d). In addition the less dense peak of ACTH in the D_2O gradient contained a substantial amount of precursor as well as processed ACTH (lanes j and k).

The bands labeled "N-terminus" migrate on SDS gels in a similar pattern and molecular weight range as the amino terminal fragments of the ACTH/endorphin precursor (Keutmann et al., 1979). These bands were immunoprecipitated by a crude antiserum (Bridget) which has anti-amino terminal titers (Fig. 7). However the control precipitation to determine specificity was not done since appropriate amino-terminal fragments were not available.

Many higher molecular weight polypeptides (indicated with an

asterisk) also copurify with granules (Fig. 5). They are not present in substantial amounts in other nearby gradient fractions. In contrast, a polypeptide that comigrates with actin (arrow) is present in major amounts in every D_2O gradient fraction. The Coomassie blue staining polypeptide pattern is essentially the same except that it sometimes shows a predominant 30 kilodalton (kd) species that also is present in every gradient fraction (Fig. 8). The 30 kd protein is a murine leukemia virus nucleocapsid protein, p30 that occasionally contaminates the granule fractions (see chapter 3).

IV. Comparison of Proteolytic Processing with Packaging into Secretory Granules

To examine the temporal relationship between ACTH/endorphin processing and intracellular transport, AtT-20 cells were labeled with ^{35}S-methionine for various lengths of time. After each labeling period, the cells were harvested and secretory granules were prepared. Secretory granule fractions from each preparation were pooled, precipitated with trichloroacetic acid and the proteins were analyzed by SDS gel electrophoresis (Fig. 9A). Densitometer scans of the autoradiograms were made and the peaks quantitated (Fig. 10A). ACTH and β-LPH abruptly accumulated in the isolated granules between 30 and 45 minutes of labeling. In contrast a band with the mobility of actin (42 kd) labeled linearly with time, a behavior which would be expected of a contaminant adsorbed to the granules. The time course of proteolytic processing of the ACTH/ endorphin precursor was determined by immunoprecipitating an aliquot of the corresponding homogenate of each granule preparation

(Figs. 9B and 10B). Mature 4.5 kd ACTH began to accumulate in the cells at 30-45 min and accumulated with continued labeling. Thus within the time resolution of this experiment, proteolytic conversion of the precursor into mature ACTH and β-LPH occurred concomitantly with packaging of the hormones into secretory granules.

Although a faint band with the mobility of β-endorphin appeared in granules after 45 minutes of labeling, it increased dramatically between 1.5 and 2 hr (Figs. 9A and 10A). Quantitation by densitometry showed that the label in β-LPH decreased by the same amount as the increase in label in β-endorphin. In a separate experiment cells were labeled for 5 hr with ^{35}S-methionine. In this case, much more label was in β-endorphin than in β-LPH (Fig. 9A, lane g). These results strongly suggest that the cleavage of β-endorphin from β-LPH occurs within the secretory granule long after the initial proteolysis of the ACTH/LPH precursor.

DISCUSSION

I have used an ACTH RIA to monitor the purification of secretory granules from AtT-20 cells. Because the ACTH detected by RIA is presumably contained in other subcellular organelles such as the rough endoplasmic reticulum and Golgi apparatus, it is not a unique marker for secretory granules. Indeed, more than one peak of ACTH is present in the D_2O density gradient (Fig. 2). I believe the dense component corresponds to secretory granules on the basis of their density, their sedimentation properties, the large amount of mature forms of hormones which they contain and the kinetics of

their labeling.

Specific activity measurements indicate that the granule preparation is very enriched in secretory product. D_2O gradient purified granules had a specific activity of 89 μg RIA-ACTH/mg protein. The granules also contain the β-LPH and amino terminal fragments of the 30 kd precursor. Since the RIA quantifies only the 4.5 kd ACTH fragment, it measures only 15% of the total secretory product. Therefore the estimated specific activity is 600 μg secretory product/mg protein which means that approximately 60% of the protein in the granule preparation consists of secretory product. Quantitative densitometry scans of SDS gel autoradiograms also show that about 50% of the radioactivity of granule fraction polypeptides is present in the fully processed secretory hormones. The granules must be therefore at least 50% pure. The purity could be greater if some of the other labeled polypeptides are also granule components.

In seven purifications, 33 to 88 fold increases in specific activity have been obtained. Since much of the cellular ACTH may not be in secretory granules, the increase in ACTH specific activity over cell homogenates is an underestimate of the actual purification factor that has been achieved. Only 6% of the starting ACTH was recovered in the secretory granule peak fractions, but losses incurred during the purification do not permit estimates of the fraction of cellular ACTH that is in secretory granules. Thus it is not possible to determine the true purification factor that has been obtained.

I have used immunoprecipitations and SDS gel electrophoresis

to confirm that the granules contain ACTH and endorphin related peptides. Previous work which included peptide mapping has shown that this approach is adequate to characterize ACTH and endorphin peptides and their precursors in AtT-20 cells. (Mains et al., 1977; Roberts & Herbert, 1977a,b; Roberts et al., 1978). All of the hormone products of AtT-20 cells including β-LPH, β-endorphin, ACTH and glycosylated ACTH are present in the purified granules. Although less rigorously demonstrated, it appears that the granules also contain the amino-terminal fragments of the 30 kd precursor.

The lack of the ACTH/endorphin precursor in the isolated granules must be reconciled with previous reports of a large proportion of precursor secreted into the medium by AtT-20 cells (Roberts et al., 1978; Mains & Eipper, 1978; Fig. 11). It appears that precursors are released via some pathway which does not involve mature secretory granules (see Chapter 3). It is unlikely that the lack of precursors in secretory granules is an experimental artifact, since substantial amounts of precursor remain in less dense D_2O gradient fractions (Fig. 7). Glembotski (1980) has claimed that 10% of the ACTH in secretory granules isolated from rat anterior pituitary is in precursor form, but he never ruled out possible contamination of the granule fraction with other membranes of the secretory apparatus. Alternatively, the discrepancy may result from using a different tissue source for granule isolation. In fact, Glembotski found that granules isolated from rat intermediate lobe have 400 times more mature endorphin than 30K precusor. Because cleavage of the precursor occurs rapidly at the time of hormone packaging into granules, the precursor may be very trans-

iently present in newly formed granules. This was found by Glembotski (1981) in pulse-chase labeling of cultured intermediate lobe cells. The lack of precursor in the secretory granules isolated from AtT-20 cells is consistent with their role as the final intracellular compartment which transports ACTH and endorphins to the outside of the cell; the role of the less dense ACTH peak is not yet known.

The isolated vesicles are homogeneous in size as determined by sedimentation analysis and are an appropriate size to be secretory granules. The radius of the granule can be calculated to be 995 Å from the sedimentation coefficient and the vesicle density (Table 2) using the following equations.

$$S = \frac{m\left(1 - \frac{\rho_{sol}}{\rho_{ves}}\right)}{6\pi\eta r} \quad \text{and} \quad m = 4/3\pi\, r^3 \rho_{ves}$$

where m = vesicle mass; r = the radius, ρ_{ves} = the vesicle density, ρ_{sol} = the density of the solution and η = the viscosity of the solution. Secretory granules of radius 1000 Å have been observed in rat pituitary corticotrophs (Siperstein & Miller, 1970) and in AtT-20 cells (Sabol et al., 1979) by electron microscopy.

The number of secretory hormone molecules in each secretory granule can be estimated using density measurements. The density of a vesicle, ρ_{ves}, can be described by the equation (Wagner et al., 1978).

$$\rho_{ves} = (1-f_s)\rho_c + f_s\rho_s$$

where ρ_c is the density of vesicle contents which includes the limiting membrane and any internal contents, ρ_s is the density of the solvent, f_s is the fractional volume of the vesicle which is exchangeable water. Since I have measured the densities of the secretory granule in both H_2O and D_2O (Table 2), I can solve two simultaneous equations to calculate that ρ_c = 1.30 gm/ml and f_s = 0.66. The high density of vesicle contents presumably reflects the large amount of secretory protein contained within the secretory granule. The granule density in aqueous solution (1.10 gm/ml) is consistent with the large proportion of water in the secretory granule. The mass of the granule contents can be calculated to be 1.85×10^{-15} gm using the measured radius, fractional volume occupied by contents ($1-f_s$) and density of vesicle contents (ρ_c). Assuming that all the membrane of the granule is made up of phospholipids, then it is possible to calculate from the diameter of the granule, the cross-sectional area and molecular weight of the phospholipid (Wagner et al., 1978) that at most 30% of the mass of the contents is taken up by lipid. If the remainder is protein and 50% of the protein is derived from ACTH precursors then there is at least 0.66×10^{-15} gm of ACTH precursor (13,000 molecules) per vesicle. If 50% of the membrane mass were proteins then there would be 16,000 molecules per vesicle. This would correspond to 64,000 processed hormone fragments per granule. Although the accuracy of this determination is limited by the aforementioned assumptions, the number of secretory molecules per vesicle is of the right order of magnitude expected for a secretory granule. For example, Narcine electric organ synaptic vesicles have 47,000 molecules of acetyl-

choline (Wagner et al., 1978) and there are 60,000 molecules of vasopressin per posterior pituitary granule (Cross et al., 1975).

Packaging of the secretory product into secretory granules is closely associated in time with proteolytic processing of the ACTH/LPH precursor. Due to the limited time resolution of the experiment it is not possible to decide whether proteolysis of the ACTH precursor occurs within the secretory granule or just prior to packaging into the granule. Proteolysis of the 30K precursor in intermediate lobe cells also occurs coincidentally with packaging into secretory granules, but a small amount of cleaved products first appears in another membrane fraction (Glembotski, 1981). A reasonable interpretation is that proteolytic processing is initiated as a result of transferring the precursor into a new intracellular compartment. The compartment is most likely either a compartment of the Golgi apparatus or the secretory granule itself. The conversion of prohormones may be initiated in the Golgi apparatus and continued in the secretory granule as it matures (see Chapters 1 and 4). The data in this chapter strongly suggest that the final cleavage of β-LPH to β-endorphin occurs within the secretory granule. This is consistent with previous findings that β-endorphin appears in AtT-20 cells with a much slower time course than the initial proteolysis of the ACTH/LPH precursor (Mains & Eipper, 1978). It is not clear why there should be such a long lag before β-LPH within the secretory granule is cleaved. This event may be a slower component of an overall cleavage process, perhaps reflecting the presence of the cleaving enzymes within the secretory granule. I have found no evidence to support the idea that β-endorphin and

ACTH are packaged into separate granules.

The packaging results demonstrate the advantage of post-translational processing activities in AtT-20 cells for studying intracellular transport and secretion. The isolated secretory granules appear to be the final intracellular compartment involved in transporting hormones out of the cell and have many of the important attributes of secretory granules from normal differentiated secretory tissues. The AtT-20 cell line thus retains normal secretory functions and is well suited for studies of molecular events involved in packaging and secretion of proteins.

TABLE 1. Secretory Granule Purification Summary

Purification Step	Total ACTH (μg)	ACTH Recovery (%)	Specific Activity (μg ACTH/mg protein)
Homogenate	41.6 ± 1.3	100	2.7 ± .084
P_2	15.5 ± 2.0	37	12 ± 1.6
Peak Secretory	2.31 ± .26	5.5	89 ± 10

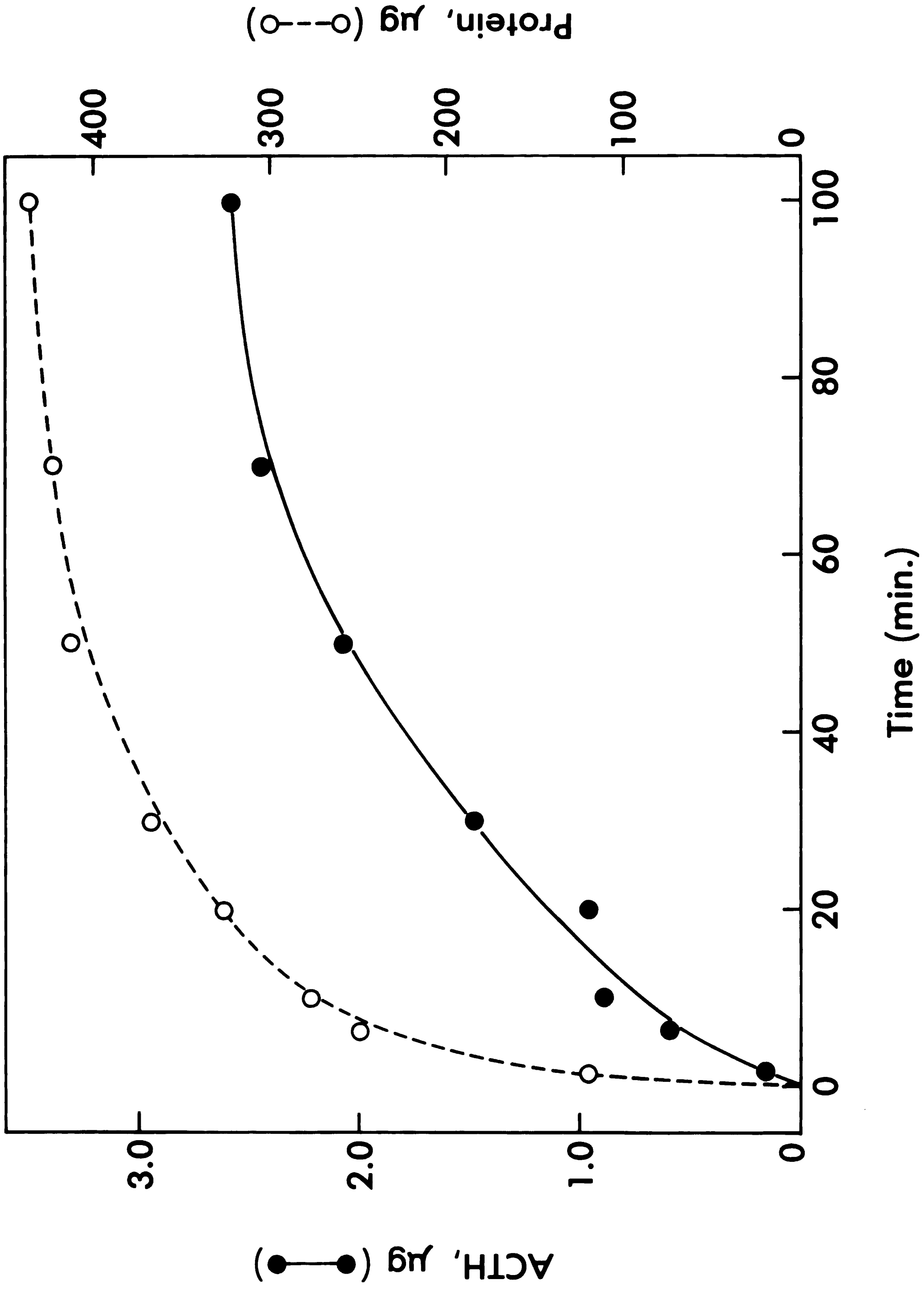
These results are from one typical purification. P_2 was the 30 kg, 30 min pellet that was fractionated on the D_2O-Ficoll density gradient. The peak secretory granule fractions were fractions 4 and 5 of the D_2O gradient shown in Figure 1. The results are expressed as the mean ± standard deviation.

Table 2. Physical Properties and Structure of the Secretory Granules

Measured Properties*	Values
Density in H_2O + Ficoll	1.10 gm/ml
Density in D_2O + Ficoll	1.17 gm/ml
Sedimentation coefficient in isoosmotic D_2O buffer	189 ± 38S
Calculated Properties	
Radius	1000 Å
Density vesicle contents	1.30 gm/ml
Fraction of vesicle volume that is exchangeable H_2O	0.66
Number of ACTH, LPH, β-endorphin and amino-terminal fragment molecules/ secretory granule	64,000

*All determinations at 4°C

FIGURE 1. Determination of conditions for differential centrifugation. A cell homogenate from 9 50 cm^2 dishes of cells was first centrifuged at 2700 rpm for 10 min in a Sorvall SS34 rotor to remove unbroken cells, nuclei and large debris. The supernate was diluted to 40 ml, divided into 8 centrifuge tubes and centrifuged at 9000 rpm in an SS34 rotor for varying lengths of time. The protein and ACTH content of each pellet was determined and plotted as a function of centrifugation time.



Protein, µg (o---o)
400
300
200
100
0
3.0
2.0
1.0
0
ACTH, µg (●—●)
0
20
40
60
80
100
Time (min.)

FIGURE 2. D_2O density gradient profile of the secretory granule purification shown in Table 1. Fractions 4 and 5 are the peak secretory granule fractions. (●——●) μg ACTH/fraction determined by radioimmunoassay; (O---O) μg protein/fraction. Each fraction was approximately 1.7 ml.

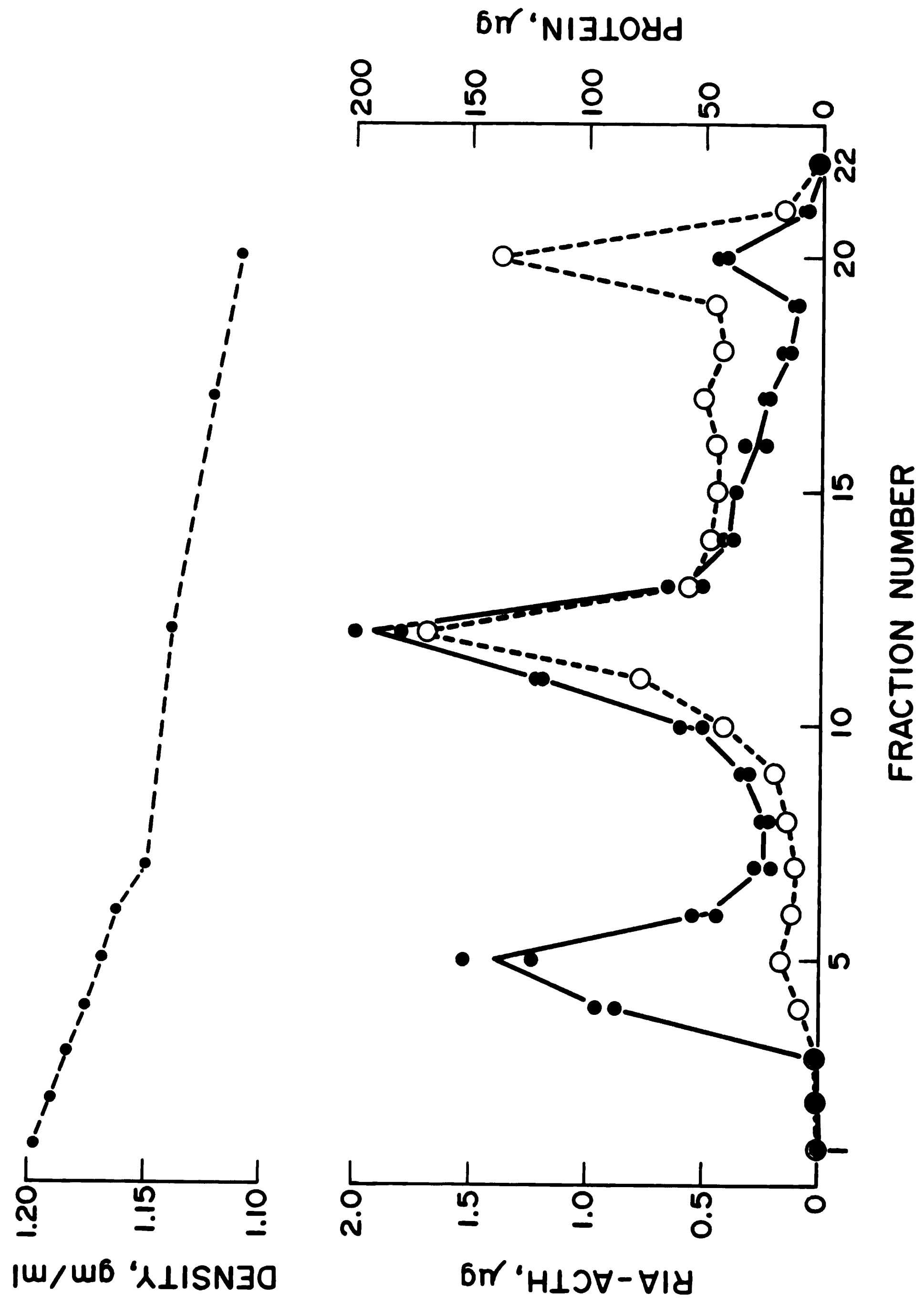

DENSITY, gm/ml
1.20
1.15
1.10
RIA-ACTH, μg
2.0
1.5
1.0
0.5
0
PROTEIN, μg
200
150
100
50
0
FRACTION NUMBER
1
5
10
15
20
22

FIGURE 3. Velocity sedimentation analysis of the P_2 fraction. A P_2 was prepared from 9 50 cm^2 dishes of cells. The resuspended P_2 (1 ml in homogenization buffer) was loaded on top of a 12 ml linear gradient of 15-35% sucrose in homogenization buffer with a 0.2 ml 60% sucrose pad. The gradient was centrifuged at 20,000 rpm in a Beckman SW41 rotor for 1 hr. 0.66 ml fractions were collected from the bottom. 47% of the ACTH and 51% of the protein were recovered using cellulose nitrate centrifuge tubes. Fraction 10 was enriched in ACTH/protein 18 fold over the homogenate and 3 fold over the P_2.

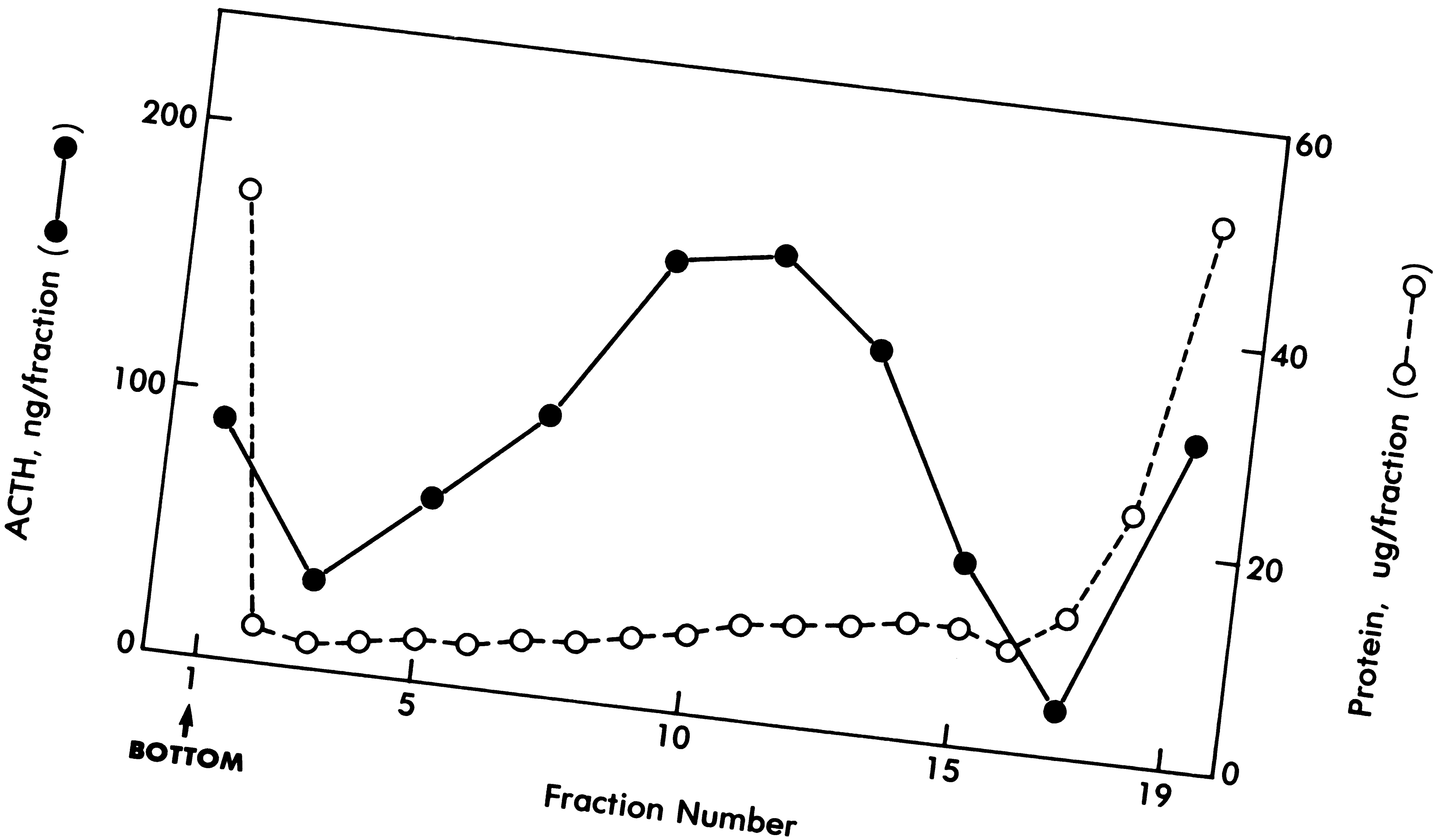

ACTH, ng/fraction
Protein, ug/fraction
Fraction Number
BOTTOM
0
100
200
0
20
40
60
1
5
10
15
19

FIGURE 4. Determination of secretory granule sedimentation coefficient. The peak fractions from a D_2O density gradient were pooled, diluted to one-fifth with isoosmotic D_2O buffer and centrifuged in an SW50.1 rotor at 18,000g average for varying lengths of time. A small amount of radiolabeled membranes from D_2O gradient side fractions were included in the samples for comparison. The amount of ACTH (●——●) and radioactivity (o--o) in each pellet were determined and expressed as the percent of maximum that could be pelleted (150,000g for 2 hr).

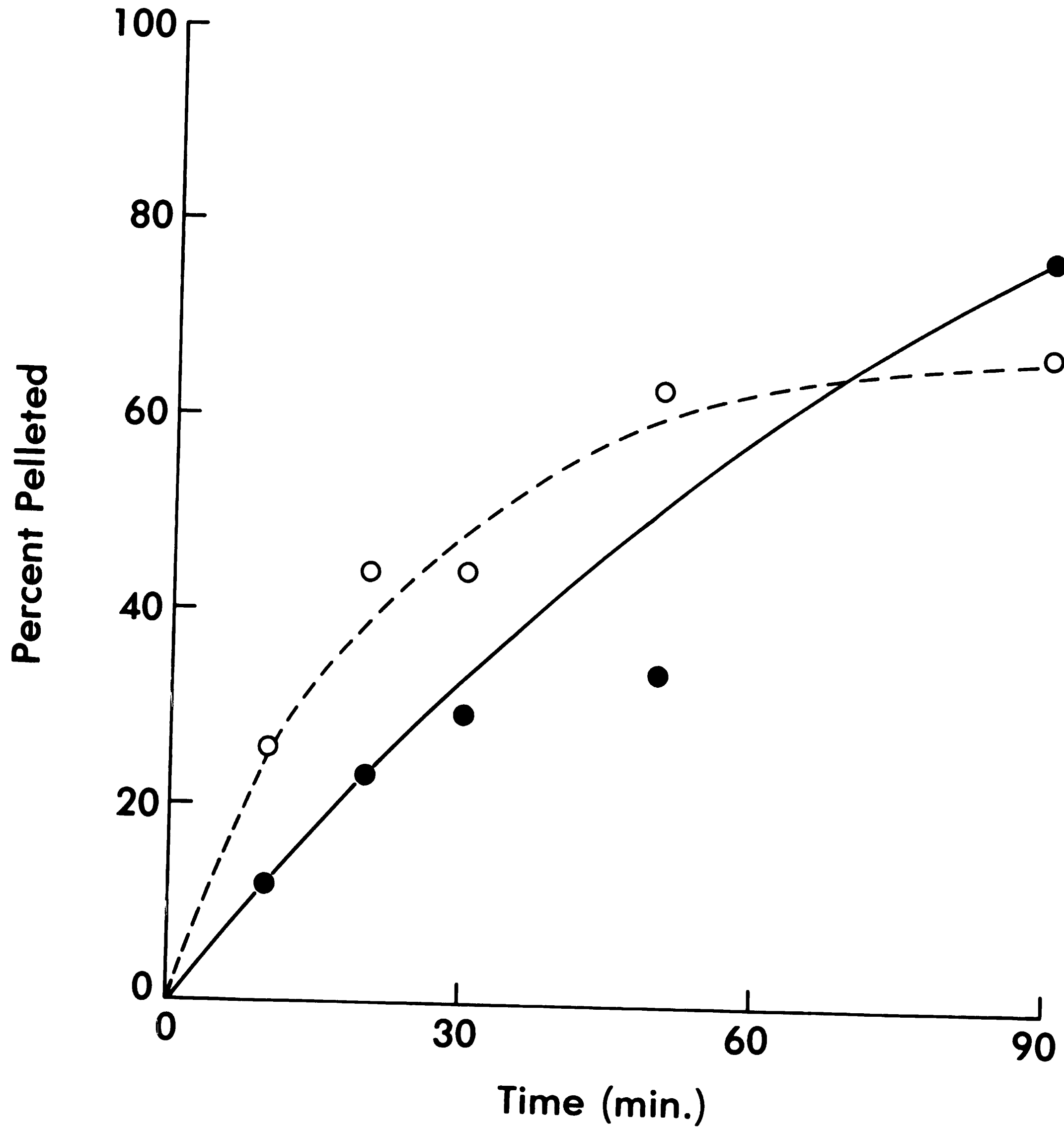

100
80
60
40
20
0
Percent Pelleted
0
30
60
90
Time (min.)

FIGURE 5. Protein analysis of D_2O density gradient purified secretory granules. Four 75 cm^2 flasks of cells were labeled with 2.5 mCi ^{35}S-methionine for 5 hr and the secretory granule purification was carried out. Equal volume aliquots of the D_2O gradient fractions were precipitated with trichloroacetic acid and examined by SDS slab gel electrophoresis. The gel was autoradiographed for 84 hr. Fractions 4, 5 and 6 were the peak secretory granule fractions as determined by ACTH radioimmunoassay. Asterisks mark unidentified polypeptides which copurify with the secretory granules. The arrow indicates a band with a mobility of actin (42 kd) that does not copurify with secretory granules.

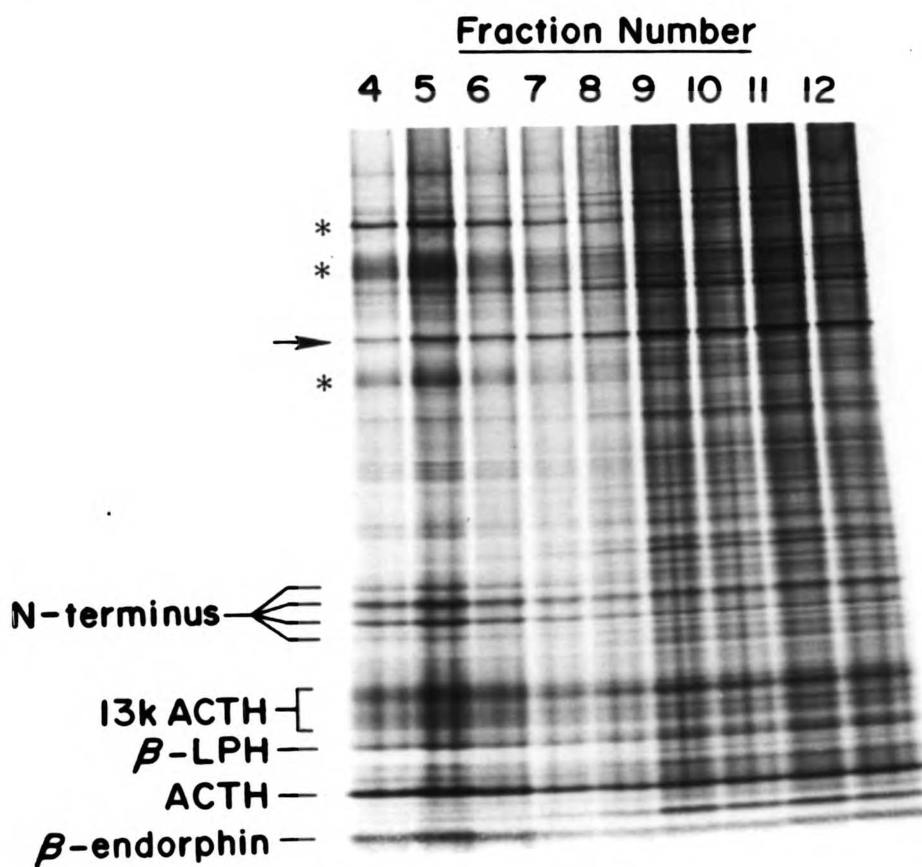

Fraction Number
4 5 6 7 8 9 10 11 12
*
*
*
N-terminus
13k ACTH
β-LPH
ACTH
β-endorphin

FIGURE 6. ACTH and endorphin immunoprecipitation of purified secretory granules analyzed by SDS gel electrophoresis. Fractions 4, 5 and 6 from the experiment shown in Fig. 2 were pooled for the sample of labeled secretory granules. The total secretory granule sample that was incubated in each precipitation is shown in lane i. Lanes e through h show the immunoprecipitation of the secretory granules; a through d, whole cell homogenate; j and k, a fraction from the light ACTH peak of the D_20 gradient (fractions 10 to 13, Fig. 2). a,b,e,f,j and k were precipitated with an ACTH antiserum in the absence (a,e,j) or the presence (b,f,k) of excess (10 μg) unlabeled ACTH to show specific precipitation. c,d,g and h were precipitated with the β-endorphin antiserum while controls d and h contained 10 μg unlabeled β-endorphin. Gels were analyzed by fluorography. Lane i was exposed one-fifth as long as lanes a through h.

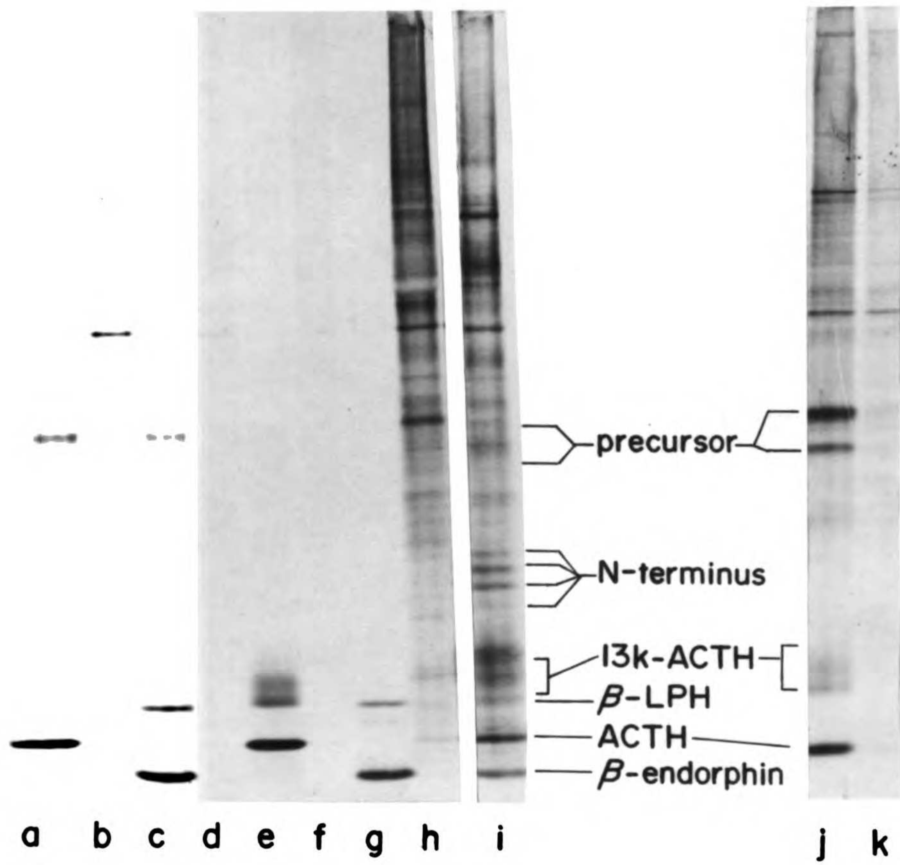

precursor
N-terminus
13k-ACTH
β-LPH
ACTH
β-endorphin
a
b
c
d
e
f
g
h
i
j
k

FIGURE 7. Amino-terminus immunoprecipitation and wheat germ agglutinin binding analysis of secretory granules on SDS gels.

a. anti-β-endorphin immunoprecipitation of total cell homogenate for reference.

b. Secretory granules from the experiment in Figure 6 were precipitated with 40 μl of antiserum Bridgett, obtained from Dr. E. Herbert. The antiserum was raised against unfractionated culture medium from AtT-20 cells. The antiserum recognized the amino-terminal fragments and ACTH portions of the common precursor and also higher molecular weight polypeptides (asterisks) which copurify with secretory granules (Fig. 5). c. The same detergent solubilized secretory granule preparation was bound to wheat germ agglutinin Sepharose. d. Wheat germ agglutinin binding components from lighter D_2O gradient fractions. The component labeled x was highly enriched in secretory granules and appears to migrate slower than glycosylated ACTH (Fig. 6). It may correspond to the major sulfate labeled component found in secretory granules and stimulated secretions of AtT-20 cells (H.P. Moore, unpublished observations).

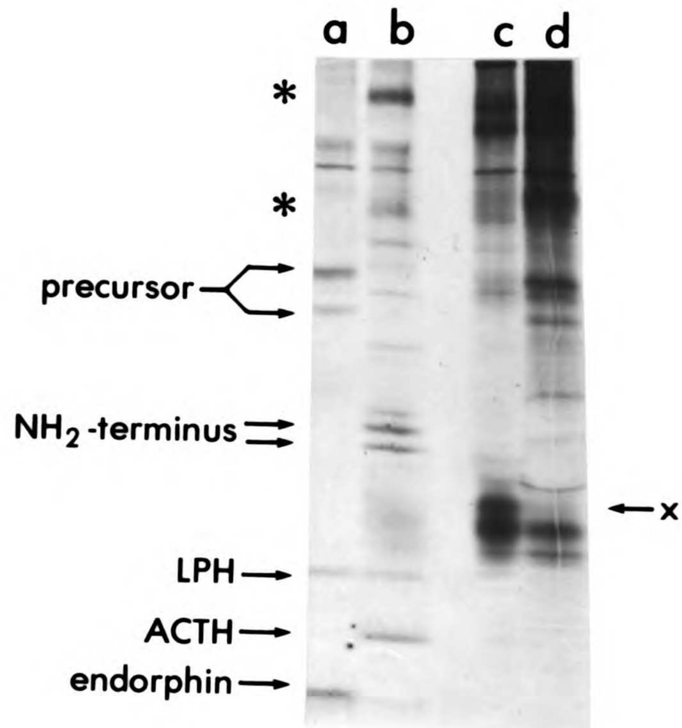

a
b
c
d
*
*
precursor
NH2 -terminus
LPH
ACTH
endorphin
x

FIGURE 8. Coomassie blue stained SDS gel of D_2O gradient purified secretory granules. Peak fractions from two separate secretory granule preparations were precipitated with trichloroacetic acid and analyzed by SDS gel electrophoresis. Arrows indicate peak fractions. Asterisks indicate polypeptides that correspond to unidentified polypeptides in Figure 5 that copurify with secretory granules. β-Endorphin was not apparent because its recovery on SDS gels is somewhat variable.

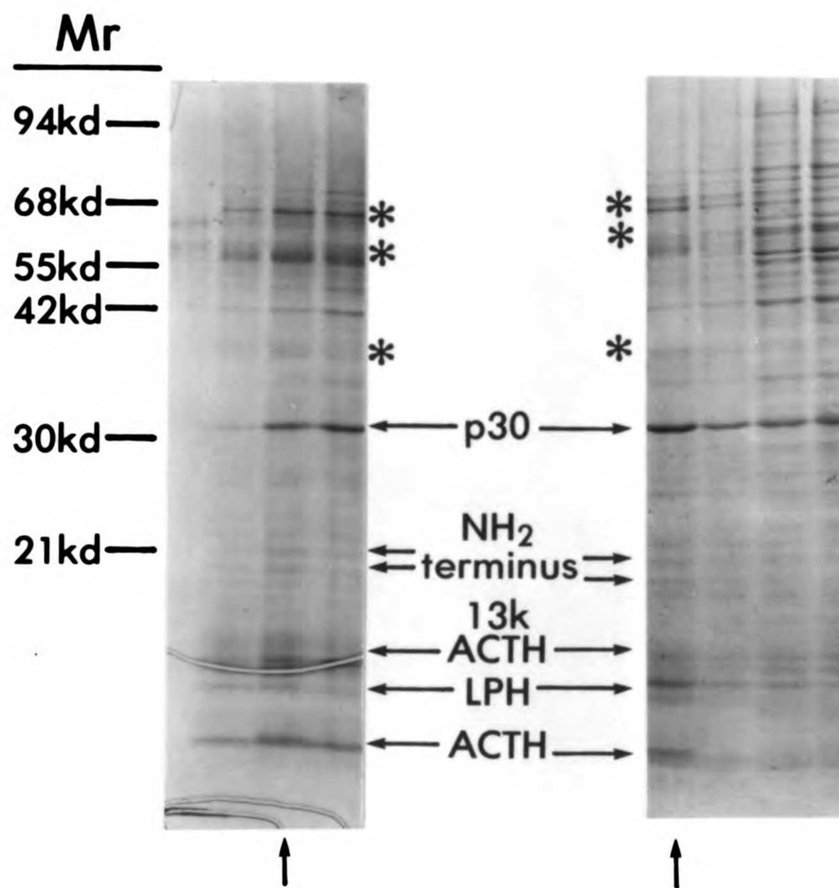
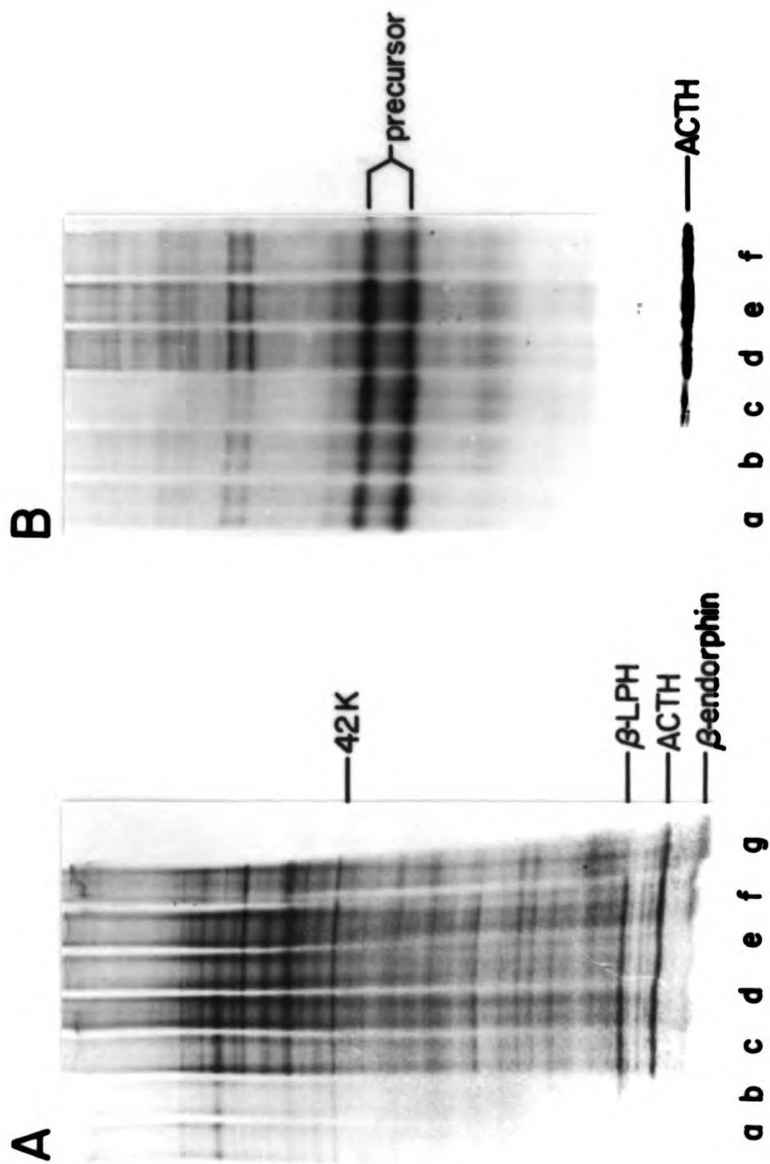

Mr
94kd
68kd
55kd
42kd
30kd
21kd
*
p30
NH2
terminus
13k
ACTH
LPH
ACTH

FIGURE 9. Correlation of ACTH/endorphin proteolytic processing with packaging into secretory granules. Each of 6 75 cm^2 flasks of cells was incubated with 800 µCi of ^{35}S-methionine for a specified time of continuous labeling. Incorporation of ^{35}S into trichloroacetic acid precipitable material per mg protein was linear with time over the full course of the experiment. The secretory granule preparation was carried out in parallel for all six sets of cells after adding some unlabeled cells to serve as carrier. D_2O gradient fractions of density greater than 1.165 gm/ml were pooled and analyzed by SDS gel electrophoresis and autoradiography (9A). Equal volume aliquots of the homogenates were immunoprecipitated with an ACTH antiserum and analyzed similarly (9B). Lanes a,b,c,d,e and f correspond to 15,30,45,60,90 and 120 min of continuous labeling, respectively. The higher molecular weight polypeptides contaminating the immunoprecipitates bind nonspecifically to SAC, since they could be pelleted in the absence of antiserum. The secretory granule preparation after 5 hr of cell labeling shown in Figure 5 is included in 9A lane g for comparison. The 42K band migrates with actin.



A
a b c d e f g
42K
β-LPH
ACTH
β-endorphin
B
a b c d e f
precursor
ACTH

FIGURE 10. Kinetics of hormone processing and packaging into secretory granules. The autoradiograms shown in Figure 9 were scanned with a densitometer. Relative peak areas were determined by weighing. 10A shows the kinetics of granule labeling from Figure 9A. (●——●) label present as 4.5 kd ACTH. (O---O) label present as β-endorphin. (Δ···Δ) appearance of label in the 42K band which comigrates with actin. 10B shows the time course of the conversion of the ACTH/endorphin 30 kd precursor to 4.5 kd ACTH. (O——O) 4.5 kd ACTH as a percentage of the sum of 4.5 kd ACTH + precursor.

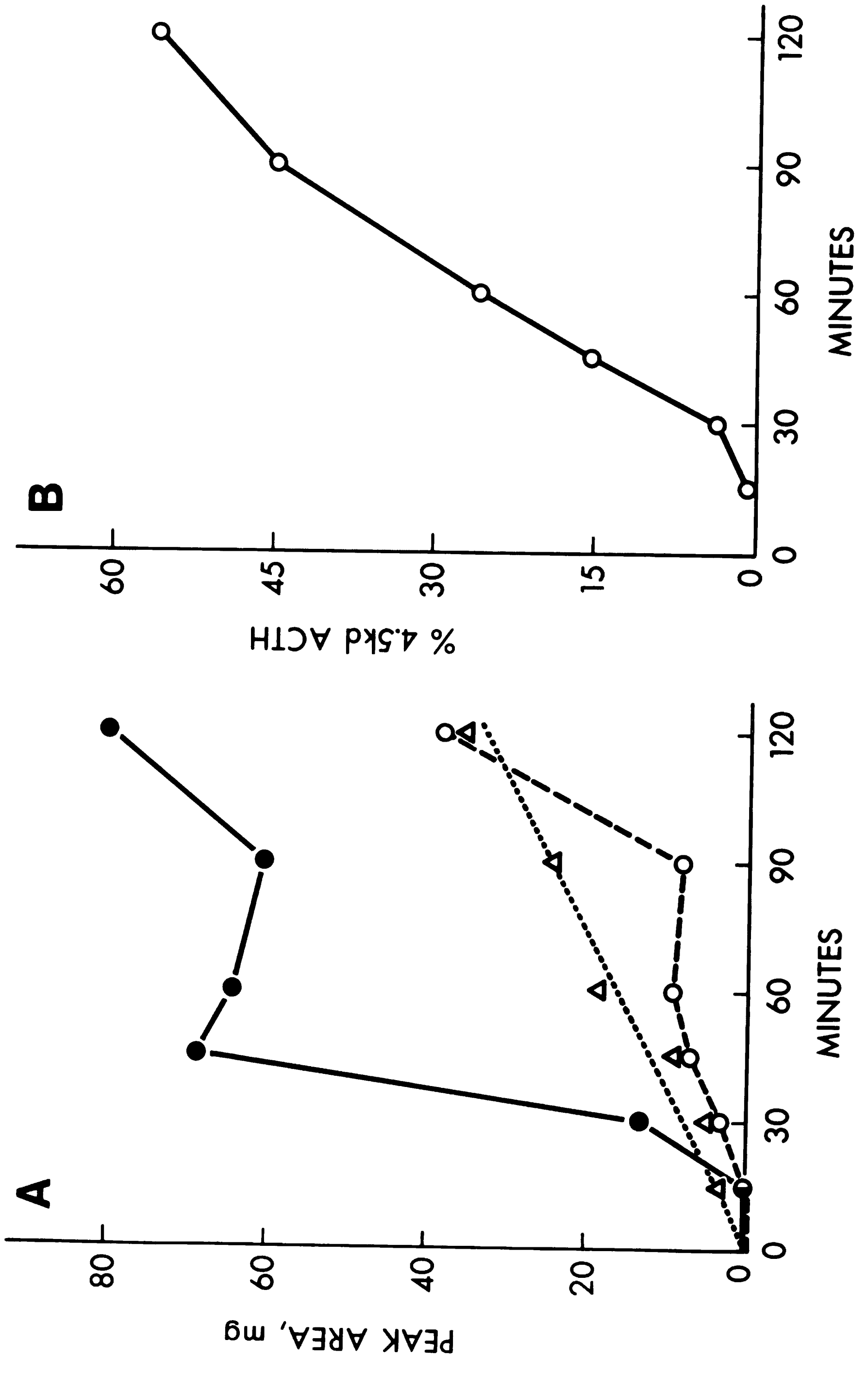
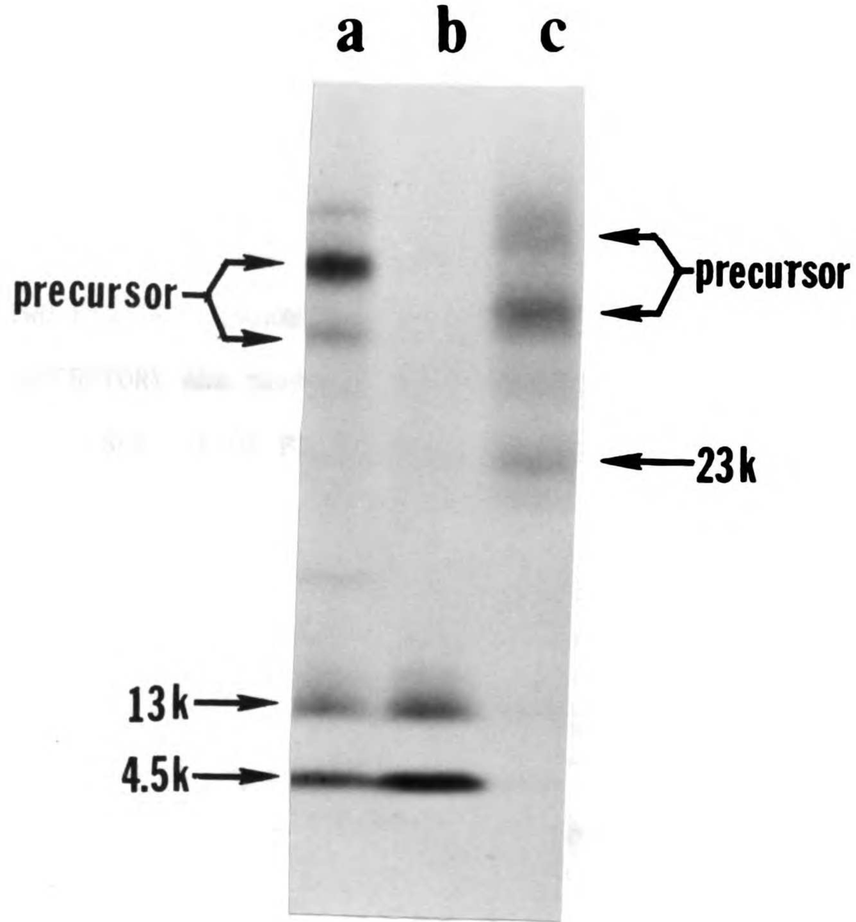

A
PEAK AREA, mg
80
60
40
20
0
0
30
60
90
120
MINUTES
B
% 4.5kd ACTH
60
45
30
15
0
0
30
60
90
120
MINUTES

FIGURE 11. Secreted forms of the ACTH precursor. ^{35}S-met labeled ACTH was immunoprecipitated from the following samples and analyzed by SDS gel electrophoresis. a. Cell homogenate: contains precursor forms of ACTH that have high mannose type oligosaccharides and mature forms of ACTH (4.5K and 13K). b. Secretory granules: contain only mature forms (4.5K and 13K) of ACTH. c. ACTH secreted into the culture medium after a short (30 min) chase interval. Only precursor forms and some intermediate (23K) ACTH were released by this time. The mobilities of the precursor forms are different than in lane a because secreted precursor molecules have complex type oligosaccharides. Presumably they have been processed in the Golgi apparatus prior to secretion.


a
b
c
precursor
precursor
23k
13k
4.5k

CHAPTER 3

TWO DISTINCT INTRACELLULAR PATHWAYS TRANSPORT SECRETORY AND MEMBRANE GLYCOPROTEINS TO THE SURFACE OF PITUITARY TUMOR CELLS

SUMMARY

The pituitary cell line, AtT-20, synthesizes adrenocorticotropin hormone (ACTH) as a glycoprotein precursor which is cleaved into mature hormones during packaging into secretory granules. The cells also produce an endogenous leukemia virus (MuLV) which is glycosylated after translation similar to the glycosylation of the ACTH precursor. The evidence presented here suggests that the envelope glycoprotein and some precursor ACTH get to the cell surface in a vesicle different from the mature ACTH secretory granule. Viral glycoproteins and ACTH precursor are released from the cells much sooner after synthesis than mature ACTH. Isolated secretory granules do not contain significant amounts of the envelope glycoprotein or ACTH precursor. Exposing cells to 8Br-cAMP stimulates release of mature ACTH 4-5 fold but has little effect on the release of the ACTH precursor or the viral glycoproteins. I propose that the viral glycoproteins and some of the ACTH precursor are transported by a constitutive pathway, while mature ACTH is stored in secretory granules where its release is enhanced by stimulation.

INTRODUCTION

Secretory proteins and plasma membrane glycoproteins are transported from the rough endoplasmic reticulum (RER) to the cell exterior by a particular set of intracellular membrane compartments (Palade, 1975; Lodish et al., 1981). In the cell types which have

been examined to date, there is no evidence that newly synthesized membrane glycoproteins and secretory proteins are segregated from each other during transport to the cell surface. Transport of acetylcholine receptor and acetylcholinesterase in muscle cells occurs with the same kinetics and cannot be dissociated by treatment with a variety of drugs that block secretion (Rotundo & Fambrough, 1980). Although transferrin and vesicular stomatitis virus (VSV) G protein are transported to the cell surface of hepatoma cells with different kinetics (Strous & Lodish, 1980), they appear to be localized to the same Golgi vesicles by immunocytochemistry (H. Lodish, personal commununication). There is also genetic evidence that the pathway for secretion and plasma membrane growth are the same in yeast (Novick et al., 1980).

If plasma membrane and secretory glycoproteins are transported by a single intracellular pathway, they should reach the plasma membrane in the same vesicle. I have already isolated and characterized the secretory granule from the pituitary cell line, AtT-20 (Gumbiner & Kelly, 1981, Chapter 2). AtT-20 cells synthesize their secretory products, adrenocorticoptropic hormone (ACTH) and endorphins, as a larger common glycoprotein precursor (Mains et al., 1977; Roberts et al., 1978), but store only mature hormones in the secretory granules (Gumbiner & Kelly, 1981, Chapter 2).

I have found that AtT-20 cells also produce an endogenous murine leukemia virus (MuLV). MuLV envelope glycoproteins are made intracellularly as precursors and are processed to a mature envelope glycoprotein (gp70) during their transport to the cell surface (Dickson et al., 1981; Fitting et al., 1981; Witte et al., 1977;

Witte & Wirth, 1979). Processing of most MuLV envelope proteins involves both proteolytic cleavage and carbohydrate modifications and is similar in nature to ACTH processing (Phillips et al., 1981). This suggests that ACTH and MuLV proteins are processed in similar or identical Golgi-like compartments.

In this report I present evidence that after leaving the Golgi the envelope glycoprotein is not transported to the cell surface by ACTH secretory granules. The route taken by the glycoprotein is faster than that of mature ACTH and not sensitive to secretagogues.

RESULTS

I. AtT-20s Synthesize and Process MuLV Glycoproteins

I have already shown that in AtT-20 cells, mature ACTH reaches the cell surface in a secretory granule. Here I examine whether the same pathway is used in AtT-20 cells for the transport of a viral envelope glycoprotein. Electron microscopy showed that AtT-20 cells contain an endogenous C-type virus (Gumbiner, unpublished observations). Immunologically the virus is very similar to Rauscher and Moloney murine leukemia viruses (MuLV). The presence of MuLV proteins in AtT-20 cells was verified by immunoprecipitating extracts of ^{35}S-methionine labeled cells with antisera specific for MuLV proteins. AtT-20 cells synthesized immunoprecipitable polypeptides which have mobilities on sodium dodecyl sulfate (SDS) gels similar to polypeptides synthesized by 3T3 fibroblasts productively infected with Moloney MuLV (Fig. 1). The major protein in the membrane envelope of MuLVs is a glycoprotein, gp70. Antiserum

raised against purified MuLV gp70 immunoprecipitated the 80-90 kd envelope glycoprotein precursors (gPr^{env}) as well as mature gp70 (Fig. 1a). Mature gp70 was identified by its mobility relative to protein standards and by its presence in released virions (Fig. 7) and at the cell surface (Fig. 6). Two lower molecular weight polypeptides coprecipitated with gp70. The larger corresponds to the p15E proteolytic fragment of Moloney MuLV envelope glycoprotein and the other may be the smaller p12E fragment of the envelope protein (Karshin et al., 1977). They coprecipitate with gp70 because a small fraction of the gp70 and p15E are disulfide bonded (Leamnson et al., 1977; Dickson et al., 1981). Immunoprecipitation with antiserum to whole Moloney virions (Fig. 1b) demonstrated that AtT-20 cells also produce the major MuLV nucleocapsid protein, p30 as well as the nucleocapsid protein precursor, Pr65.

The precursors to gp70 in AtT-20 cells were identified by a pulse-chase experiment (Fig. 2, no endo-H). Two polypeptides (80 kd and 90 kd) that could be precipitated by antibody to gp70 were synthesized during a 15 minute pulse. These polypeptides disappeared with a halftime of 37 minutes and there was a coordinate appearance of gp70 (Fig. 3). Although it has been reported that cells infected with different strains of MuLVs usually produce either a 90 kd or an 80 kd envelope glycoprotein precursor (gPr^{env}), it has been suggested that the 80 kd polypeptide results from rapid cleavage of the 90 kd precursor (Dickson et al., 1981). The presence of both polypeptides suggests that AtT-20 cells either express two different MuLV envelope glycoprotein genes or contain both forms of envelope protein precursor.

The envelope glycoprotein oligosaccharides were characterized in two ways. The high mannose type carbohydrates on the envelope glycoprotein precursors and gp70 were detected by their susceptibility to digestion with endoglycosidase-H (endo-H) (Robbins et al., 1977) and galactose-terminating carbohydrates were detected by binding to the lectin ricin (Nicholson & Blaustein, 1972). The mobilities of the 80 kd and 90 kd precursors on SDS gels were significantly increased by digestion with endo-H (Fig. 2). It is therefore probable that the envelope glycoprotein precursors in AtT-20 cells contain high mannose type oligosaccharides as do envelope glycoprotein precursors of all MuLVs (Dickson et al., 1981; Witte & Wirth, 1979). In contrast the mobility of gp70 was only slightly increased by digestion with endoglycosidase-H. Endo-H treated gp70 electrophoresed between the endo-H treated precursor polypeptides, unlike untreated samples, where it electrophoresed faster (Fig. 2). The mobility of endo-H digested gp70 was confirmed with iodinated samples (Fig. 6). To determine whether some carbohydrate chains of gp70 were resistant to endo-H digestion, the viral glycoproteins were also labeled directly with ^{3}H-mannose. Although the ^{3}H-mannose labeled oligosaccharides were completely digested from the envelope glycoprotein precursor with endo-H, label remained associated with gp70 (Fig. 4). Since complex carbohydrates are not substrates for endo-H (Robbins et al., 1977), these results suggest that many but not all of the carbohydrate chains in gp70 are of the complex type. To determine whether gp70 actually does contain sugars characteristic of complex type oligosaccharides, labeled cell extracts and culture media were

fractionated by binding to ricin agarose prior to immunoprecipitation (Fig. 5). Ricin specifically binds terminal galactosyl residues (Nicholson & Blaustein, 1972) which are found in complex but not high mannose oligosaccharides (Robbins et al., 1977; Tabas & Kornfeld, 1979). Most of the envelope glycoprotein precursor polypeptides did not bind to the ricin column. However, gp70 from both cell extracts and culture medium bound to the column and was eluted with galactose. A small amount of a 90 kd polypeptide bound to ricin and it may represent an intermediate envelope glycoprotein form which has undergone carbohydrate processing prior to proteolytic conversion. These results indicate that AtT-20 MuLV envelope glycoprotein precursors are made with high mannose oligosaccharides and are converted to a mature gp70 containing complex type carbohydrate. Thus oligosaccharide processing is similar to the processing of MuLV and other viral envelope glycoproteins (Robbins et al., 1977; Witte & Wirth, 1979) and the same as that found for ACTH (Phillips et al., 1981; Gumbiner, unpublished observations). I conclude that the viral envelope glycoproteins and ACTH pass through functionally equivalent RER and Golgi-like compartments.

II. Gp70 is Transported to the Exterior of AtT-20 Cells

The cell surface localization of gp70 was demonstrated in two ways. Gp70 was accessible to cell surface radioiodination by lactoperoxidase (Fig. 6). It amounted to approximately 3% of the cell surface protein susceptible to iodination. Neither the nucleocapsid protein, p30, nor the envelope glycoprotein precursor were accessible to iodination. Also, gp70 in intact cells that had been labeled metabolically with a 15 min pulse of ^{35}S-methionine and 60

min of chase was susceptible to digestion with chymotrypsin. To quantify the label remaining in various polypeptides after digestion, fluorograms of SDS gels of immunoprecipitates from chymotrypsin treated and control cells were scanned with a densitometer. The cells remained intact during protease treatment since 105% of the p30 was recovered (p30 was digested in cell lysates). Only 37% of the gp70 remained associated with cells after chymotrypsin treatment even though 78% of the gPr^{env} was not digested.

Although gp70 is made as a membrane bound precursor (gPr^{env}), after proteolytic cleavage most of it is only weakly attached to membrane bound p15E and is easily sloughed from virions during handling (McGrath et al., 1978). It is presumably lost from the cell surface in the same way since it is released from cultured cells in soluble form (Bolognesi et al., 1975) and is found in excess of virions in mouse serum (Strand & August, 1976). AtT-20 cells released ^{35}S-methionine labeled gp70 into the culture medium in a soluble form and in a pelletable form that we assume to be virus particles (Fig. 7A,B). During short chase intervals (45 min), gp70 was released but not detectable in the virus pellet (Fig. 7A). Since p30 was found in the particulate fraction, it is clear that centrifugation effectively removed virions from the culture medium. By 6 hr of chase, gp70 was released as a pelletable virus (Fig. 7B). Even so, half of the gp70 was still soluble (not shown). Antibodies to gp70 also precipitated the membrane bound p15E component of the envelope glycoprotein from the pellet fraction (Fig. 7B) whereas coprecipitation was not observed with the soluble material (Fig. 7A). The slow release of pelletable gp70

probably results from the budding of virions from the plasma membrane. As most of the gp70 reaches the surface of AtT-20 cells by 60 minutes of chase, the rapid release of soluble gp70 probably reflects its appearance at the surface of AtT-20 cells. Release of soluble gp70 therefore provides a convenient measure of its transport to the cell surface.

AtT-20s also released glycosylated forms of the nucleocapsid protein precursor (glycosylated gag) which are not found in virions (Fig. 7A). Antiserum raised against p30 immunoprecipitated a 50 kd and 35 kd polypeptide from culture medium. In many MuLV infected cells, the glycosylated gag resides on the cell surface as a 90 kd precursor, is cleaved into the two fragments and released from the cell (Edwards & Fan, 1979). I was unable to detect glycosylated gags at the cell surface (Fig. 6), but it has been reported that the 90 kd surface form is sometimes not susceptible to lactoperoxidase catalyzed iodination (Fitting et al., 1981).

III. Kinetics of Viral Glycoprotein and ACTH Release

The intracellular transport times for viral glycoproteins and ACTH were compared by examining the kinetics of their release. Gp70 and glycosylated gag fragments were released from AtT20 cells with similar kinetics in pulse-chase experiments (Fig. 8). Both proteins appeared in the medium after a 10-15 min lag and attained half-maximal release by approximately 40 min. Mature ACTH is released from AtT20 cells much more slowly with a half-time of 3-4 hr (Mains & Eipper, 1978).

It has been frequently noted that resting AtT-20 cells release precursor forms of ACTH (Mains & Eipper, 1981; Roberts et al.,

1978). Precursor release is not due to cell lysis because only the minor intracellular endo-H resistant form is found in the culture medium (Phillips et al., 1981). I have confirmed this observation. Approximately 20% of the ACTH molecules initially labeled were released in precursor form during the first hour of chase. By this time the remaining ACTH molecules were almost completely converted to the mature form and accumulated intracellularly (unpublished observations, Mains & Eipper, 1978; Roberts et al., 1978). At one hour, only a small fraction (10-15%) of the mature ACTH was released. Unlike the release of mature ACTH, the release of ACTH precursor occurred with the same kinetics as the viral glycoproteins (Fig. 8). Thus, release of the viral glycoproteins and the 30K ACTH precursor occurred simultaneously during the time when most of the ACTH precursor was being cleaved to mature forms for intracellular storage. The site of mature ACTH storage is the secretory granule (Gumbiner & Kelly, 1981, Chapter 2).

IV. Viral Envelope Glycoproteins do not Accumulate in ACTH Secretory Granules

The kinetic data suggest that precursor ACTH and viral glycoproteins are released by a pathway distinct from that involved in the secretion of mature ACTH. Secretory granules contain little or no ACTH precursor (Gumbiner & Kelly, 1981, Chapter 2), but store mature ACTH intracellularly with a half-life of 3 hr before they fuse with the plasmamembrane. If newly synthesized viral membrane glycoproteins are transported to the plasma membrane by secretory granules, they should accumulate in the granules along with mature ACTH. To determine whether gp70 accumulates in granules, AtT-20

cells were pulse labeled with ^{35}S-methionine and chased for 60 min to achieve maximal labeling of secretory granule proteins. Secretory granules were then isolated as described earlier (Gumbiner & Kelly, 1981, Chapter 2). Secretory granules obtained in this way are purified greater than 80 fold with respect to starting material and contain about 5% of the ACTH in the homogenate. The labeled secretory granules were analyzed by immunoprecipitation for their contents of ACTH and viral envelope glycoproteins (Fig. 9). 4-5% of the total cellular radiolabeled ACTH was recovered in granules. However less than 0.15% of the total cellular radiolabeled gp70 could be detected in secretory granules by immunoprecipitation. In another similar experiment a small amount (0.41% of the total cellular gp70) was recovered in isolated secretory granules which contained 5% of the cellular radiolabeled ACTH. This was probably due to the contamination of the secretory granules with viral particles, that I occasionally find. The envelope glycoprotein precursor could not be immunoprecipitated from isolated secretory granules even though it was present in cell extracts. Therefore, the percent recovery of total cellular ACTH in secretory granules was at least 12-25 fold greater than the recovery of gp70 or gPr^{env}. This suggests that at most 8% of the viral glycoproteins reach the surface via ACTH-containing secretory granules. The majority of the viral envelope glycoproteins and precursor ACTH must reach the cell surface by a different pathway.

V. Effect of ACTH Secretagogues on the Release of Viral Glycoproteins

To further discriminate between the pathways of release I

examined the cellular response to secretagogues. Total ACTH secretion from AtT-20 cells (determined by radioimmunoassay) was stimulated 4-5 fold by treatment with 5mM 8-bromoadenosine 3':5'-cyclic monophosphate (8Br-cAMP) (Fig. 10A,B). To examine its effects on the release of viral glycoproteins and the different forms of ACTH, 8Br-cAMP was added to cells either immediately following ^{35}S-methionine labeling (Fig. 10B) or after a 45 min chase period (Fig 10A). In both cases, secretion of mature ACTH was stimulated 4-5 fold compared to untreated cells, while release of precursor (30K) and intermediate (23K) forms of ACTH was stimulated less than two fold. Similarly it has been shown that norepinephrine stimulates the release of mature but not precursor forms of ACTH from AtT-20 cells (Mains & Eipper, 1981). Depolarization of the plasma membrane with KCl was less effective than 8Br-cAMP in triggering the release of mature ACTH (Fig. 10B). The limited effectiveness of KCl depolarization has been reported previously (Mains & Eipper, 1981). Release of both gp70 and glycosylated gags was stimulated less than twofold by 8Br-cAMP and paralleled the release of the ACTH precursor but not the release of mature ACTH. I conclude that mature ACTH is released via a pathway which is regulated differently from the pathways for the release of viral glycoproteins and the ACTH precursor.

DISCUSSION

The MuLV envelope glycoprotein produced in AtT-20 cells provides an excellent cell surface marker. It seems to be made as

a N-asparagine-linked high mannose precursor (gPr^{env}) which disappears with a half time of about 37 minutes. As gPr^{env} is cleaved to gp70, it is modified to acquire complex type oligosaccharides. Thus it seems likely that the envelope glycoprotein, like many other membrane glycoproteins, is processed by enzymes in the RER and Golgi membranes as it is transported to the cell surface (Bretz et al., 1980; Fries & Rothman, 1980; Lodish et al., 1981; Robbins et al., 1977; Strous & Lodish, 1980, see Chapter 1). A very similar pattern of glycosylation and cleavage is found for precursor ACTH (Phillips et al., 1981). Thus precursor ACTH and the gPr^{env} are in functionally equivalent compartments as they move from RER to Golgi.

Three types of evidence demonstrate that the viral envelope glycoprotein does not utilize ACTH secretory granules in its transport to the plasma membrane. Release of gp70 occurred much more rapidly than release of mature ACTH. In fact, release of gp70 was essentially completed within 90 minutes after its synthesis, while the majority of the mature ACTH was predominantly still in its intracellular stores. Physiological dissociation of gp70 export and secretory granule exocytosis was also evident. Exocytosis of secretory granules was stimulated by treatment with 8Br-cAMP, since release of mature ACTH increased 4-5 fold. Release of gp70 was not significantly increased and therefore was not coupled to exocytosis of ACTH containing secretory granules. Finally, isolated secretory granules were analyzed directly for the presence or absence of gp70. Pulse-chase radiolabeling conditions were employed such that a considerable fraction of the labeled ACTH would be packaged into

secretory granules (Gumbiner & Kelly, 1981, Chapter 2). It was then possible to use the recovery of radiolabeled ACTH in isolated granules to normalize for losses incurred in the purification. Recovery of radiolabeled gp70 in isolated secretory granules from the starting cells was 12-25 fold less than the recovery of ACTH. Therefore, most of the cellular gp70 was localized to another cellular compartment and very little if any of the gp70 could have accumulated in secretory granules. It is plausible therefore to propose that a different exocytotic vesicle transports gp70 to the cell surface.

Since the purification of granules emphasizes purity over recovery it is possible that I have only isolated a subpopulation of secretory granule types. Even if this is true, these results show that the viral glycoprotein is transported by a vesicle different from the one we have isolated. The kinetic and physiological differences between mature ACTH and gp70 release also support the conclusion that they take separate pathways.

Infection with virus could perturb hormone processing and release by AtT-20 cells. In the extreme case, some cells could be releasing virus and others hormone. This is unlikely since cells which lack the gp70 molecule cannot be detected by immunofluorescence (Trina Schroer, unpublished observations). Also electron micrographs show viruses budding from cells which contain secretory granules (unpublished observations). Perhaps perturbing effects of viral production are less likely to occur when a cloned cell line containing an endogenous virus is used in preference to introducing an exogenous virus.

Tartakoff et al. (1978) have suggested previously that cells use two kinds of pathways for exocytosis. One, which is typified by zymogen secretion from pancreatic acinar cells, is dependent upon extracellular calcium ions and regulated over short times by physiological stimuli. The other is unregulated, not dependent on extracellular calcium and is found in cells such as macrophages and fibroblasts. The evidence presented here suggests that AtT-20 cells use both types of pathways (Fig. 11). One is a constitutive pathway which releases viral glycoproteins rapidly after synthesis. This pathway is insensitive to regulation by cAMP and could be the one used by the cell for plasma membrane protein insertion. If so, this constitutive pathway would presumably be essential for growth and characteristic of all cells. The second pathway is more complex. The mature hormones are concentrated into specialized secretory granules which are stored in the cell. Exocytosis is dependent on extracellular calcium ions (Mains & Eipper, 1981) and is stimulated by appropriate regulators such as norepinephrine or cyclic AMP. This kind of pathway would not be required for cell viability and is only present in specialized secretory cells such as endocrine and exocrine cells or neurons.

In several other systems more than one post-Golgi pathway has not been seen. Thus acetylcholinesterase and acetylcholine receptor in muscle cells (Rotundo & Fambrough, 1980), acid phosphatase and sulfate permease in yeast (Novick et al., 1980), and albumin and vesicular stomatitis virus G protein in hepatoma cells (Lodish, personal communication) seem to reach the cell surface by identical routes. Perhaps such cells contain only the constitutive pathway

and lack the specialized condensing pathway of AtT-20 cells.

It has been suggested that ACTH precursor is secreted from AtT20 cells by an entirely different pathway than the mature hormones (Mains & Eipper, 1981; Gumbiner & Kelly, 1981, Chapter 2). Only the release of the mature hormone is regulated by secretatogues (Mains & Eipper, 1981; Figure 10). Since precursor ACTH and the viral glycoproteins are released from AtT-20 cells with the same kinetics, I propose that the ACTH precursor is also secreted by the constitutive pathway (Pathway 1, Fig. 11). Proteolytic conversion of the precursor may be specifically associated with hormone packaging into secretory granules (Pathway 2, Fig. 11). Proof of this hypothesis would require purifying the post-Golgi precursor ACTH and viral glycoprotein transport vesicle or vesicles.

Alternatively, it has been proposed that the ACTH precursor is released from pituitary cells by exocytosis of newly formed "immature" secretory granules (Allen et al., 1981). This explanation is tenable only if immature secretory granules have distinct physical properties which cause them to be purified away from mature granules (Gumbiner & Kelly, 1981, Chapter 2). The immature granules must also acquire the machinery for regulated exocytosis only after complete proteolytic processing of the granule contents. It seems more likely that the precursor is secreted constitutively by a different pathway, but proof will require the direct demonstration of a separate transport vesicle.

Secretion of proteins by an alternate pathway due to incomplete packaging into secretory granules may also occur in other

secretory tissues. Basal secretion has been reported to differ in composition from stimulated secretion and the secretory granule content in several tissues, including for example the exocrine pancreas (Dagorn, 1978; Rothman, 1975). In this case, basal secretion of enzymes is very small compared to stimulated secretion (0.35%) (Dagorn, 1978) and could easily result from inefficient packaging into zymogen granules and direct release via a constitutive pathway. There are also many examples of tumor or cultured cells which contain very few, if any, dense core secretory granules but secrete large amounts of hormones (Oliver et al., 1975; Vila-Porcile & Oliver, 1980). Numerous small clear vesicles have been observed in such cells and it has been proposed that they mediate secretion by bypassing a concentration step.

Secretion of a large proportion of protein by a rapid, constitutive pathway in addition to a conventional regulated storage pathway would greatly complicate the kinetic analysis of secretion. Preferential release of newly synthesized protein has been reported for many cells (Rothman, 1975; Vila-Porcile & Oliver, 1980), and could result from a large constitutive secretion relative to storage in secretory granules. It seems important to consider whether the extent of packaging into storage granules has a role in the regulation of secretion by different types of cells.

The segregation of secretory granule proteins from the viral envelope glycoproteins in AtT20 cells provides an interesting example of intracellular protein and membrane sorting. It will be important to determine the intracellular site of this sorting event. The mechanism for concentrating hormones into the secretory

granule may underlie the sorting of mature hormones away from the constitutive pathway of membrane transport.

FIGURE 1. Comparison of MuLV proteins synthesized by AtT-20 and Moloney MuLV infected fibroblasts. AtT-20 cells and Moloney Leukemia Virus infected 3T3 fibroblasts were grown to confluency in 50 cm^2 dishes and labeled for 3 hr with 0.5 mCi ^{35}S-methionine. Each cell extract was divided into 3 equal portions for immunoprecipitations with the following antisera. (a) anti-gp70; (b) anti-whole Moloney MuLV; (c) nonimmune goat serum. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and the fluorogram was exposed for 8 hr.

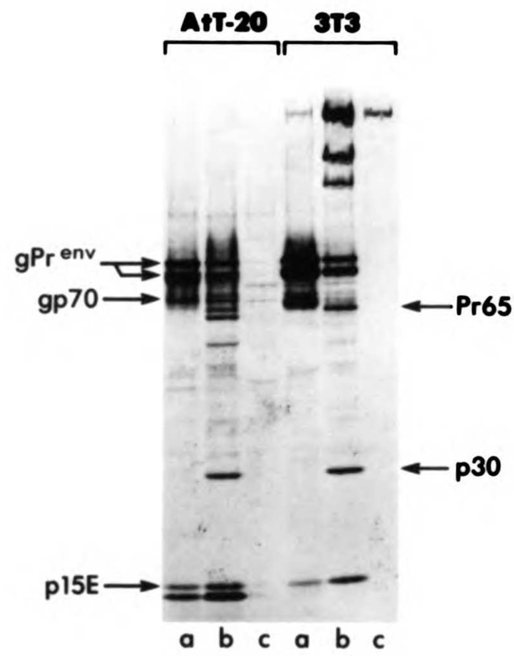
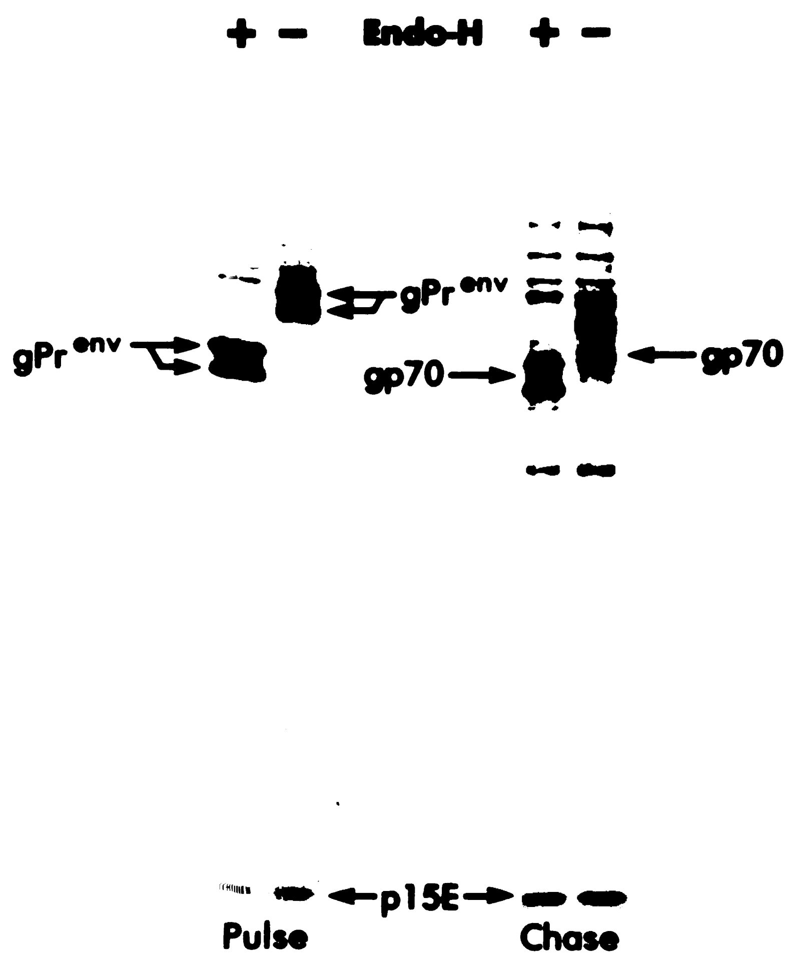

AtT-20
3T3
gPr env
gp70
p15E
Pr65
p30
a b c a b c

FIGURE 2. Processing of MuLV envelope glycoproteins by AtT-20 cells. Seven equally confluent 75 cm^2 flasks of AtT-20 cells were pulse labeled for 15 min with 0.5 mCi ^{35}S-methionine and chased with unlabeled medium for times varying between 0 and 120 min. Shown here are the 0 and 90 min chase samples. One third of each cell extract was immunoprecipitated with anti-gp70 serum. Half of each immunoprecipitate was digested with endo-H (+) and run next to the untreated control (-) on SDS-PAGE. Note that endo-H treated gp70 electrophoresed between the endo-H treated precursor polypeptides, unlike untreated samples, where it electrophoresed faster. The fluorogram was exposed for 3 days. To determine the time course of processing all of the lanes from the 7 time points were scanned with a densitometer.



+ − Endo-H + −
gPr env
gPr env
gp70
gp70
p15E
Pulse
Chase

FIGURE 3. Kinetics of MuLV envelope glycoprotein processing. The fluorogram from the experiment described in Figure 2 was scanned by densitometry and the label appearing in viral proteins was plotted for each time point. (O——O) gPr^{env}; (●——●) gp70 in cell extracts; (□——□) gp 70 released into the culture medium. The radiolabel disappearing from gPr^{env} was not completely recovered in gp70 because most of the methionine residues are contained in the cleaved p15E fragment. In virions released from cells, the ratio of label in p15E to gp70 was about 3:1. Similarly, the nucleotide sequence of the Moloney MuLV envelope gene revealed only 2 methionines in gp70 out of 7 in gPr^{env} (Shinnick et al., 1981). Assuming a ratio of 4:1 methionines in gPr^{env} to gp70, all of the envelope glycoprotein molecules could be accounted for in this pulse-chase experiment.

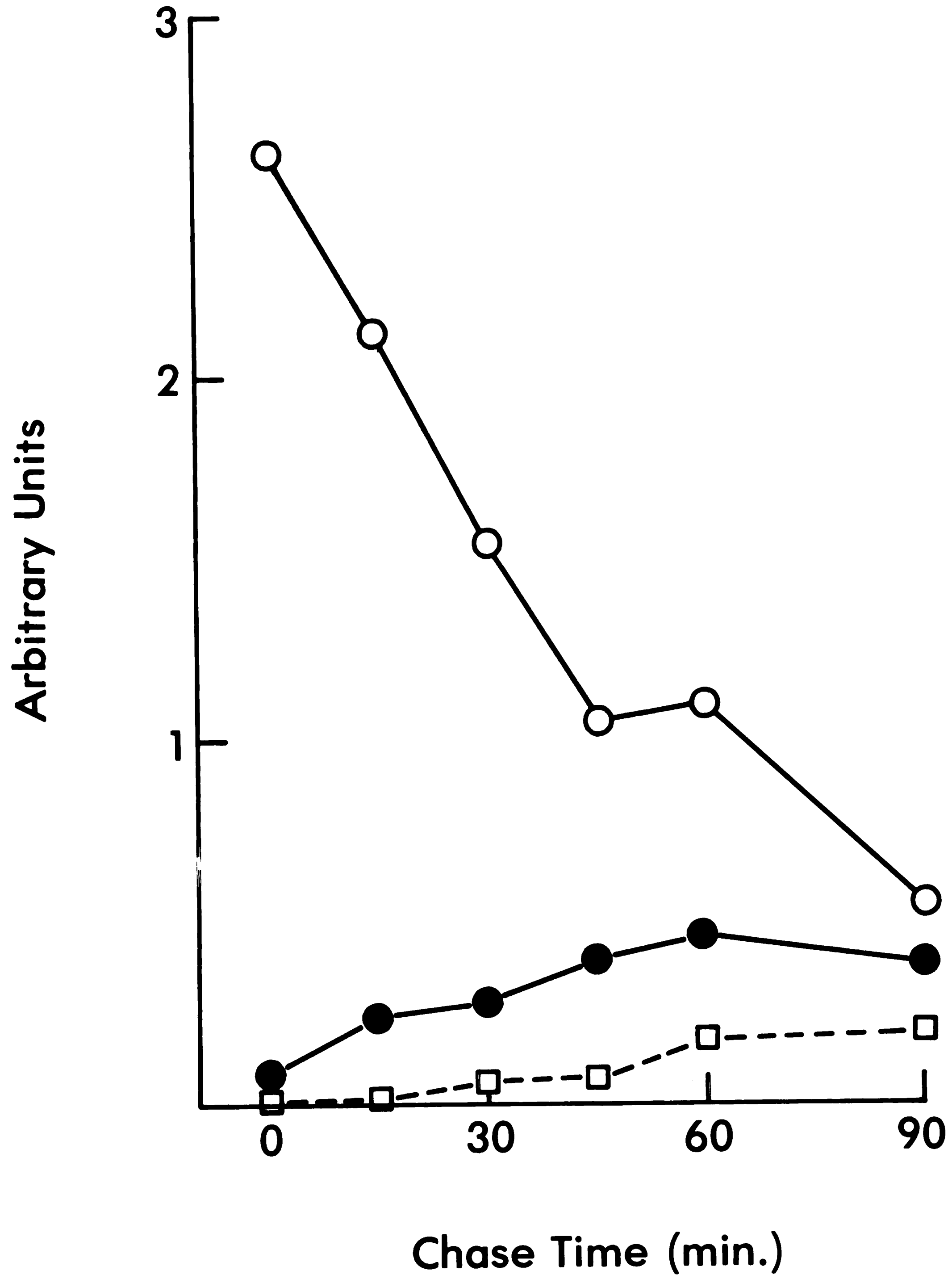

3
2
1
Arbitrary Units
0
30
60
90
Chase Time (min.)

FIGURE 4. Endoglycosidase-H digestion of ^{3}H-mannose labeled viral glycoproteins. Three confluent 6 cm^2 dishes of cells were rinsed and preincubated for 30 min in culture medium lacking glucose. They were each pulse labeled in 1 ml of the same medium containing 170 μCi of ^{3}H-2-mannose and chased in several milliliters of normal medium containing glucose (4.5 g/l). One-half of each cell extract was immunoprecipitated with anti-gp70. Half of each immunoprecipitate was digested with endo-H (+) and electrophoresed alongside the untreated half (-). The fluorogram was exposed for two months. a. 30 min pulse. b. 30 min pulse, 40 min chase. c. 30 min pulse, 2 hr chase. Although all of the label in gPr^{env} was removed by endo-H treatment, some label remained in gp70 (arrows).

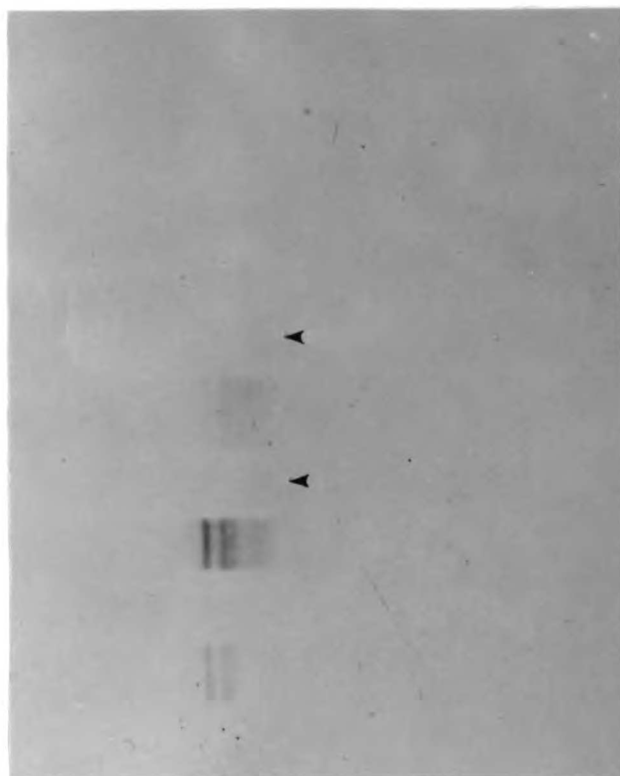

gPr env
gp70
− + − + − + : endo-H
a b c

FIGURE 5. Fractionation of envelope glycoproteins by ricin-agarose affinity chromatography. One 75 cm^2 flask of AtT-20 cells was pulse labeled for 15 min with 0.5 mCi of ^{35}S-methionine and chased for 60 min with normal medium. (a) One-half of the cell extract and one-fourth of the chase medium were immunoprecipitated directly with anti-gp70 to show the composition of the entire sample (the high background for total cell extract was due to overloading compared with the other samples). The rest of each sample was fractionated on the ricin column prior to immunoprecipitation with anti-gp70. (b) Immunoprecipitation of material that bound to the column and was eluted with 3% galactose. (c) Immunoprecipitation of one-half of the material which did not bind to the column. The fluorogram of the SDS gel were exposed 11 days for the cell extracts and 40 days for the chase medium. The major unidentified polypeptide in the immunoprecipitates of culture medium is probably due to cross-reacting antibodies in the anti-whole Moloney anti-serum.

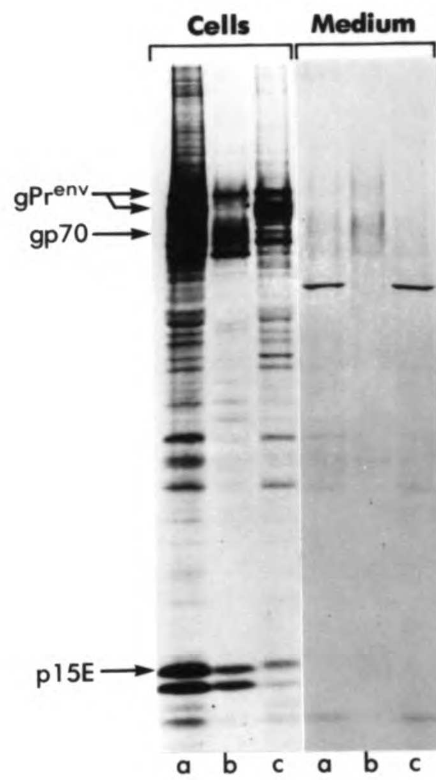

Cells
Medium
gPr^env
gp70
p15E
a b c a b c

FIGURE 6. Cell surface iodination of gp70. One confluent 75 cm^2 flask of AtT-20 cells was labeled with 1 mCi ^{125}I by lactoperoxidase catalyzed iodination. The cell extract was divided into 3 equal portions and immunoprecipitated with the following antisera: (a) anti-gp70; (b) anti-whole Moloney MuLV; (c) nonimmune goat serum. The arrows for gPr^{env}, Pr65, and p30 indicate where these polypeptides would migrate on this SDS-gel. A small portion of the immunoprecipitated iodinated gp70 was digested with endo-H (+) and electrophoresed alongside untreated samples (-).

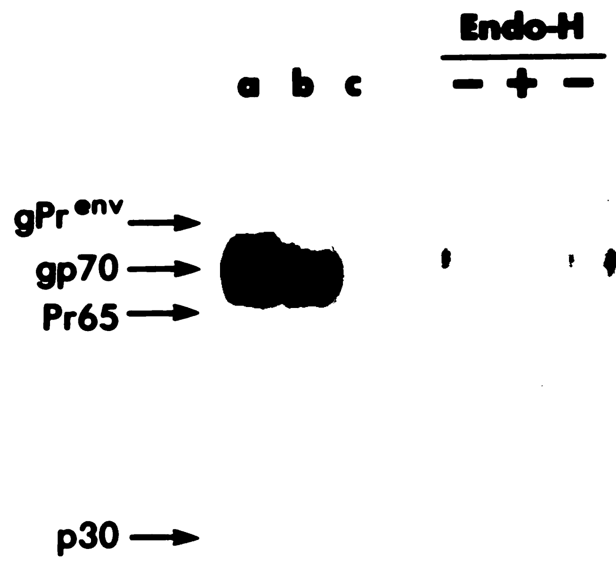

Endo-H
a
b
c
−
+
−
gPr env
gp70
Pr65
p30

FIGURE 7. Separation of virus particles from soluble viral proteins released into the culture medium. A. AtT-20 cells were pulse labeled for 15 min with 0.5 mCi ^{35}S-methionine per 75 cm^2 flask and chased with complete medium for 45 min. The chase medium collected from two 75 cm^2 flasks of cells was centrifuged at 12,000g for 10 min to remove cell debris and then centrifuged for 1 hr at 35,000 rpm in a Beckman SW40 rotor to pellet virus particles. The pellet and supernate each were divided into 3 equal portions and immunoprecipitated with (a) anti-gp70 serum; (b) anti-p30 serum, and (c) anti-whole Moloney MuLV serum and analyzed by SDS-PAGE and fluorograph. The 50 kd and 35 kd polypeptides correspond to released fragments of the glycosylated gag precursor. B. One 75 cm^2 flask of AtT-20 cells was labeled with 0.4 mCi ^{35}S-methionine for 2 hr and then incubated in normal medium for 6 hr. The medium was collected, centrifuged and analyzed in the same way discussed in A. 50% of the labeled gp70 was not pelletable (data not shown).

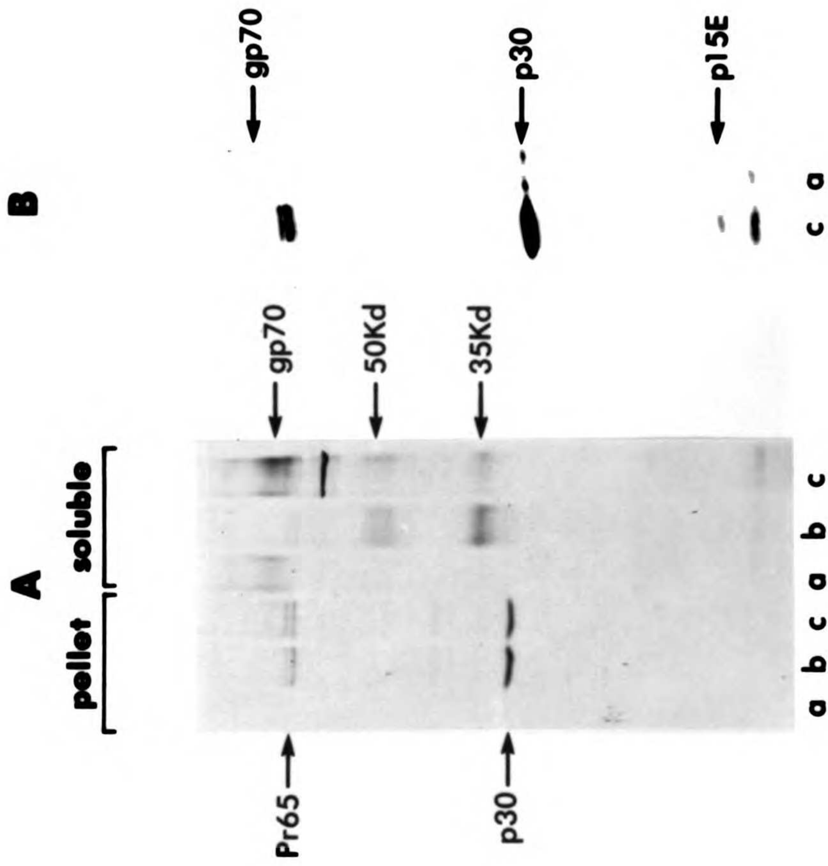

A
pellet
soluble
a b c a b c
Pr65
p30
gp70
50Kd
35Kd
B
gp70
p30
p15E
c a

FIGURE 8. Kinetics of viral glycoprotein and ACTH precursor release. The chase media from the experiment described in Fig. 2 were collected and immunoprecipitated with antiserum to ACTH and with antiserum to whole Moloney MuLV. The immunoprecipitates were analyzed by SDS-PAGE. Fluorograms were scanned with a densitometer to quantify the label present in each polypeptide. Release of each protein was expressed as the percentage of the total amount released in 120 min in order to normalize for differential incorporation of label into various polypeptides. Δ - ACTH precursor; ● - gp70; O - glycosylated gag polypeptides. At 60 min chase approximately 70% of the total (cells and medium) precursor molecules were present in the culture medium, but only 10-15% of the total mature ACTH molecules were released.

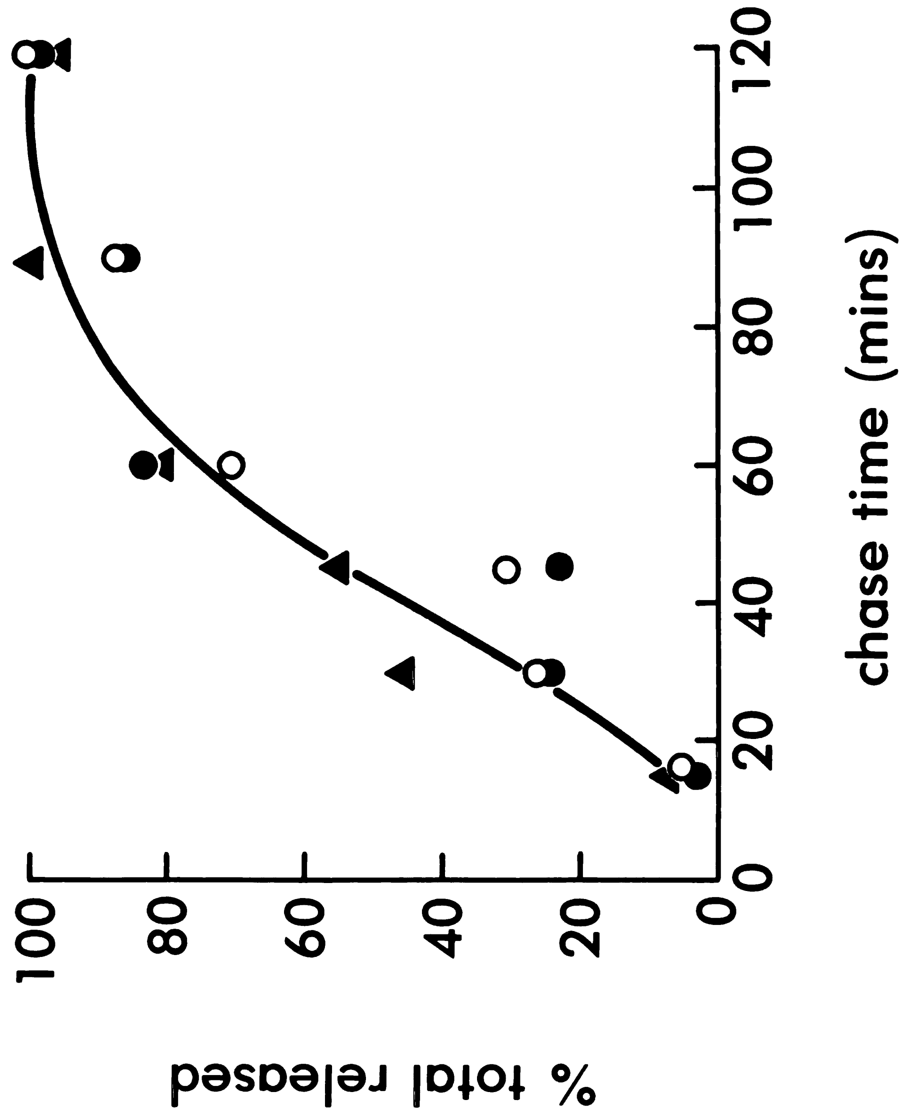
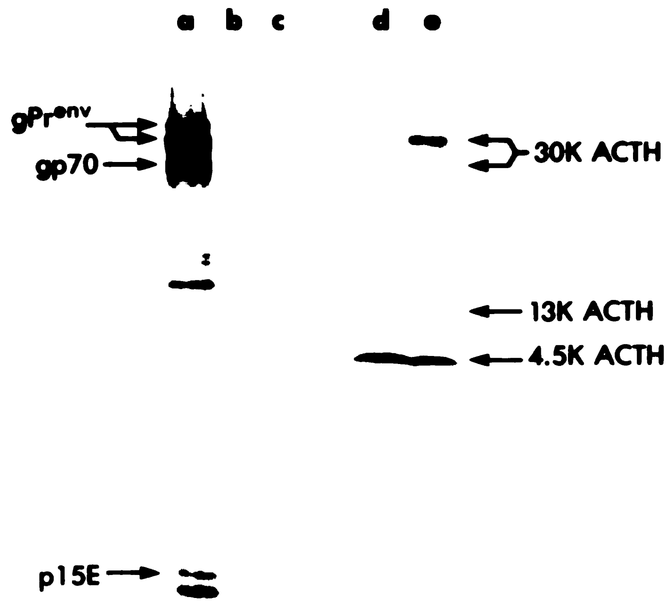
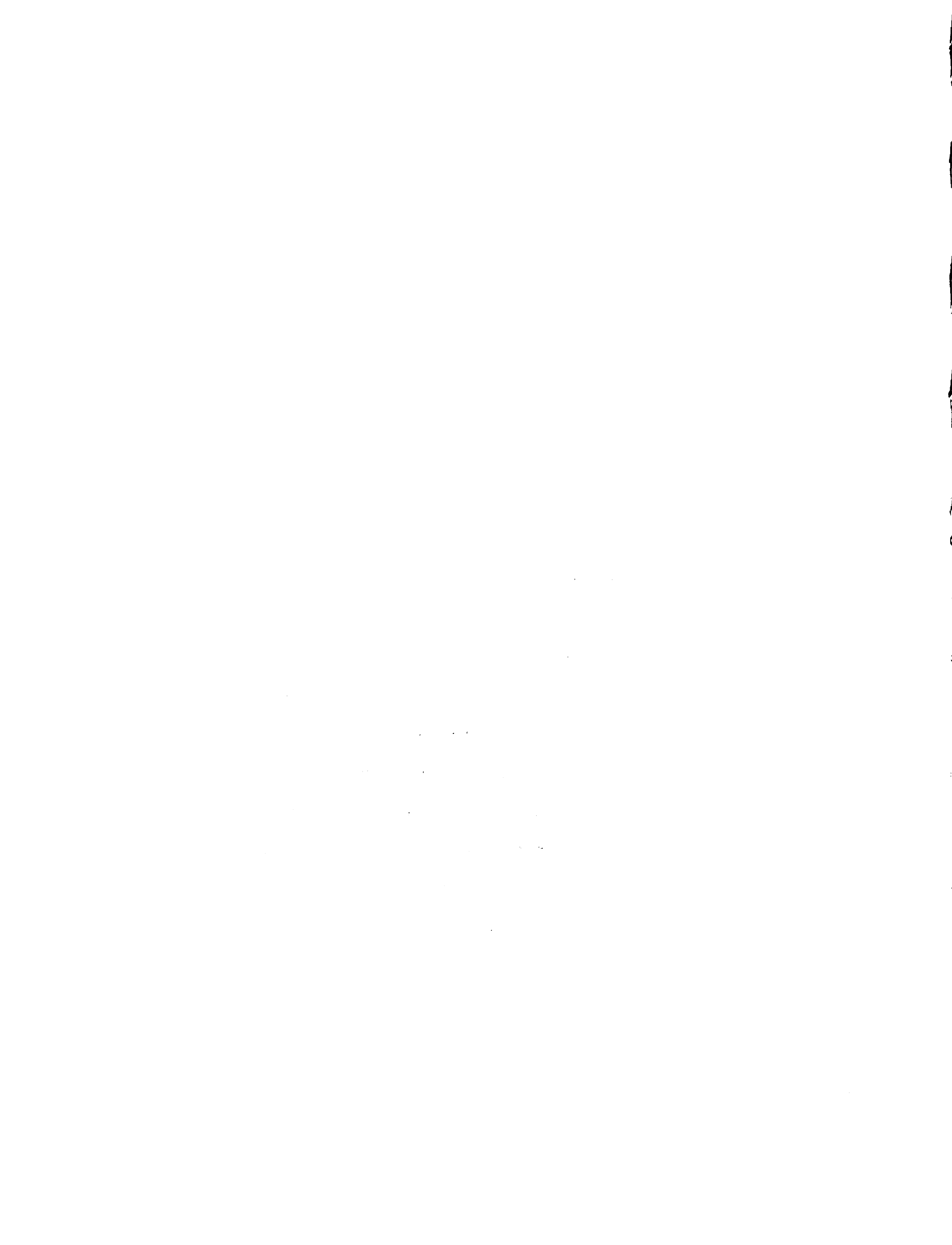

% total released
100
80
60
40
20
0
0
20
40
60
80
100
120
chase time (mins)

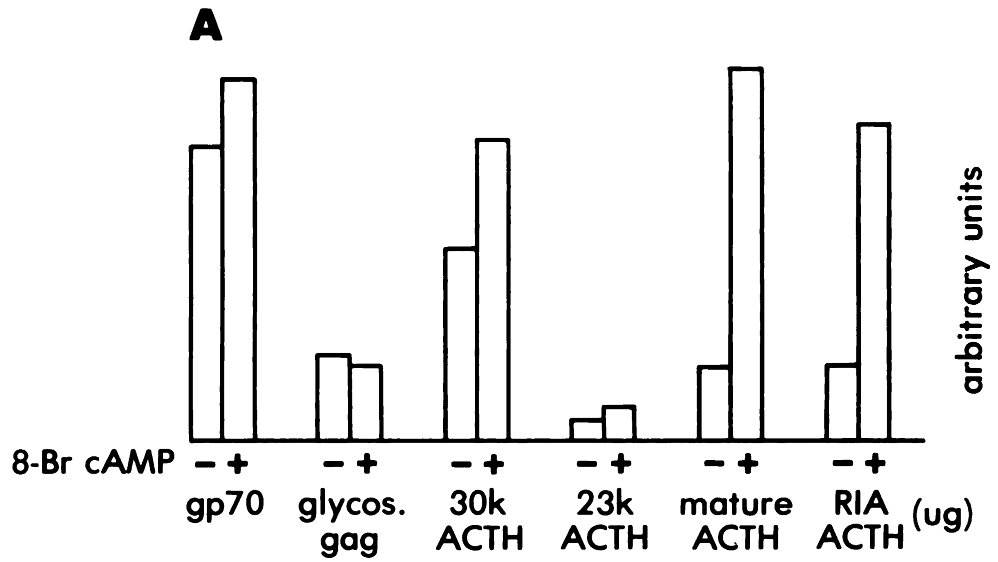
FIGURE 9. Lack of viral envelope glycoprotein accumulation in ACTH secretory granules. Four confluent 75 cm^2 flasks of AtT-20 cells were pulse labeled for 30 min with a total of 2.0 mCi ^{35}S-methionine and chased for 60 min with normal medium. One-tenth of the harvested cells was extracted immediately to measure the total label incorporated into ACTH and the viral glycoproteins and the remainder was used for the secretory granule isolation. ACTH and gp70 were immunoprecipitated from extracts of cells and secretory granules and analyzed by SDS-PAGE and fluorography with prefogged film. (a) anti-gp70 immunoprecipitation of one-third of the cell extract; (b) nonimmune control precipitation of one-third of the cell extract; (c) anti-gp70 immunoprecipitation of one-half of the isolated secretory granules. The limit of detection of gp70 at this exposure was 1/50 of sample a; (d) anti-ACTH immunoprecipitation of one-tenth of the secretory granules; (e) anti-ACTH immunoprecipitation of 1/30 of the cell extract.



a b c d e
gPr^env
gp70
30K ACTH
13K ACTH
4.5K ACTH
p15E

FIGURE 10. Effect of secretagogues on the release of ACTH and viral glycoproteins. A. Two equally confluent 75 cm^2 flask of cells were pulse labeled for 15 min with 0.5 mCi ^{35}S-methionine each and chased for 45 min with normal medium. The chase media were removed and cells were incubated in either normal medium (-) or medium containing 0.005M 8Br-cAMP (+) for 90 min. These media were immunoprecipitated with antiserum to ACTH and antiserum to whole Moloney MuLV and analyzed by SDS-PAGE. Fluorograms were scanned by densitometry to quantify the release of labeled polypeptides. (The label in each form of ACTH was divided by the number of methionine residues in that form in order to normalize their release on a molar basis. The units plotted for the viral glycoproteins cannot be compared directly to the units for ACTH.) B. Three 75 cm^2 flasks of cells were labeled continuously for 3 hr with 0.5 mCi ^{35}S-methionine each. The labeling media were removed and cells were incubated for 15 min in normal medium, medium containing 0.005M 8Br-cAMP, or medium in which .08M KCl was isotonically substituted for NaCl. These media were immunoprecipitated and analyzed in the same way as A.




A
arbitrary units
8-Br cAMP - + - + - + - + - + - +
gp70
glycos. gag
30k ACTH
23k ACTH
mature ACTH
RIA ACTH
(ug)

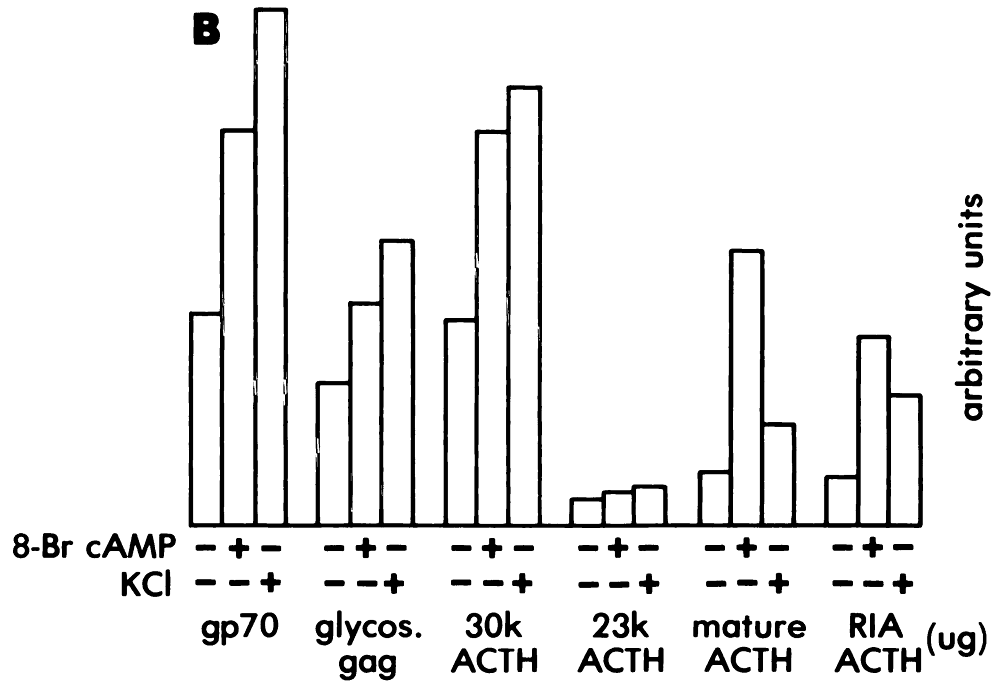
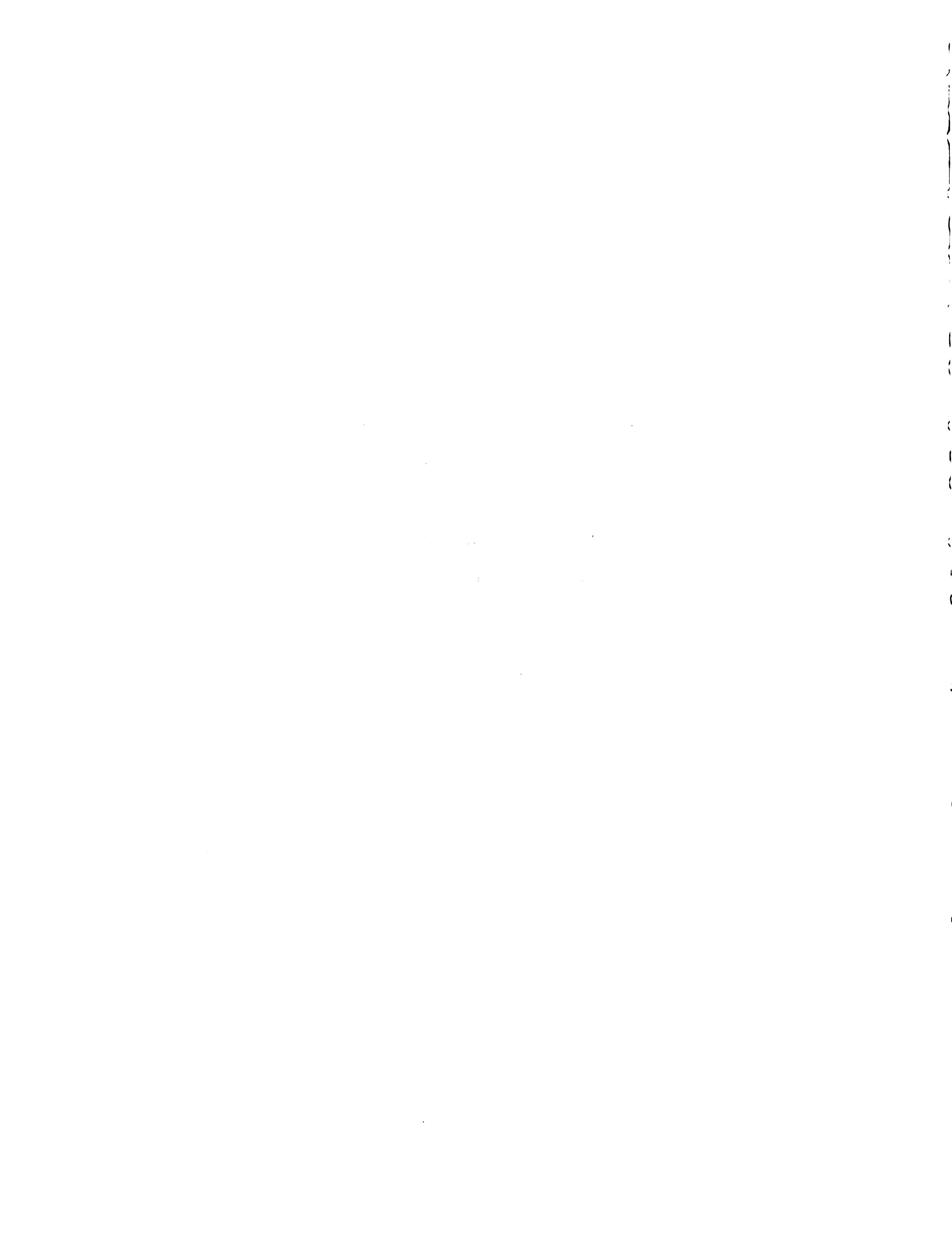
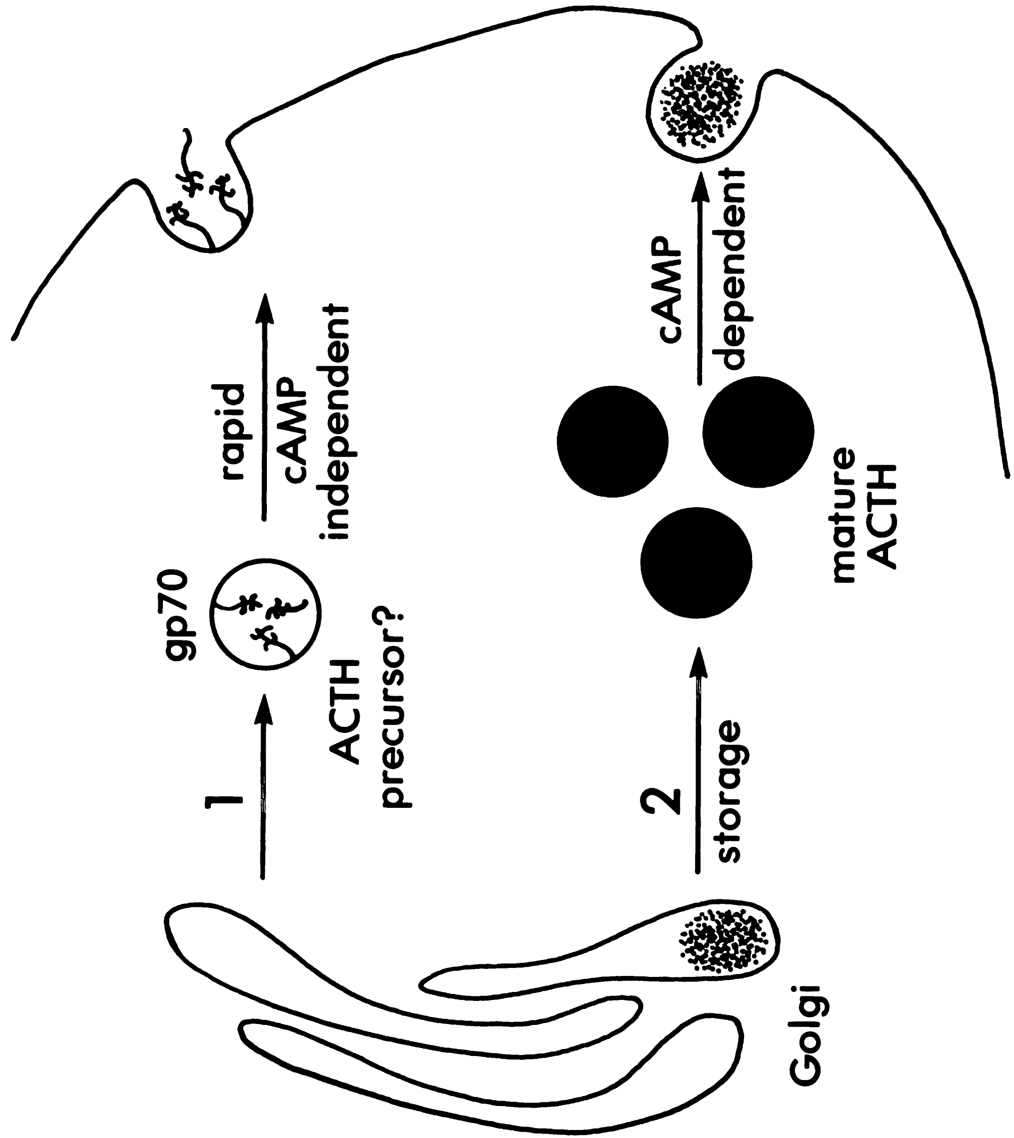

B
arbitrary units
8-Br cAMP - + - - + - - + - - + - - + - - + -
KCl - - + - - + - - + - - + - - + - - +
gp70
glycos. gag
30k ACTH
23k ACTH
mature ACTH
RIA ACTH
(ug)

FIGURE 11. Model for two pathways for the transport of glycoproteins to the surface of AtT-20 cells. Newly synthesized ACTH and viral glycoproteins are transported from the Golgi apparatus to the plasma membrane via two distinct exocytotic vesicles. Pathway 1 is constitutive. Vesicles containing gp70 fuse with the plasma membrane rapidly in a cAMP independent manner. This may be the pathway used for plasma membrane insertion and thus essential for cell growth. Pathway 2 is specialized for the physiologically regulated release of mature hormones. Mature hormones are condensed and stored in dense core secretory granules which accumulate in the cytoplasm. Secretory granule exocytosis occurs continuously but is greatly stimulated by increased intracellular levels of cAMP or Ca^{++}. Some of the ACTH precursor may be secreted by pathway 1 because it escapes condensation into secretory granules and proteolytic cleavage.



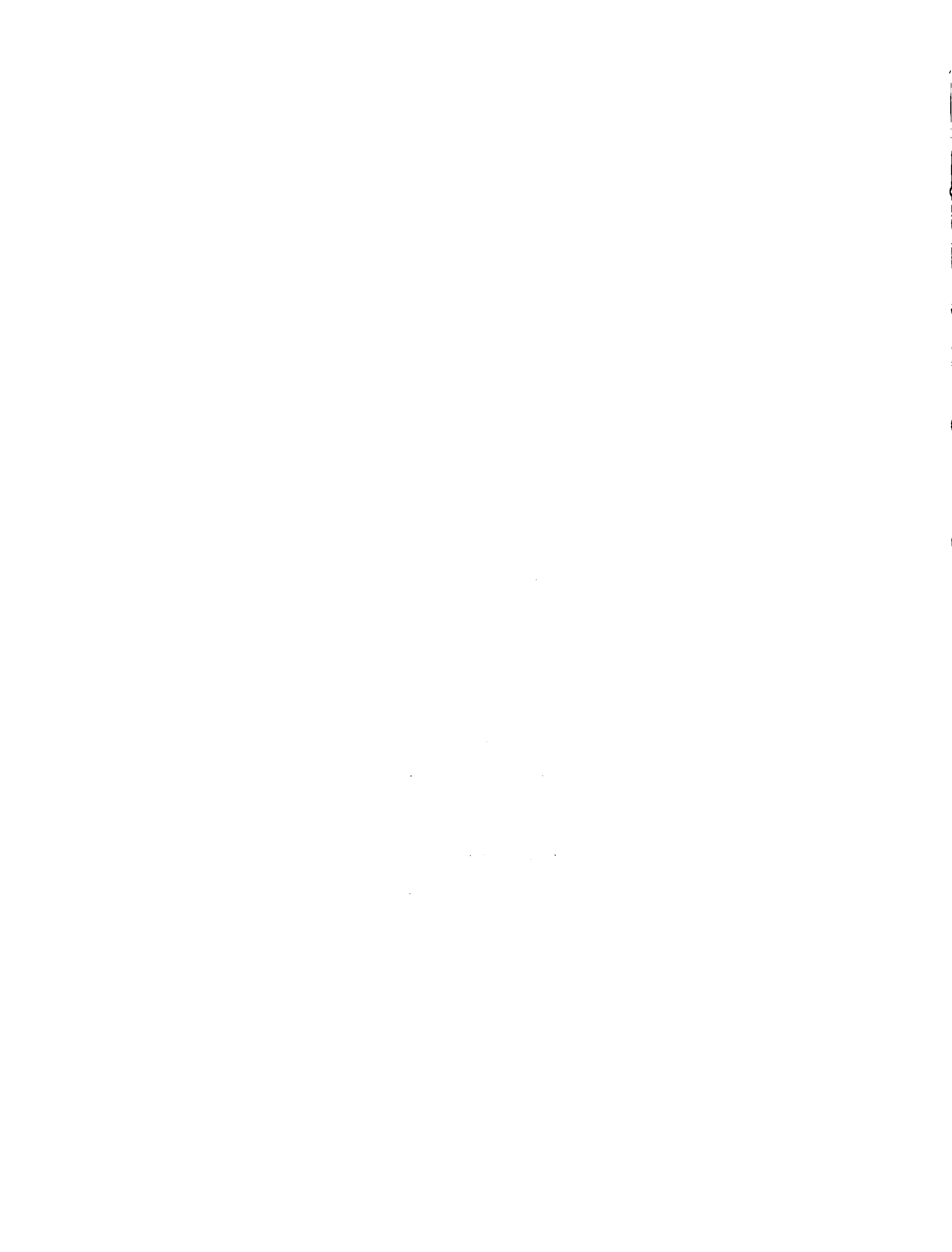

1
ACTH
precursor?
gp70
rapid
cAMP
independent
2
storage
mature
ACTH
cAMP
dependent
Golgi

CHAPTER 4

THE PROTEOLYTIC PROCESSING OF PRO-ACTH/ENDORPHIN AND ITS TRANSPORT INTO SECRETORY GRANULES ARE INHIBITED BY NIGERICIN

SUMMARY

To determine the intracellular location of the proteolytic conversion of the ACTH/endorphin precursor, the processing of the precursor and its packaging into secretory granules was examined in AtT-20 cells treated with the ionophore, nigericin. Carboxylic cation ionophores, including monensin and nigericin, block intracellular transport of plasma membrane and secretory proteins in the Golgi apparatus. Proteolytic cleavage of the ACTH precursor was inhibited 75% by 10^{-6}M nigericin. The ACTH precursor that accumulated in treated cells had undergone terminal glycosylation since half of it was resistant to digestion with endoglycosidase-H. Acquisition of endoglycosidase-H resistance of ACTH in nigericin treated cells was 70% of normal cells. Incorporation of all hormone fragments derived from the precursor and several other presumptive secretory granule proteins into isolated secretory granules was also blocked by 10^{-6}M nigericin. Inhibition of hormone incorporation into secretory granules by nigericin was dose dependent, becoming maximal between 10^{-7}M and 10^{-6}M. Gross cell morphology, cellular ATP levels, protein synthesis and galactosyl transferase activity were not significantly affected by 10^{-6}M nigericin, but the characteristic dilation of the Golgi saccules was evident. The results indicate that proteolysis of the ACTH precursor does not occur when transport from the Golgi to the secretory granule is blocked. Therefore, prohormone cleavage may occur in a compartment distal to the glycosyltransferase containing Golgi elements; either the secretory granule itself or a condensing vacuole.



INTRODUCTION

Many polypeptide hormones are synthesized as prohormones and proteolytically cleaved during their intracellular transport. Prohormone cleavage occurs late during intracellular transport and is often associated in time with hormone packaging into secretory granules (Kemmler, 1976; Glembotski, 1981; Gumbiner & Kelly, 1981; chapter 2). Proinsulin conversion to insulin takes place either in the Golgi apparatus or the secretory granule, because cleavage is prevented by inhibiting export from the RER with metabolic energy poisons (Steiner, 1976). Proteolytic cleavage may continue to some extent within the secretory granules that contain endorphins (Gumbiner & Kelly, 1981, Chapter 2), insulin (Steiner, 1976) and vasopressin (Gainer et al., 1977). There is also preliminary evidence that the membranes of isolated secretory granules have activities which accurately process proinsulin and proglucagon (Fletcher et al., 1981) and pro-ACTH/endorphin (Loh & Gainer, 1982). Although these results indicate that the proteases are located within the secretory granule, there is also evidence that cleavage begins in the Golgi apparatus (Steiner, 1976 Glembotski, 1981). In fact, it has been proposed that proteolytic cleavage provides an irreversible modification that underlies the packaging or concentration of hormones into secretory granules (Steiner, 1976; see Chapter 1, section VII).

Several results suggest that the ACTH/endorphin precursor in AtT-20 cells is cleaved in a special late compartment that is either the secretory granule itself or its immediate precursor, the

condensing vacuole. The precursor acquires its terminal sugars, presumably in the Golgi, before it is cleaved (Phillips et al., 1981). Isolated secretory granules contain only cleaved hormones (Gumbiner & Kelly, 1981; Chapter 2). Also, AtT-20 cells apparently secrete fully glycosylated precursor molecules by a different exocytotic vesicle than the secretory granule (Gumbiner & Kelly, 1982; Chapter 3). This implies that precursor molecules are cleaved only during segregation into the secretory granules after completion of glycosylation in the Golgi apparatus. Proteolysis either is required for hormone segregation and packaging or else occurs after segregation and terminal glycosylation. Thus it is interesting to determine whether proteolysis occurs in the Golgi apparatus itself.

The ionophores, monensin and nigericin block the transport of secretory and membrane proteins in the Golgi apparatus in all examples reported so far (Tartakoff & Vassalli, 1977; Tartakoff & Vassalli, 1979; Tartakoff et al., 1978; Strous & Lodish, 1980; Johnson & Schleisinger, 1980; Tartakoff et al., 1981; Tartakoff, 1982), except for influenza maturation in MDCK cells (Alonso & Compans, 1981). Autoradiography, cell fractionation and immunofluorescence studies showed that proteins drained from the RER and accumulated in the Golgi of monensin treated cells (Tartakoff & Vassalli, 1977; Tartakoff et al., 1978; Kaarianen et al., 1980). Therefore experiments were undertaken to determine whether the ACTH/endorphin precursor would be cleaved when its transport is blocked in the Golgi by ionophores. The data show that both proteolytic processing and incorporation into secretory granules is

inhibited with nigericin treatment. Because carbohydrate processing of the precursor still occurs to a large extent, I conclude that inhibition by nigericin occurs between the Golgi and the secretory granules. The site of transport inhibition by ionophores is discussed critically.

RESULTS

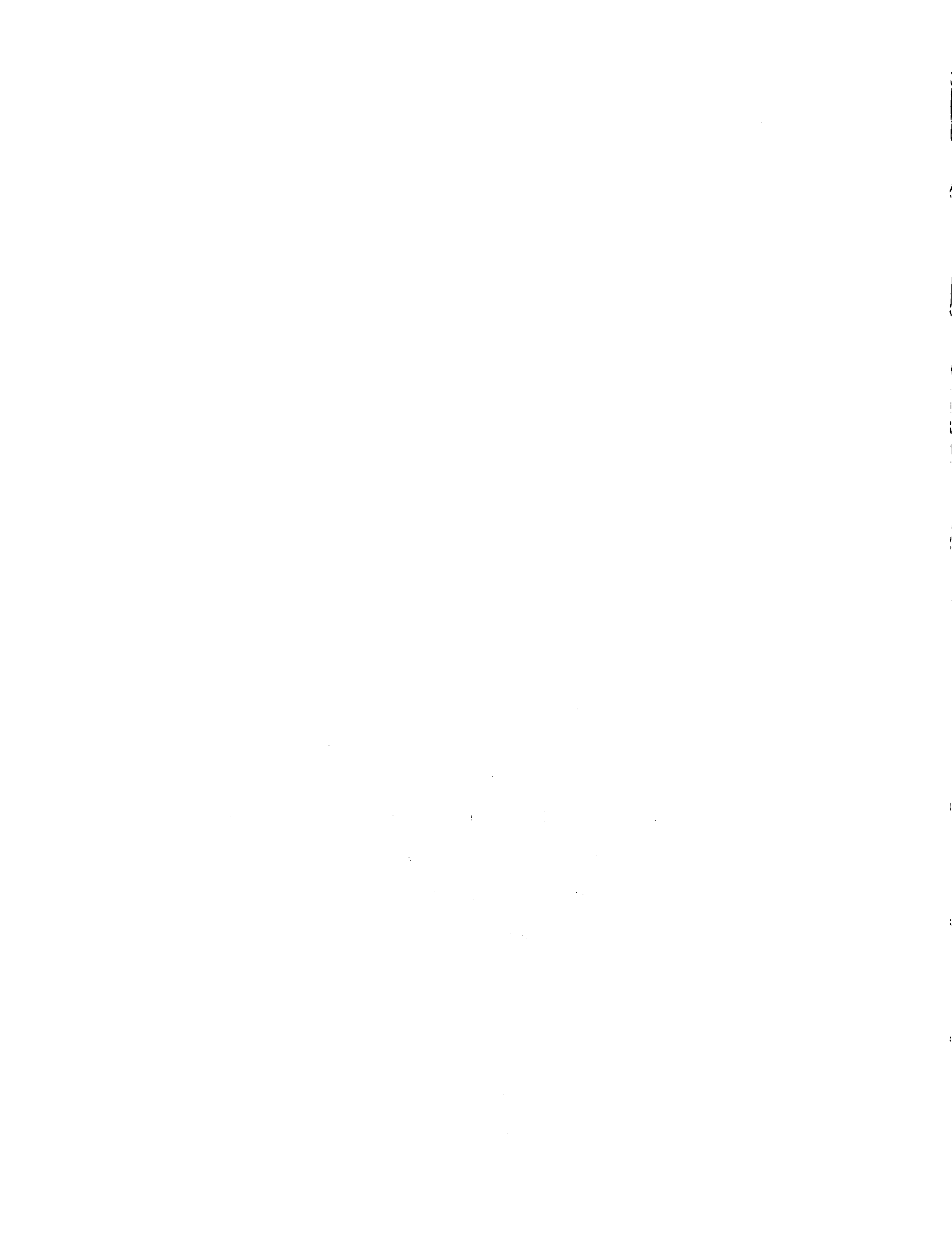
I. Nigericin Inhibits the Processing of Pro-ACTH/Endorphin

The effect of 10^{-6}M nigericin on the proteolytic processing of ACTH is shown in Figure 1A. After a 50 min pulse of ^{35}S-methionine untreated cells had converted some precursor to mature ACTH (lane a), but conversion was more complete after a 60 min chase (lane b). When 10^{-6}M nigericin was included throughout the same pulse and chase intervals the appearance of mature ACTH was reduced significantly (lane c). Similarly after 110 min continuous labeling mature ACTH appeared in controls (Lane d) but not in the presence of 10^{-6}M nigericin (lane e). Since the precursor accumulated in cells relative to controls (compare lanes b and c), the absence of mature ACTH was due to inhibition of proteolytic cleavage.

The autoradiogram was scanned with a densitometer to quantify the extent of proteolytic conversion. The radiolabel in the various forms of ACTH was normalized to a molecular basis by dividing by the number of methionine contained in each form (three in precursor, two in intermediate and one in mature ACTH). In the experiment shown in Figure 1A, 26% of the molecules was converted to mature ACTH after a 50 min pulse, 80% was converted after a 60

min chase in control cells, but only 23% was converted after the same pulse-chase interval in the presence of nigericin. In the experiment shown in Figure 1B, 21% of the molecules was converted to mature ACTH during a 30 min pulse, 53% was converted after a 60 min chase in control cells, but only 13% was converted after the same pulse-chase in the presence of nigericin. Therefore, proteolytic conversion of pro-ACTH after pulse-chase labeling in the presence of 10^{-6}M nigericin was inhibited to 25% of normal conversion.

The effect of nigericin on the carbohydrate processing of ACTH was determined by digestion with endoglycosidase-H (endo-H) (Fig. 1B). Endo-H removes high mannose oligosaccharides from glycoproteins, but not complex type oligosaccharides that are obtained through processing in the Golgi apparatus (Robbins et al., 1977; Strous & Lodish, 1980). Polypeptide 3 is the deglycosylated form of the ACTH precursor; polypeptides 1 and 2 are the endo-H resistant forms of the precursor which have undergone terminal glycosylation. Only 11% of the precursor molecules became endo-H resistant after a 30 min pulse labeling (lanes a), but 51% of the precursor molecules became endo-H resistant after a 30 min pulse and 60 min chase in the presence of nigericin. Similarly, 48% of the precursor molecules remaining in normal cells after a pulse-chase were endo-H resistant. To compare the extent of terminal glycosylation in nigericin treated and control cells, the percent of all forms of ACTH with processed carbohydrates was calculated. All cleavage fragments of the ACTH/endorphin precursor including the 23K intermediate and the amino-terminus have terminally glycosylated carbohy-



drates (Phillips et al., 1981). The small amounts of 23K ACTH and 13K ACTH present in nigericin treated cells appeared to be endo-H resistant (not shown). Therefore, all cleaved forms of ACTH and the endo-H resistant precursor were included in the total amount molecules with processed carbohydrates. 80% of the ACTH molecules remaining in control cells after 1 hr chase had undergone carbohydrate processing. If the proportion of molecules that were probably secreted during the hour chase interval (see Chapter 3; Mains & Eipper, 1978) are included in the completely processed forms, 89% of the ACTH molecules initially labeled had undergone terminal glycosylation. In the presence of nigericin 62% of the ACTH molecules had undergone terminal glycosylation. Therefore carbohydrate processing of the pro-ACTH/endorphin derived molecules in the presence of nigericin was 70% of the processing in control cells.

II. Nigericin Blocks the Incorporation of Newly Synthesized Proteins Into Secretory Granules

To determine whether the inhibition of ACTH precursor processing was associated with a block in intracellular transport, secretory granules were isolated from normal cells and cells treated with 10^{-6}M nigericin (Fig. 2). Cells were pulse labeled for 90 min with ^{35}S-methionine to label ACTH in the secretory granules (Chapter 2). Nigericin was included throughout the pulse-chase period. Unlabeled carrier cells were added to both sets of labeled cells and secretory granules were isolated by a modification of the procedure described in Chapter 2. In this modification, an attempt was made to recover all of the secretory granules on a 20% Ficoll pad at the bottom of the D_2O gradient rather than allow-

ing them to equilibrate as usual in the Ficoll step gradient. The distribution of ACTH, determined by radioimmunoassay, across the D_2O density gradients was similar for both sets of cells, but it indicated that not all of the secretory granules were pelleted. Therefore, the six bottom fractions from each gradient were precipitated with TCA and analyzed by SDS gel electrophoresis and autoradiography (Fig. 2). Although there was more contamination of granule fractions with nongranule polypeptides compared to the usual isolation procedure (compare with Fig. 5, Chapter 2), the densest fractions (1 and 2) obtained from control cells were enriched in hormone products as well as other presumptive granule polypeptides (*). The contamination with nonspecific polypeptides afforded an interesting comparison between nigericin treated and untreated cells. The patterns of contaminating polypeptides across both sets of gradient fractions appeared similar. In contrast, all of the polypeptides which copurify with secretory granules, including the mature hormone fragments and the higher molecular weight polypeptides (indicated by an asterisk), were present only in the dense fractions obtained from control cells. Therefore, nigericin apparently blocks the transport of the major newly synthesized secretory granule proteins into the secretory granule. The block in hormone incorporation could not be explained solely by the inhibition of proteolytic cleavage, since precursor forms of ACTH did not accumulate in the secretory granule fractions. This result has been repeated two additional times using a miniature version (5 ml) of the similar D_2O density gradient. I have not eliminated the possibility that nigericin caused lysis or a density perturbation

of the secretory granules such that they could not be recovered on the D_2O density gradient.

The miniaturized D_2O density gradient was used to determine the nigericin dose dependence for the inhibition of radiolabeled ACTH incorporation into secretory granules (Fig. 3). The granule fractions were analyzed by SDS gel electrophoresis and autoradiograms were scanned with a densitometer to quantify the radiolabeled polypeptides. The label that appeared in the LPH and ACTH peptides was normalized to the label that appeared in a major contaminant, the p30 viral nucleocapsid protein. (The label in p30 varied only slightly between samples.) The inhibition of LPH and ACTH appearance in secretory granules occurred over a range of 10^{-8}M to 10^{-6}M nigericin. The dose dependence was similar to that reported for externalization of acetylcholine receptor and acetylcholinesterase in muscle cells treated with nigericin (Rotundo & Fambrough, 1980).

III. <u>General Effects of Nigericin on AtT-20 Cells</u>

No gross morphological changes in the AtT-20 cells was evident after two hour treatment with 10^{-6}M nigericin. However electron microscopy showed that a 90 min incubation with 10^{-6}M nigericin caused the Golgi saccules to vacuolate (B. Drees & F. Novak, unpublished observations). The mitochondria appeared somewhat denser than normal and the RER may have swelled slightly. These changes are the same as those that have been described previously from treatment with the ionophores monensin and nigericin (Tartakoff, 1982; Tartakoff & Vassalli, 1977; Tartakoff et al., 1981).

It is important to be certain that nigericin had no major metabolic effects on the cells, because depletion of metabolic

energy inhibits the export of secretory proteins from the RER (Jamieson & Palade, 1968). The ATP content of normal cells and cells treated for 90 min with 10^{-6}M nigericin was 30 nanomoles/mg protein and 31 nm/mg protein, respectively. Protein synthesis was not altered significantly by 10^{-6}M nigericin. Incorporation of ^{35}S-methionine into TCA precipitable counts was 87±10% of controls. Galactosyltransferase activity assayed on exogenous substrates in detergent lysates of AtT-20 cells was unaffected by 10^{-6}M nigericin treatment for 90 min (B. Drees, unpublished observations). Thus, nigericin caused no dramatic metabolic changes in AtT-20 cells. Its effects on ultrastructure and intracellular transport are probably the same as those produced by monensin.

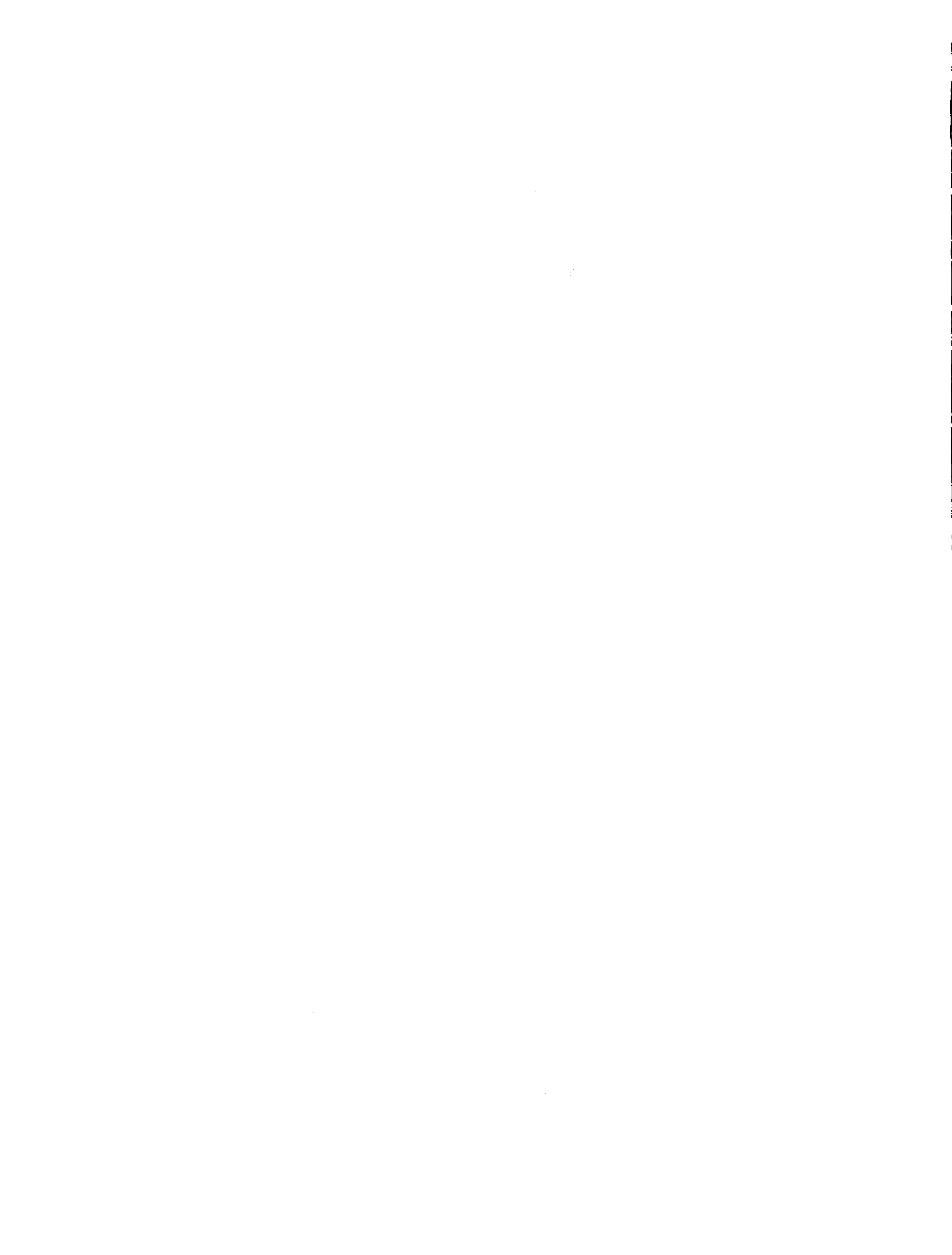
DISCUSSION

Nigericin treatment of AtT-20 cells probably blocks the intracellular transport of ACTH into the secretory granule. Incorporation of hormones into isolated secretory granules was inhibited in a dose dependent fashion. It is unlikely that the lack of hormone recovery in the secretory granule fractions was due to intracellular lysis or perturbation of the secretory granules. Tartakoff et al. (1978) showed that exocytosis of zymogen granules in the exocrine pancreas was unaffected by monensin treatment. Rather an earlier step in intracellular transport was blocked.

Proteolytic cleavage of the ACTH/endorphin precursor was inhibited by ionophore treatment to a greater extent than carbohydrate processing. Assuming that the precursor accumulated in the

vacuolated Golgi membranes like secretory proteins in other cells (Tartakoff and Vassalli, 1977; Tartakoff et al., 1978), one interpretation is that proteolysis occurs in a compartment distal to the Golgi saccules that are responsible for terminal glycosylation. The distal compartment could either be the secretory granule itself or a special concentration compartment that forms secretory granules (see Chapter 1, section VI). This interpretation is consistent with the localization of the pro-ACTH/endorphin converting protease in the secretory granule (Loh & Gainer, 1982) and my suggestion that precursor molecules are cleaved only during segregation into secretory granules after completion of terminal glycosylation (Gumbiner & Kelly, 1982; Chapter 3).

Terminal glycosylation of pro-ACTH/endorphin was partially inhibited by nigericin treatment. Perhaps inhibiting transport from the Golgi to the secretory granule causes secretory proteins to "back-up" proximal to the glycosyltransferase containing Golgi membranes. Nigericin may also directly inhibit transport into the glycosylation compartment, but to a lesser extent than transport into secretory granules. It is also possible that all of the ACTH precursor accumulates in membranes that contain glycosyltransferases, but is inefficiently glycosylated because dilation of Golgi by ionophores dilutes secretory proteins away from the enzymes. This interpretation is favored by Lodish (personal communication) to explain why monensin inhibited the glycosylation of transferrin, but not the VSV G protein in hepatoma cells (Strous & Lodish, 1980). Both proteins have been localized to the same dilated Golgi vesicles by immunocytochemistry (Lodish, personal



communication). Of course, this interpretation could also explain the greater inhibition of pro-ACTH/endorphin cleavage by nigericin. The proteases could be located within the same Golgi membranes as the glycosyltransferases, but proteolysis could be more sensitive to vacuolization caused by ionophores.

Tartakoff & Vassalli (1979) and Tartakoff (1982) proposed that transport between two sequential processing compartments within the Golgi is inhibited. In the presence of monensin, fatty acylation of the VSV G protein and phosphorylation of lysosomal enzymes persist (Johnson & Schleisinger, 1980; Tartakoff, 1982). These activities were therefore attributed to a proximal Golgi compartment. Because many proteins are incompletely glycosylated during ionophore treatment (see below), Tartakoff (1982) concluded that terminal glycosylation occurs in a distal compartment.

The evidence that monensin inhibits intracellular transport proximal to a Golgi compartment for terminal glycosylation is inconclusive. The VSV G protein acquires resistance to endo-H digestion even in the presence of monensin (Johnson & Schlesinger, 1980; Strous & Lodish, 1980). Tartakoff (1982) suggested that the G protein was a special case due to its very rapid rate of transport. However, tranferrin has been found in the same dilated Golgi vacuoles of monensin treated hepatoma cells as the VSV G protein (Lodish, personal communication), even though it remained sensitive to endo-H digestion. Tartakoff & Vassalli (1979) also showed that glycosylation of secretory IgM was inhibited in monensin treated plasma cells. Although monensin inhibited ^{3}H-galactose incorporation into secretory IgM to 20% of control, ^{3}H-galactose incorpora-

tion into total cellular protein was 80% of control. This indicates that most of the cellular glycoproteins being transported through the Golgi of monensin treated cells were still accessible to galactosyltransferase. IgM may be poorly glycosylated because it is a more sensitive substrate, not because of its compartmentalization.

Terminal glycosylation of membrane proteins may not go entirely to completion in ionophore treated cells. Membrane bound IgM and H-2 antigens did not acquire sialic acid residues (Tartakoff & Vassalli, 1981). Because their sensitivity to endo-H digestion was not determined, it is not known whether they acquired any terminal sugars. Although the VSV G protein did become resistant to endo-H, its mobility on SDS gels was slightly greater than normal (Johnson & Scheisinger, 1980). This difference in mobility may result from an absence of sialic acids on the G protein (Johnson & Schleisinger, 1980).

These results indicate that processing activities of the Golgi apparatus are differentially affected by alteration of the Golgi with ionophores. Proteolytic conversion of the Sindbis virus glycoprotein was also inhibited with monensin (Johnson & Schleisinger, 1980). Proteolytic cleavage and sialic acid addition may be more sensitive to inhibition than galactose addition or mannose trimming which may be in turn more sensitive than fatty acylation or phosphorylation. It is interesting that the latest steps in processing are more completely inhibited by the ionophores. This is consistent with a progressive block increasing distally through the Golgi.

There is morphological evidence that transport of Semliki Forest virus envelope proteins are inhibited in a subset of Golgi

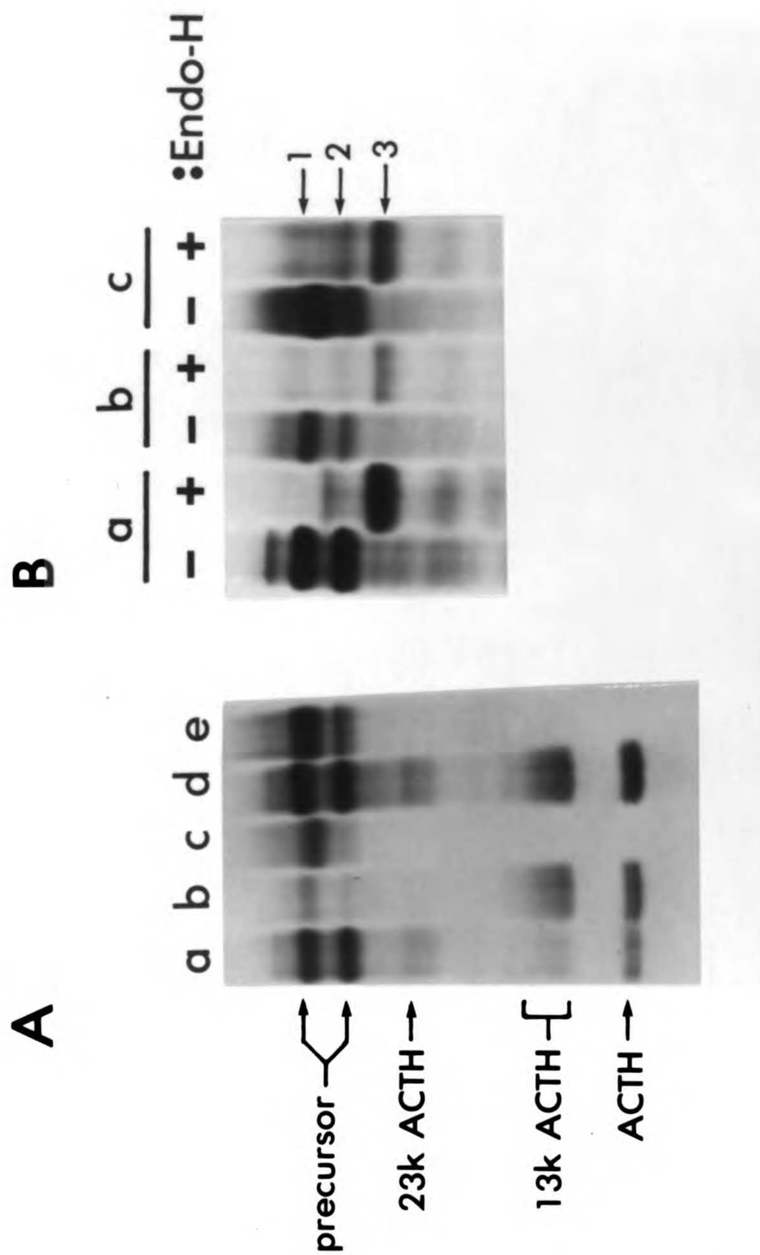
membranes by monensin. Viruses budded into some of the dilated vacuoles, presumably because the envelope glycoproteins accumulated there. Apparently, viruses never budded into vacuoles which stained histochemically for the enzyme thiamine pyrophosphatase. Since TPPase is usually segregated in the trans Golgi saccule, these findings imply that viral glycoproteins accumulated in TPPase negative cis Golgi saccules during monensin treatment. However, it is possible that viruses budded into the acid phosphatase staining GERL compartment that is distal to the TPPase staining Golgi saccules (Broadwell & Oliver, 1981; see Chapter 1, section VI).

Tartakoff & Vassalli (1979) also claimed that IgM labeled with ^{3}H-galactose prior to monenin treatment was efficiently exported from the Golgi and secreted during monensin treatment of plasma cells. However, their data showed that secretion of ^{3}H-galactose labeled IgM was unaffected only in the first 15 minutes, but was inhibited greater than 50% at later times. Perhaps the molecules initially secreted came from exocytotic vesicles that have already left the Golgi. Exocytosis appears to be unaffected by monensin (Tartakoff et al., 1978). The results I present here support the interpretation that ionophores block transport from the Golgi to the secretory vesicle.

FIGURE 1. Effect of nigericin on the processing of ACTH.

A. Five confluent 2.0 cm^2 wells of cells were labeled with 100 μCi of ^{35}S-methionine each. After the appropriate pulse-chase protocol, the cells were extracted, immunoprecipitated with anti-ACTH and analyzed by SDS-PAGE and autoradiography. a) 50 min pulse; b) 50 min pulse, 60 min chase; c) 50 min pulse, 60 min chase all in the presence of $10^{-6}M$ nigericin. d) 110 min pulse; e) 110 min pulse with $10^{-6}M$ nigericin.

B. Three confluent wells of cells were labeled and analyzed as in (A) except that half of each immunoprecipitate was treated with endo-H (+) and electrophoresed next to the untreated half (-). a) 30 min pulse; b) 30 min, pulse, 60 min chase; c) 30 min pulse, 60 min chase all in the presence of $10^{-6}M$ nigericin. Polypeptides 1 and 2 are the endo-H resistant forms of the precursor. Polypeptide 3 is the endo-H digestion product of the sensitive forms.



A
a b c d e
precursor
23k ACTH
13k ACTH
ACTH
B
a b c
- + - + - + :Endo-H
1
2
3

FIGURE 2. Inhibition of protein incorporation into secretory granules by nigericin. Two 150 cm^2 flasks of cells were labeled for 90 min with 1.0 mCi ^{35}S-methionine each. One flask was treated with $10^{-6}M$ nigericin throughout the labeling period. The cells were harvested and each separately diluted with 2 flasks of unlabeled cells. Secretory granules were isolated from each set by a modified procedure (see text). An equal portion of each of the six most dense fractions (out of 22 fractions total) from each gradient were TCA precipitated and analyzed by SDS-PAGE and autoradiography. Asterisks indicate polypeptides which have previously been found to copurify with secretory granules on the D_2O gradient (see Chapter 2).

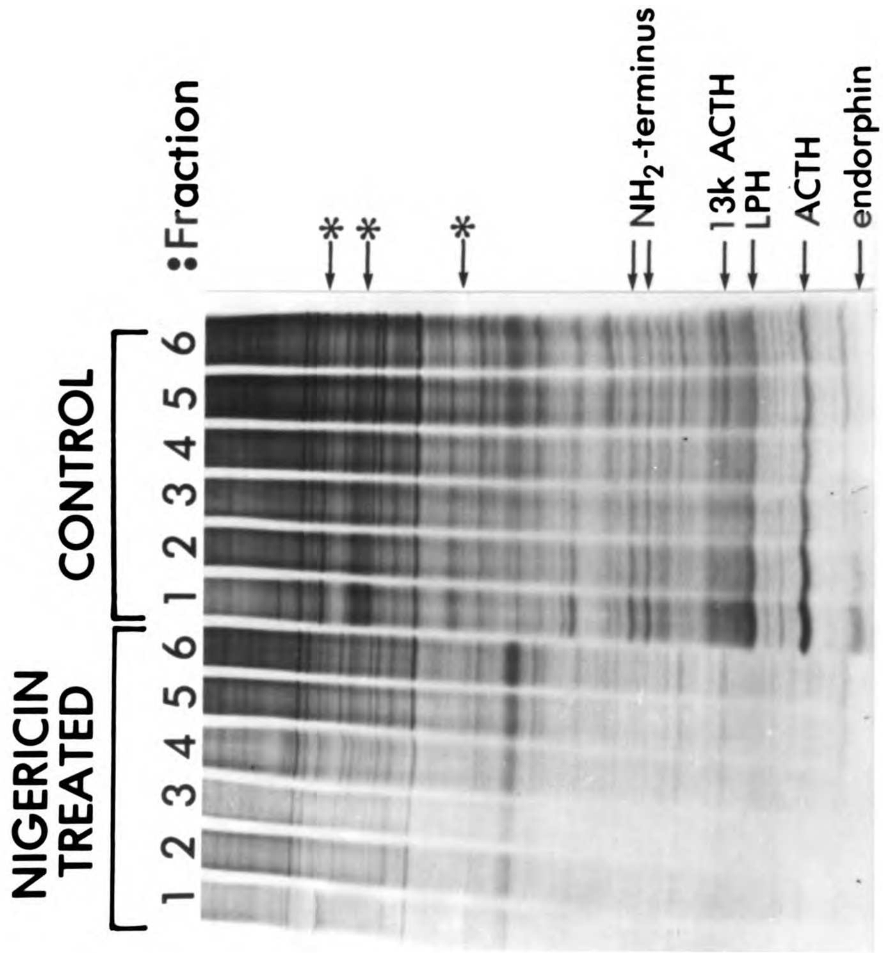
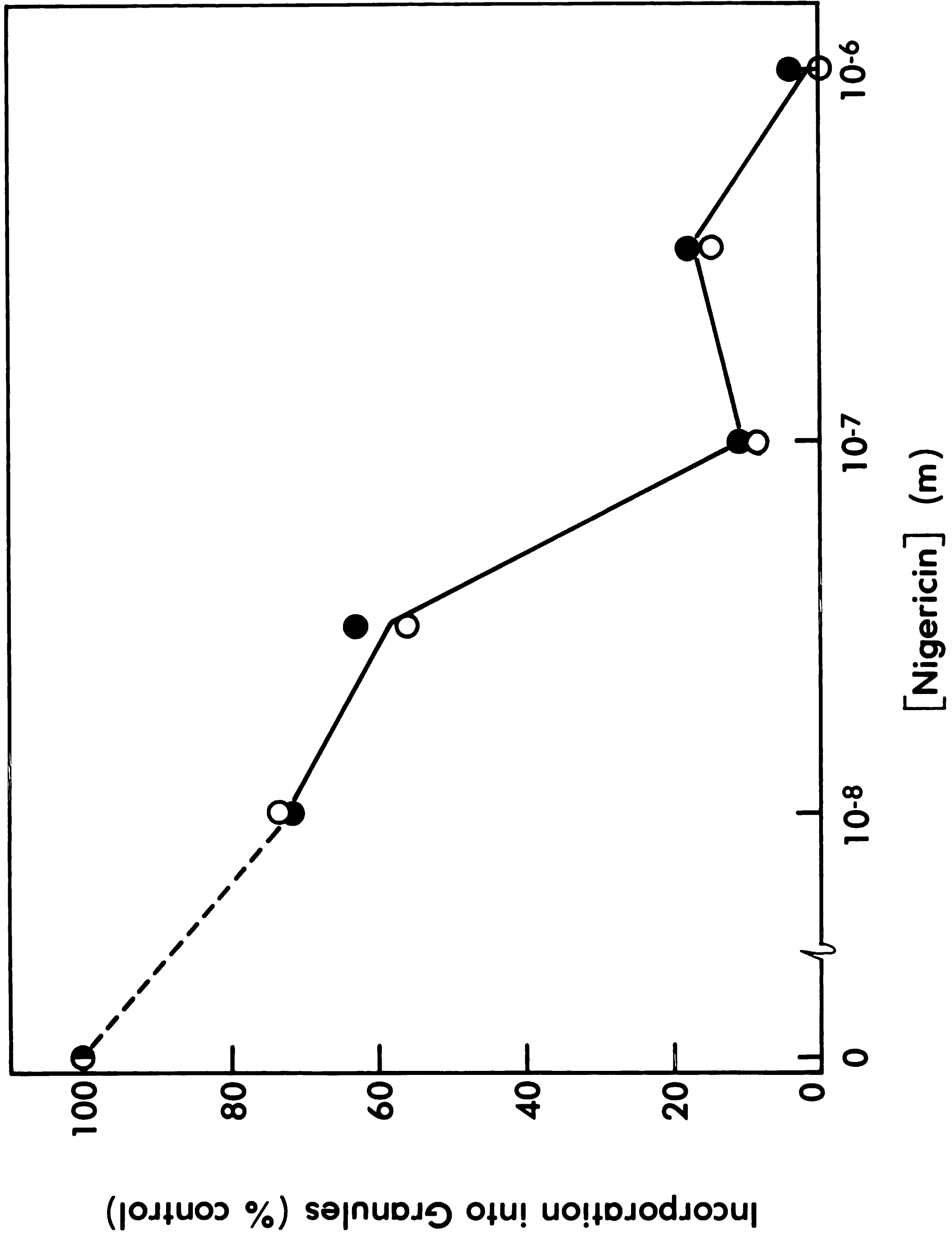

NIGERICIN
TREATED
CONTROL
1 2 3 4 5 6 1 2 3 4 5 6
: Fraction
*
*
*
NH2-terminus
13k ACTH
LPH
ACTH
endorphin

FIGURE 3. Dose dependence for the inhibition of hormone incorporation into secretory granules by nigericin. Six confluent 75 cm^2 flasks of cells were each labeled for 90 min with 300 μCi ^{35}S-methionine. Each flask contained the indicated concentration of nigericin throughout the labeling period. Secretory granules were isolated from each flask by a modified procedure using a 5 ml D_2O density gradient. The pelleted (Ficoll pad) granules were TCA precipitated and analyzed by SDS-PAGE. Autoradiograms were scanned by densitometry. The label that appeared in the LPH (●) and the ACTH (O) peptides was normalized to the amount of label that appeared in the contaminating viral nucleocapsid protein, p30.



Incorporation into Granules (% control)
100
80
60
40
20
0
0
10-8
10-7
10-6
[Nigericin] (m)

CHAPTER 5

EXPERIMENTAL PROCEDURES

1. Cell Culture

AtT-20/D-16v cell monolayers were grown in Eagle's minimal essential medium (Dulbecco's modification) supplemented with 10% horse serum and glutamine. Cells were normally used just before or at confluency. Cells were always harvested by incubating at 37°C in phosphate buffered saline (0.02M sodium phosphate, pH 7.4, 0.15M NaCl) containing 4mM ethyleneglycol-bis-β-aminoethylether)N,N'-tetraacetic acid (EGTA). They were then pelleted at low speed in the cold before use. Approximately 5×10^6 cells were obtained from each 75 cm^2 T-flask. 3T3 fibroblasts productively infected with Moloney MuLV were obtained from Dr. Charlotte Hammond, Department of Microbiology, UCSF.

2. Secretory Granule Purification

Cell pellets harvested from five to ten 75 cm^2 T-flasks were resuspended in 15 ml of cold homogenization buffer [250mM sucrose, 10mM Hepes, pH 7.4, 2mM EGTA, 1mM ethylenediamine tetraacetic acid (EDTA)]. They were then homogenized on ice with six strokes of a Kontes Dounce homogenizer (type B pestle).

Large debris and organelles were removed by pelleting at 10,000g for 5 min in a Sorvall SS34 rotor (P_1). The supernatant (S_1) was then centrifuged in the same rotor at 30,000g for 35 min to obtain a crude pellet (P_2). This pellet was resuspended in 1-2 ml of homogenization buffer by vigorous repeated pipetting with a 1 ml automatic Pipetman until it appeared as a homogeneous milky suspension. The resuspended P_2 was loaded on top of a D_2O-Ficoll density gradient. All density gradient solutions contained 250mM sucrose, 20mM KCl, 10mM Hepes, pH 7.4, 1mM ethylenediamine tetra-

acetic acid (EDTA). From bottom to top in a polyallomer centrifuge tube there was 1 ml 20% Ficoll in D_2O; 2 ml each 17%, 14%, 11% Ficoll in D_2O; 1 ml 9-10% Ficoll in D_2O; then a 29 ml linear gradient of 40% D_2O-100% D_2O was poured in which the Ficoll was constant at 8% to 10% (see below). The D_2O gradient was centrifuged at 26,000 rpm in a Beckman SW27 rotor for 12-15 hr. 1.7 ml fractions were collected by dripping through a puncture hole in the bottom of the centrifuge tube. To concentrate secretory granules, peak fractions were pooled and diluted 5 to 10 fold with iso-osmotic buffer in H_2O in order to reduce the solution density. The secretory granules were pelleted in a polyallomer tube in a Beckman SW27 rotor; centrifuging for 3 hr at 27,000 rpm.

The proper concentration of Ficoll throughout the D_2O gradient was extremely critical for effective separation of secretory granules from contaminating membranes and organelles. The effective concentration varied between 8% and 10% depending on the batch of Ficoll used. Simply monitoring the gradient solutions by refractive index was not adequate. Therefore it was necessary to adjust the concentration of Ficoll (mixing in some 20% Ficoll solution) by trying the entire secretory isolation procedure. Once adjusted, the gradient solutions worked consistently. If the concentration of Ficoll was too low, contaminating membranes overlapped considerably with the peak of secretory granules. If the concentration of Ficoll was too high, the secretory granules did not equilibrate in a distinct peak within the Ficoll step gradient at the bottom. The best separation was obtained when the major peak of light scattering material equilibrated exactly in the

middle fractions of the entire density gradient (see Fig. 2, Chapter 2).

3. Radioimmunoassay

ACTH radioimmunoassay (RIA) was performed by a modification of the procedure described by Rees et al. (1971). ACTH antiserum Violet was a gift of Dr. E. Herbert. ^{125}I-ACTH was provided by Dr. S. Hane. ACTH for standards (porcine 1-39 ACTH) was a gift of Dr. Ramachandran. ACTH and samples containing ACTH were always stored in plastic tubes and pipetted with plastic tips. RIA buffer contained 25mM sodium phosphate, pH 7.4, 0.25% bovine serum albumin and 0.5% by volume β-mercaptoethanol. Prior to assay samples were diluted into RIA buffer containing 0.5% NP40 in plastic test tubes and heated in a volume of 100-200 μl for 1 min in a 100°C temperature block. The heated samples and ACTH standards were then diluted (2 to 20 fold) with RIA buffer up to a total volume of 100 μl in conical tipped 4 ml polystyrene tubes (Sarstedt). The standard curve was obtained from 12 twofold serial dilutions, with a final concentration of 12 ng ACTH/100 μl in the first tube. To each tube 100 μl of RIA buffer containing a 1000 fold dilution of antiserum Violet (200 fold of a 5 fold diluted stock) and $\sim 3 \times 10^5$ cpm ^{125}I-ACTH/ml. The assay was incubated overnight, at least 12 hr, at 4°C. Bound and free ACTH were separated by the addition of a charcoal suspension (2 g Norit-A activated charcoal, 0.5 g dextran-Sigma No. D-4751, 10 ml horse serum and 13 ml 0.25M sodium phosphate, pH 7.4--brought to a final volume of 100 ml) that was stored at 4°C. 100 μl of charcoal solution was added to each tube beginning at time 0 and finishing in less than 5 min. At 15 min

the charcoal was pelleted at 1500g for 5 min in a swingout rotor. 200 µl of each supernate was counted to determine the amount of free ^{125}I-ACTH. Cpm were plotted as a function of log ACTH concentration for the standard curve.

The assay incubation time can be shortened significantly by a simple modification, but the RIA becomes three times less sensitive. The concentration of antiserum Violet is doubled and incubations shortened to 2-3 hr. Since the binding of ACTH to the antibodies at this time interval has not reached completion, it is important to add the antiserum to all of the samples within a 10 min period.

4. Labeling with ^{35}S-Methionine

Cells were rinsed and incubated for 20 min in modified Eagle's medium lacking methionine and serum to deplete them of methionine. For pulse labeling, cells were incubated in the same medium containing ^{35}-methionine (1000 Ci/mmol). To chase, the labeling medium was removed and the cells were rinsed once and incubated in complete modified Eagle's medium without serum. For continuous labeling periods between 1 and 3 hr, cells were incubated in medium containing 1/50 the normal amount of methionine along with the ^{35}S-methionine. The labeling volume was always equivalent to 3 ml per 75 cm^2 of cells.

5. Cell Surface Iodination

Lactoperoxidase catalyzed radioiodination of the surface of AtT-20 cells was performed by a modification of the procedure of Hynes (1973). The following iodination buffer was used for the iodination and all cell rinses: 0.15M NaCl, 0.002M $CaCl_2$, 0.03M

Hepes, pH 7.4. (Phosphate buffer causes cells to detach slowly during the course of iodination.) Monolayers of cells were iodinated in the flasks in which they were grown. Cells were rinsed in iodination buffer three times prior to iodination. Iodination was carried out in the equivalent of 3 ml iodination buffer per 75 cm^2 flask of cells either at room temperature or at 4°C (to prevent endocytosis if necessary). 3 ml of buffer containing 50 µg lactoperoxidase, 20 µl of a 40 fold dilution of glucose oxidase and 0.3 mCi Na ^{125}I was added to the cells. The reaction was initiated by the addition of 50 µl of a 6% glucose solution which had been allowed to mutarotate overnight. The reaction was continued for 20 min and then cells were rinsed 5 times in iodination buffer in which 20mM NaI replaced 20mM of NaCl.

6. Sample Preparation and Immunoprecipitation

All samples to be analyzed by immunoprecipitation were treated with iodoacetamide and phenylmethanesulfonyl fluoride (final concentration of 1mM each) to prevent proteolysis. Viral proteins were extracted directly from cell monolayers with 2 ml ice cold extraction buffer (1% Nonidet P-40, 0.5% Na deoxycholate, 0.1% SDS, 0.4M NaCl, 0.02M $NaPO_4^-$, pH 7.4) per 75 cm^2 flask. Samples were incubated 30 min on ice and then centrifuged at 15,000 rpm for 20 min in a Sorvall SS34 rotor to remove insoluble material. Prior to addition of antiserum, the extracts were preincubated with Staphylococcus aureus Cowan I (SAC) (50 µl of a 10% (w/v) suspension per ml). ACTH was extracted from whole cells with 5N acetic acid (~50 µl/cm^2 of cells). Acid extracts were frozen and thawed 3 times, incubated at 4°C overnight, centrifuged for 30 min at 12,000g to

remove debris, and lyophilized. Culture media collected from labeled cells were concentrated by lyophilization. Lyophilized samples were resuspended in extraction buffer that contained 0.1M NaCl instead of 0.4M NaCl, and precleared by centrifugation for 30 min at 12,000xg prior to addition of antisera. Secretory granule and virus pellets were resuspended directly in extraction buffer and precleared by centrifugation at 12,000xg for 30 min.

All samples (0.2-1.0 ml) were incubated with antisera for 12-15 hr at 4°C. Antisera were always added in excess to ensure complete immunoprecipitation. ACTH content was measured by radioimmunoassay to determine the amount of the ACTH antiserum required. The amount of each antiserum required to completely immunoprecipitate the viral proteins from cell extracts and culture media was determined empirically by titration of the immunoprecipitation reaction (see antisera below). Immune complexes were precipitated with 50 µl of a 10% suspension (w/v) of Staphylococcus aureus (SAC) (Kessler, 1975). The SAC was then washed 3 times in 1 ml extraction buffer and once in 1 ml distilled water. Immunoprecipitates were eluted from the SAC for gel electrophoresis by boiling for 2 min in SDS gel sample buffer.

7. Antisera

The ACTH antiserum was obtained from J. Ramachandran at UCSF and purified on an ACTH affinity column as described (Mains & Eipper, 1976). The β-endorphin antiserum and the anti-amino terminal antiserum Bridget were generous gifts of Dr. E. Herbert (University of Oregon, Eugene). The antiserum raised to Rauscher MuLV gp70, the antiserum raised to Moloney MuLV p30 and the anti-

serum raised to intact Moloney MuLV virions were obtained from the Division of Cancer Cause and Prevention, National Cancer Institute. Iodinated cells were used for titrating the amount of anti-gp70 required for cell extracts, and culture medium obtained from ^{35}S-methionine labeled cells was used to determine the amount of anti-serum required for released viral proteins. 50 μl of anti-Rauscher-gp70 (lot no. 79S-771) per 25 cm^2 flask of confluent cells completely (>95%) immunoprecipitated envelope glycoproteins from cell extracts. 20 μl of anti-intact Moloney Mulv (lot no. 77S-186) was in excess for the immunoprecipitation of viral envelope proteins released from 75 cm^2 of confluent cells over a 45 min period. 20 μl of anti-Moloney-p30 (77S-280) was sufficient to immunoprecipitate p30 from the equivalent of 10 cm^2 of confluent cells.

8. Endoglycosidase-H Digestion

Immunoprecipitates were eluted from SAC by boiling in 40 μl of 0.1M Na citrate pH 5.5 containing 1% SDS. Half of each sample was treated overnight at 37° with 3 μl of 4 μg/ml endo-H (gift of Dr. Phillip Robbins, Department of Biology, MIT). Treated and untreated samples were prepared for SDS polyacrylamide gel electrophoresis by diluting 2-fold with gel sample buffer containing 12 μl/ml of 1.5M Tris, pH 8.8.

9. Protease Digestion of Intact Cells

After ^{35}S-methionine labeling, cells in a 75 cm^2 flask were rinsed and digested with 2 ml of 1 mg/ml α-chymotrypsin in serum free culture medium for 5 min at 37°C. Phenylmethanesulfonyl fluoride was added at a final concentration of 5mM to the detached

cells to inactivate the protease. The cells were pelleted at 12,000xg for 5 min at room temperature and extracted with 1 ml extraction buffer containing 30 μg of pancreatic trypsin inhibitor.

10. Ricin-Agarose Affinity Chromatography

Samples of cell extracts and culture media (adjusted to 0.2% NP40) were loaded onto a 0.5 ml column of ricin-agarose and run at a flow rate of 20 ml/hr. The column was then washed with 5 ml of 0.2% NP40, 0.15M NaCl, 0.02M $NaPO_4$, pH 7.4. Bound material was eluted with 3% galactose in the same buffer.

11. SDS Gel Electrophoresis and Fluorography

SDS polyacrylamide slab gel electrophoresis was performed with the buffer system originally described by Laemmli (1970). Exponential gradient gels from 10-18% acrylamide were used for analysis of ACTH immunoprecipitates, and 11% acrylamide gels were used for analysis of viral proteins. Exponential gradients were formed by maintaining a constant volume in the mixing chamber which initially contained the 18% acrylamide solution. This was achieved by modifying a 50 ml syringe, using the plunger as the stopper for the mixing chamber. The 10% acrylamide solution was fed into the mixing chamber through a hole in the plunger. The 18% acrylamide solution was 11 ml and contained 12.5% glycerol to facilitate formation of the gradient. The 10% acrylamide solution was 16.5 ml. A few ml of the acrylamide solution was left over after pouring the gels. For fluorography, gels containing ^{35}S-methionine were treated with Enhance (New England Nuclear), dried and exposed to Kodak XAR-5 film at -80°C. Fluorograms were scanned with a densitometer. The film response was directly proportional to the quantity

of ^{35}S-methionine in the gel over the range used in these experiments. For detection of faint bands, the film was prefogged as described (Laskey & Mills, 1975).

12. Other Assays

Protein was determined by the Amido Schwarz staining (Schaffner & Weissmann, 1973) using bovine serum albumin as a standard. Ficoll interferes with the assay but can be degraded at low pH and high temperatures. Therefore, samples containing Ficoll were heated for 15 min at 100°C before they were spotted on the filter. For routine assays of protein samples which did not contain D_2O or detergents, the BioRad protein assay reagent was used. With a BSA standard, it resulted in similar determinations as the Amido Schwarz procedure.

Viscosities of solutions were determined with an Ostwald viscometer (capillary 90mm x 0.4mm). The viscometer was calibrated using sucrose solutions of known viscosity at 4°C.

13. Materials

^{35}S-methionine and ^{125}I were obtained from Amersham. Nonidet P40 was from Particle Data Labs (Elmhurst, Ill.). Glucose oxidase (type V) for iodination, Na deoxycholate, phenylmethanesulfonyl fluoride, pancreatic trypsin inhibitor, 8-bromoadenosine 3':5'-cyclic monophosphate (8Br-cAMP), Ficoll and Hepes (N-2-hydroxyethylpiperazine-N'-2 ethanesulfonic acid) were purchased from Sigma. α-Chymotrypsin was from Worthington and Staphylococcus aureus Cowan I cells were purchased from The Enzyme Center (Boston, Mass., product name IgSORB). Ricin-agarose (Agarose-Castor Bean Lectin-120) was obtained from P-L Biochemicals and D_2O was obtained from

BioRad. All media and reagents for cell culture were provided by the UCSF cell culture facility.

REFERENCES

Allen, R.G., Herbert, E., Hinman, M., Shibuya, H. & Pert, C.B. (1978). Coordinate control of corticotropin, β-lipotropin and β-endorphin release in mouse pituitary cell cultures. Proc. Nat. Acad. Sci. USA 75, 4972-4976.

Allen, R., Hinman, M. & Herbert, E. (1982). Forms of corticotropin and β-endorphin released by partially purified CRF in mouse anterior pituitary cultures. Endocrinology, in press.

Alonso, F.B. & Compans, R.W. (1981). Differential effect of monensin on enveloped viruses that form at distinct plasma membrane domains. J. Cell Biol. 89, 700-705.

Bainton, D.F. (1981). The discovery of lysosomes. J. Cell Biol. 91, part 2, 66s-76s.

Bainton, D.F. & Farquhar, M.G. (1970). Segregation and packaging of granule enzymes in eosinophilic leukocytes. J. Cell Biol. 45, 54-73.

Ballas, L., Bell, R.M. & Coleman, R. (1981). Lipid topogenesis: Glycerolipid synthesis occurs on cytoplasmic surface of the endoplasmic reticulum. Personal communication.

Banerjee, D., Manning, C.P. & Redman, C.M. (1976). The in vivo effect of colchicine on the addition of galactose and sialic acid to rat hepatic serum glycoproteins. J. Biol. Chem. 251, 3887-3892.

Bartelt, D. & Scheele, G. (1981). Calmodulin and calmodulin binding proteins in the exocrine pancreas. J. Cell Biol. 91,

398a.

Bennett, G., LeBlond, C.P. & Haddad, A. (1974). Migration of glycoproteins from the Golgi apparatus to the surface of various cell types as shown by autoradiography after labeled fucose injection into rats. J. Cell Biol. 60, 258-284.

Bergeron, J.J.M., Ehrenreich, J.H., Siekevitz, P. & Palade, G.E. (1973). Golgi fractions prepared from rat liver homogeniates: II. Biochemical characterization. J. Cell Biol. 59, 73-88.

Bergeron, J.J.M., Rachubinski, R.A., Sikstrom, R.A., Posner, B.I.& Paiement, J. (1982). Galactose transfer to endogenous acceptors within Golgi fractions of rat liver. J. Cell Biol. 92, 139-146.

Bergmann, J.E. (1981). Asymmetric addition of VSV glycoprotein to the Golgi apparatus. Personal communication.

Bergmann, J.E., Tokuyasu, K.T. & Singer, S.J. (1981). Passage of an integral membrane glycoprotein, the vesicular stomatitis virus glycoprotein, through the Golgi apparatus en route to the plasma membrane. Proc. Nat. Acad. Sci. USA 78, 1746-1750.

Blobel, G. (1980). Intracellular protein topogenesis. Proc. Nat. Acad. Sci. USA 77, 1496-1500.

Blobel, G. & Dobberstein, B. (1975). Transfer of proteins across membranes. J. Cell Biol. 67, 835-862.

Bolognesi, D.P., Langlois, A.J. & Schafer, W. (1975). Polypeptides of mammalian oncornaviruses. IV. Structural components of murine leukemia virus released as soluble antigens in cell culture. Virology 68, 550-555.

Braell, W.A. & Lodish, H.F. (1982). The erythrocyte anion transport protein is cotranslationally inserted into microsomes. Cell 28, 23-31.

Bretscher, M.S., Thomson, J.N. & Pearse, B. (1980). Coated pits act as molecular filters. Proc. Nat. Acad. Sci. USA 77, 4156-4159.

Bretz, R., Bretz, H. & Palade, G.E. (1980). Distribution of terminal glycosyltransferases in hepatic Golgi fractions. J. Cell Biol. 84, 87-101.

Broadwell, R.D. & Oliver, C. (1981). Golgi apparatus, GERL, and secretory granule formation within neurons of the hypothalamo-neurohypophysial system of control and hyperosmotically stressed mice. J. Cell Biol. 90, 474-484.

Broadwell, R.D., Oliver, C. & Brightman, M.W. (1979). Localization of neurophysin within organelles associated with protein synthesis and packaging in the hypothalamo-neurohypophysial system: An immunocytochemical study. Proc. Nat. Acad. Sci. USA 76, 5999-6003.

Brown, R.M. & Willison, J.H.M. (1977). Golgi apparatus and plasma membrane involvement in secretion and cell surface deposition, with special emphasis on cellulose biogenesis. In: International Cell Biology 1976-77, B.R. Brinkley & K.R. Porter, eds, Rockefeller University Press, pp. 267-283.

Camier, M., Alazard, R. & Cohen, P. (1973). Hormonal interactions at the molecular level. A study of oxytocin and vasopressin binding to bovine neurophysins. Eur. J. Biochem. 32, 207-214.

Carbonetto, S. & Fambrough, D.M. (1979). Synthesis, insertion into the plasma membrane and turnover of α-bungarotoxin receptors in chick sympathetic neurons. J. Cell Biol. 81, 555-569.

Carlson, S.S., Wagner, J.A. & Kelly, R.B. (1978). Purification of synaptic vesicles from elasmobranch electrical organ and the use of biophysical criteria to demonstrate purity. Biochemistry 17, 1188-1199.

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

Castle, J.D., Jamieson, J.D. & Palade, G.E. (1975). Secretion granules of the rabbit parotid gland. J. Cell Biol. 64, 183-210.

Cheng, H. & Farquhar, M.G. (1976). Presence of adenylate cyclase activity in Golgi and other fractions from rat liver. J. Cell Biol. 70, 681-684.

Claude, A. (1970). Growth and differentiation of cytoplasmic membranes in the course of lipoprotein granule synthesis in the hepatic cell. I. Elaboration of elements of the Golgi complex. J. Cell Biol. 47, 745-766.

Comb, M., Seeburg, P.H., Adelman, J., Eiden, L. & Herbert, E. (1982). Primary structure of the human met- and leu- enkephalin precursor and its mRNA. Nature, in press.

Cotman, C., Brown, D.H., Harrell, B.W. & Anderson, N.G. (1970). Analytical differential centrifugation: An analysis of the sedimentation properties of synaptosomes, mitochondria and

lysosomes from rat brain homogenates. Arch. Biochem. Biophys. 136, 436-447.

Creutz, C.E., Pazoles, C.J. & Pollard, H.B. (1978). Identification and purification of an adrenal medullary protein (synexin) that causes calcium-dependent aggregation of isolated chromaffin granules. J. Biol. Chem. 253, 2858-2866.

Cross, B.A., Dyball, R.E.J., Dyer, R.G., Jones, C.W., Lincoln, D.W., Morris, J.F. & Pickering, B.T. (1975). Endocrine neurons. Rec. Progr. Hormone Res. 31, 243-286.

Croze, E.M. & Morre, D.J. (1981). Flow kinetics of mouse histocompatibility antigens. Proc. Nat. Acad. Sci. USA 78, 1547-1551.

Czichi, U. & Lennarz, W.J. (1977). Localization of the enzyme system for glycosylation of proteins via the lipid-linked pathway in rough endoplasmic reticulum. J. Biol. Chem. 252, 7901-7904.

Dagorn, J.C. (1978). Non-parallel enzyme secretion from rat pancreas: in vivo studies. J. Physiol. 280, 435-448.

DeCamilli, P., Peluchetti, D. & Meldolesi, J. (1976). Dynamic changes in the lumenal plasmalemma in stimulated parotid acinar cells. A freeze-fracture study. J. Cell Biol. 70, 59-74.

deDuve, C., Pressman, B.C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat liver tissue. Biochem. J. 60, 604-617.

deDuve, C. & Wattiaux, R. (1966). Functions of lysosomes. Ann. Rev. Physiol. 28, 435-492.

Dickson, C., Eisenman, R., Fan, H. & Hunter, E. (1982). Protein biosynthesis and assembly, Chapter 6, in: Molecular Biology of Tumor Viruses, part III, RNA Tumor Viruses, eds. R. Weiss, N. Teich, H.E. Varmus & J. Coffin. Cold Spring Harbor Press, Cold Spring Harbor, N.Y., in press.

Dimond, R.L., Burns, R.A. & Jordan, K.B. (1981). Secretion of lysosomal enzymes in the cellular slime mold, Dictyostelium discoideum. J. Biol. Chem. 256, 6565-6572.

Dunphy, W.G., Fries, E., Urbani, L.J. & Rothman, J.E. (1981). Early and late functions associated with the Golgi apparatus reside in distinct compartments. Proc. Nat. Acad. Sci. USA 78, 7453-7457.

Edwards, S.A. & Fan, H. (1979). Gag-related polyproteins of Moloney murine leukemia virus: Evidence for independent synthesis of glycosylated and unglycosylated forms. J. Virol. 30, 551-563.

Ehrenreich, J.H., Bergeron, J.J.M., Siekevitz, P. & Palade, G.E. (1973). Golgi fractions prepared from rat liver homogenates. I. Isolation procedure and morphological characterization. J. Cell Biol. 59, 45-72.

Elhammer, A., Svensson, H., Autori, F. & Dallner, G. (1975). Biogenesis of microsomal membrane glycoproteins in rat liver. III. Release of glycoproteins from the Golgi fraction and their transfer to microsomal membranes. J. Cell Biol. 67, 715-724.

Erickson, A.H. & Blobel, G. (1979). Early events in the biosynthesis of the lysosomal enzyme cathepsin D. J. Biol. Chem. 254, 11771-11774.

Esmon, B., Novick, P. & Schekman, R. (1981). Compartmentalized assembly of oligosaccharides on exported glycoproteins in yeast. Cell 25, 452-460.

Fambrough, D.M. & Devreotes, P.N. (1978). Newly synthesized acetylcholine receptors are located in the Golgi apparatus. J. Cell Biol. 76, 237-244.

Farquhar, M.G. (1971). Processing of secretory products by cells of the anterior pituitary gland. Mem. Soc. Endocrinol. 19, 79-124.

Farquhar, M.G. (1978). Recovery of surface membrane in anterior pituitary cells. Variations in traffic detected with anionic and cationic ferritin. J. Cell Biol. 77, R35-R42.

Farquhar, M.G. & Palade, G.E. (1963). Junctional complexes in various epithelia. J. Cell Biol. 17, 375-412.

Farquhar, M.G. & Palade, G.E. (1981). The Golgi apparatus (complex)-1954-1981)-from artifact to center stage. J. Cell Biol. 91, part 2, 77s-103s.

Fawcett, D.W. (1966). The Cell, An Atlas of Fine Structure. W.B. Saunders Co.

Field, C. & Schekman, R. (1980). Localized secretion of acid phosphatase reflects the pattern of cell surface growth in S. cerevisiae. J. Cell Biol. 86, 123-128.

Fischer, H.D., Gonzalez-Noriega, A., Sly, W.S. & Morre, D.J. (1980). Phosphomannosyl-enzyme receptors in rat liver. J. Biol. Chem. 255, 9608-9615.

Fitting, T., Ruta, M. & Kabat, D. (1981). Mutant cells that abnormally process plasma membrane glycoproteins encoded by murine leukemia virus. Cell 24, 847-858.

Fletcher, D.J., Quigley, J.P., Bauer, G.E. & Noe, B.D. (1981). Characterization of proinsulin and proglucagon converting activities in isolated islet secretory granules. J. Cell Biol. 90, 312-322.

Franke, W.W., Luder, M.R., Kartenbeck, J. & Zerban, H. (1976). Involvement of vesicle coat material in casein secretion and surface regeneration. J. Cell Biol. 69, 173-195.

Friend, D.S. & Farquhar, M.G. (1967). Functions of coated vesicles during protein absorption in the rat vas deferens. J. Cell Biol. 35, 357-376.

Fries, E. & Rothman, J.E. (1980). Transport of vesicular stomatitis viral glycoprotein in a cell free extract. Proc. Nat. Acad. Sci. USA 77, 3870-3874.

Fries, E. & Rothman, J.E. (1981). Transient activity of Golgi-like membranes as donors of vesicular stomatitis viral glycoprotein in vitro. J. Cell Biol. 90, 697-704.

Fuchs, S., DeLorenzo, F. & Anfinsen, C. (1967). Studies on the mechanism of the enzymatic catalysis of disulfide interchange in proteins. J. Biol. Chem. 242, 398-402.

Gainer, H., Sarne, Y. & Brownstein, M.J. (1977). Biosynthesis and axonal transport of rat neurohypophysial proteins and peptides. J. Cell Biol. 73, 366-381.

Garoff, H., Frischauf, A.M., Simons, K., Lehrach, H. & Delius, H. (1980). Nucleotide sequence of cDNA coding for Semliki Forest virus membrane glycoproteins. Nature 288, 236-241.

Gasser, S.M., Ohashi, A., Daum, G., Bohni, P., Gibson, J., Reid, G.A., Yonetani, T. & Schatz, G. (1982). Imported mitochondrial proteins cytochrome b2 and cytochrome cl are processed in two steps. Proc. Nat. Acad. Sci. USA 79, 267-271.

Geisow, J., Hart, P.D. & Young, M.R. (1981). Temporal Changes of lysosome and phagosome pH during phagolysosome formation in macrophages: Studies by fluorescence spectroscopy. J. Cell Biol. 89, 645-652.

Geissler, D., Martinek, A., Margolis, R.U., Margolis, R.K., Skrivanek, J.A., Leden, R., Konig, P. & Winkler, H. (1977). Composition and biogenesis of complex carbohydrates of ox adrenal chromaffin granules. Neuroscience 2, 685-693.

Geuze, J.J. & Kramer, M.F. (1974). Function of coated membranes and multivesicular bodies during membrane regulation in stimulated exocrine pancreas cells. Cell Tissue Res. 156, 1-20.

Geuze, J.J., Slot, J.W. & Tokuyasu, K.T. (1979). Immunocytochemical localization of amylase and chymotrypsin in the exocrine pancreatic cell with special attention to the Golgi complex. J. Cell Biol. 82, 697-707.

Geuze, H.J., Slot, J.W., vanderLey, P.A. & Scheffer, R. (1981). Use of colloidal gold particles in double-labeling immunoelectron microscopy of ultrathin frozen tissue section. J. Cell Biol. 89, 653-665.

Giannattasio, G., Zanini, A. & Meldolesi, J. (1975). Molecular organization of prolactin granules. I. In vitro stability of intact and membraneless granules. J. Cell Biol. 64, 246-251.

Gibson, R., Kornfeld, S. & Schlesinger, S. (1980). A role for oligosaccharides in glycoprotein biosynthesis. Trends Biochem. Sci. 5, 290-293.

Gibson, R., Schlesinger, S. & Kornfeld, S. (1979). The nonglycosylated glycoprotein of vesicular stomatitis virus is temperature-sensitive and undergoes intracellular aggregation at elevated temperatures. J. Biol. Chem. 254, 3600-3607.

Glembotski, C.C. (1980). Post-translational processing of pro-ACTH/endorphin in rat pituitary. In: Biosynthesis, Modification and Processing of Cellular and Viral Polyproteins (eds. D. Richter & G. Koch), Academic Press, N.Y., pp. 139-150.

Glembotski, C.C. (1981). Subcellular fractionation studies on the posttranslational processing of pro-adrenocorticotropic hormone/endorphin in rat intermediate pituitary. J. Biol. Chem. 256, 7433-7439.

Godelaine, D., Spiro, M.J. & Spiro, R.G. (1981). Processing of the carbohydrate units of thyroglobulin. J. Biol. Chem. 256, 10161-10168.

Goldberg, D.E. & Kornfeld, S. (1981). The kinetics of phosphorylation of β-glucuronidase oligosaccharides in mouse macrophages. Fed. Proc. 40, 1861.

Goldman, B.M. & Blobel, G. (1978). Biogenesis of peroxisomes: Intracellular site of synthesis of catalase and uricase. Proc. Nat. Acad. Sci. 75, 5066-5070.

Goldstein, J.L., Anderson, R.G.W. & Brown, M.S. (1979). Coated pits, coated vesicles and receptor mediated endocytosis (review). Nature 279, 679-684.

Gonzalez-Noriega, A., Grubb, J.H., Talkad, V. & Sly, W.S. (1980). Chloroquine inhibits lysosomal enzyme pinocytosis and enhances lysosomal enzyme secretion by impairing receptor recycling. J. Cell Biol. 85, 839-852.

Green, J., Griffith, G., Louvard, D., Quinn, P. & Warren, G. (1981). Passage of viral membrane proteins through the Golgi complex. J. Mol. Biol. 152, 663-698.

Green, R.F., Meiss, H.K. & Rodriques-Boulan, E. (1981b). Glycosylation does not determine segregation of viral envelope proteins in the plasma membrane of epithelial cells. J. Cell Biol. 89, 230-239.

Grieder, M.H., Howell, S.L. & Lacy, P.E. (1969). Isolation and properties of secretory granules from rat islets of Langerhans. II. Ultrastructure of the β granule. J. Cell Biol. 41, 162-166.

Grinna, L.S. & Robbins, P.W. (1979). Glycoprotein biosynthesis. Rat liver microsomal glucosidases which process oligosaccharides. J. Biol. Chem. 254, 8814-8818.

Gumbiner, B. & Kelly, R.B. (1981). Secretory granules of an anterior pituitary cell line, AtT-20, contain only mature forms of corticotropin and β-lipotropin. Proc. Nat. Acad. Sci. USA 78, 318-322.

Gumbiner, B.G. & Kelly, R.B. (1982). Two distinct intracellular pathways transport secretory and membrane glycoproteins to the surface of pituitary tumor cells. Cell 28, 51-59.

Hand, A.R. & Oliver, C. (1977). Relationship between the Golgi apparatus, GERL and secretory granules in acinar cells of the rat exorbital lacrimal gland. J. Cell Biol. 74, 399-413.

Hanover, J.A. & Lennarz, W.J. (1981). Transmembrane assembly of membrane and secretory glycoproteins. Arch. Biochem. Biophys. 211, 1-19.

Hasilik, A. & Neufeld, E.F. (1980a). Biosynthesis of lysosomal enzymes in fibroblasts. J. Biol. Chem. 255, 4937-4945.

Hasilik, A. & Neufeld, E.F. (1980b). Biosynthesis of lysosomal enzymes in fibroblasts. Phosphorylation of mannose residues. J. Biol. Chem. 255, 4946-4950.

Haymovits, A. & Scheele, G.A. (1976). Cellular cyclic nucleotides and enzyme secretion in the pancreatic acinar cell. Proc. Nat. Acad. Sci. USA 73, 156-160.

Helenius, A., Kartenbeck, J., Simons, K. & Fries, E. (1980). On the entry of Semliki Forest virus into BHK-21 cells. J. Cell Biol. 84, 404-420.

Herbert, E. (1981). Discovery of pro-opiomelanocortin--a cellular polyprotein. Trends in Biochem. Sci. 6, 184-188.

Herbert, E., Allen, R.G. & Paquette, T.L. (1978). Reversal of dexamethasone inhibition of adrenocorticotropin release in a mouse pituitary tumor cell line either by growing cells in the absence of dexamethasone or by addition of hypothalamic extract. Endocrinology 102, 218-226.

Herzog, V. & Farquhar, M.G. (1977). Luminal membrane retrieved after exocytosis reaches most Golgi cisternae in secretory cells. Proc. Nat. Acad. Sci. USA 74, 5073-5077.

Herzog, V. & Miller, F. (1972). The localization of endogenous peroxidase in the lacrimal gland of the rat during postnatal development. J. Cell Biol. 53, 662-680.

Herzog, V. & Miller, F. (1979). Membrane retrieval in epithelial cells of isolated thyroid follicles. Eur. J. Cell Biol. 19, 203-215.

Herzog, V. & Reggio, H. (1980). Pathways of endocytosis from luminal plasma membrane in rat exocrine pancreas. Eur. J. Cell Biol. 21, 141-150.

Heuser, J.E. & Reese, T.S. (1973). Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. J. Cell Biol. 57, 315-344.

Hickman, S., Kulczycki, A., Lynch, R.G. & Kornfeld, S. (1977). Studies of the mechanism of tunicamycin inhibition of IgA and IgE secretion by plasma cells. J. Biol. Chem. 252, 4402-4408.

Hino, Y., Asano, A. & Sato, R. (1978). Biochemical studies on rat liver Golgi apparatus I and II. J. Biochem. (Tokyo) 83, 925-942.

Hong, K., Duzgunes, N. & Papahadjopoulos, D. (1982). Modulation of membrane fusion by calcium binding proteins. Biophys. J. 37, 297-305.

Hortnagl, H. (1976). Membranes of adrenal medulla: A comparison of membranes of chromaffin granules and endoplasmic reticulum. Neuroscience 1, 9-18.

Howell, S.L., Kostianovsky, M.K. & Lacy, P.E. (1969). β granule formation in isolated islets of Langerhans: a study by electron microscopic radioautography. J. Cell Biol. 42, 695-705.

Howell, S.L., Young, D.A. & Lacy, P.E. (1969). Isolation and properties of secretory granules from rat islets of Langerhans. III. Studies of the stability of the isolated β granule. J. Cell Biol. 41, 167-176.

Hubbard, S.C. & Ivatt, R.J. (1981). Synthesis and processing of asparagine-linked oligosaccharides. Ann. Rev. Biochem. 50, 555-583.

Hubbard, S.C. & Robbins, P.W. (1979). Synthesis and processing of protein-linked oligosaccharides in vivo. J. Biol. Chem. 254, 4568-4576.

Hymer, W.C. (1975). Fractionation of anterior pituitary secretory granules. In: The Anterior Pituitary (ed. A. Tixier-Vidal & M.G. Farquhar), Academic Press, N.Y., pp. 137-180.

Hynes, R.O. (1973). Alteration of cell surface proteins by viral transformation and by proteolysis. Proc. Nat. Acad. Sci. USA 70, 3170-3174.

Ito, A. & Palade, G.E. (1978). Presence of NADPH-cytochrome P-450 reductase in rat liver Golgi membranes. J. Cell Biol. 79, 590-597.

Ivatt, R.J. (1981). Regulation of glycoprotein biosynthesis by formation of specific glycosyltransferase complexes. Proc. Nat. Acad. Sci. USA 78, 4021-4025.

Jamieson, J.D. & Palade, G.E. (1967). Intracellular transport of secretory proteins in the pancreatic exocrine cell. J. Cell Biol. 34, 577-615.

Jamieson, J.D. & Palade, G.E. (1968). Intracellular transport of secretory proteins in the pancreatic exocrine cell. IV. Metabolic requirements. J. Cell Biol. 39, 589-603.

Jamieson, J.D. & Palade, G.E. (1971a). Condensing vacuole conversion and zymogen granule discharge in pancreatic exocrine cells: Metabolic studies. J. Cell Biol. 48, 503-522.

Jamieson, J.D. & Palade, G.E. (1971b). Synthesis, intracellular transport and discharge of secretory proteins in stimulated pancreatic exocrine cells. J. Cell Biol. 50, 135-158.

Johnson, D.C. & Schlesinger, M.J. (1980). Vesicular stomatitis virus and Sindbis virus glycoprotein transport to the cell surface is inhibited by ionophores. Virology 103, 407-424.

Johnson, L.K., Eberhardt, N.L., Spindler, S.S., Martial, J.A., Dallman, M.F., Jones, M.T. & Baxter, J.D. (1980). Regulation of the genes for ACTH and growth hormone by glucocorticoid hormones. in Endocriology 1980 (ed. I.A. Cummings, J.W. Funder & F.A.O. Meldelson), Australian Academy of Sciences, Canberra, pp. 70-73.

Kaarianen, L., Hashimoto, K., Saraste, J., Virtanen, I. & Penttinen, K. (1980). Monensin and FCCP inhibit the intracellular transport of alphavirus membrane glycoproteins. J. Cell Biol. 87, 783-791.

Kaplan, A., Achord, D.T. & Sly, W.S. (1977). Phosphohexosyl components of a lysosomal enzyme are recognized by pinocytosis

receptors on human fibroblasts. Proc. Nat. Acad. Sci. USA 74, 2026-2030.

Karshin, W.L., Arcement, L.J., Naso, R.B. & Arlinghaus, R.B. (1977). Common precursor of Rauscher leukemia virus gp69/71, p15(E) and p12(E). J. Virol. 23, 787-798.

Katona, E. (1976). Incorporation of inorganic sulfate in rat-liver Golgi. Eur. J. Biochem. 63, 538-590.

Katz, F.N., Rothman, J.E., Lingappa, V.R., Blobel, G. & Lodish, H.F. (1977). Membrane assembly in vitro: Synthesis, glycosylation and asymmetric insertion of a transmembrane protein. Proc. Nat. Acad. Sci. USA 74, 3278-3282.

Kennan, T.W. & Morre, D.J. (1970). Phospholipid class and fatty acid composition of Golgi apparatus isolated from rat liver and comparison with other cell fractions. Biochemistry 9, 19-25.

Kennan, T.W., Morre, D.J., Olson, D.E., Yunghans, W.N. & Patton, S. (1970). Biochemical and morphological comparison of plasma membrane and milk fat globule membrane from bovine mammary gland. J. Cell Biol. 44, 80-93.

Kelly, R.B., Deutsch, J.W., Carlson, S.S. & Wagner, J.A. (1979). Biochemistry of neurotransmitter release. Ann. Rev. Neurosci. 2, 399-446.

Kemmler, W., Peterson, J.D. & Steiner, D.F. (1971). Studies on the conversion of proinsulin to insulin. I. Conversion in vitro with trypsin and carboxypeptidase B. J. Biol. Chem. 246, 6786-6791.

Kemmler, W., Steiner, D.F. & Borg, J. (1973). Studies on the conversion of proinsulin to insulin. III. Studies in vitro

with a crude secretion granule fraction isolated from rat islets of Langerhans. J. Biol. Chem. 248, 4544-4551.

Kessler, S.W. (1975). Rapid isolation of antigens from cells with a Staphylococcus protein A antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. 115, 1617-1624.

Keutmann, H.T., Eipper, B.A. & Mains, R.E. (1979). Partial characterization of a glycoprotein comprising the NH_2-terminal region of mouse tumor cell pro-ACTH/endorphin. J. Biol. Chem. 254, 9204-9208.

Kiely, M., McKnight, G. & Schimke, R. (1976). Studies on the attachment of carbohydrate to ovalbumin nascent chains in hen oviduct. J. Biol. Chem. 251, 5490-5495.

Kuhn, N.J. & White, A. (1977). The role of nucleoside diphosphatase in a uridine nucleotide cycle associated with lactose synthesis in rat mammary gland Golgi apparatus. Biochem. J. 168, 423-433.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Laskey, R.A. & Mills, A.D. (1975). Quantitative film detection of ^3H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. 56, 335-341.

Leamnson, R.N., Shander, M.H.M. & Halpern, M.S. (1977). A structural protein complex in Moloney leukemia virus. Virology 76, 437-439.

Leavitt, R., Schleisinger, S. & Kornfeld, S. (1977). Impaired intracellular migration and altered solubility of nonglyco-

sylated glycoproteins of vesicular stomatitis virus and Sindbis virus. J. Biol. Chem. 252, 9018-9023.

Leduc, E.H., Avrameas, S. & Bouteille, M. (1968). Ultrastructural localization of antibody in differentiating plasma cells. J. Exptl. Med. 127, 109-118.

Lehrner, L.M. & Malacinski, G.M. (1975). Biochemical genetics of α-amylase isozymes of the chicken pancreas. Biochem. Genet. 13, 145-173.

Lingappa, V.R., Katz, F.N., Lodish, H.F. & Blobel, G. (1978). A signal sequence for the insertion of a transmembrane glycoprotein. Similarities to the signals of secretory proteins in primary structure and function. J. Biol. Chem. 253, 8667-8670.

Lodish, H.F., Braell, W.A., Schwartz, A.L., Strous, G.J.A.M. & Zilberstein, A. (1981). Synthesis and assembly of membrane and organelle proteins. in: Membrane Research, Classic Origins and Current Concepts, Internatl Rev. Cytol., suppl. No. 12 (ed. A.L. Muggleton-Harris), Academic Press, N.Y. pp. 247-309.

Loh, Y.P. & Gainer, H. (1982). Characterization of pro-opiocortin-converting activity in purified secretory granules from rat pituitary neurointermediate lobe. Proc. Nat. Acad. Sci. USA 79, 108-112.

Mains, R.E. & Eipper, B.A. (1976). Biosynthesis of adrenocorticotropic hormone in mouse pituitary tumor cells. J. Biol. Chem. 251, 4115-4120.

Mains, R.E. & Eipper, B.A. (1978). Coordinate synthesis of corticotropins and endorphins by mouse pituitary tumor cells. J.

Biol. Chem. 253, 651-655.

Mains, R.E. & Eipper, B.A. (1981a). Coordinate, equimolar secretion of smaller peptide products derived from pro-ACTH/endorphin by mouse pituitary tumor cells. J. Cell Biol. 89, 21-28.

Mains, R.E. & Eipper, B.A. (1981b). Differences in the post-translational processing of β-endorphin in rat anterior and intermediate pituitary. J. Biol. Chem. 256, 5683-5688.

Mains, R.E., Eipper, B.A. & Ling, N. (1977). Common precursor to corticotropins and endorphins. Proc. Nat. Acad. Sci. USA 74, 3014-3018.

Matthew, W.D., Tsavaler, L. & Reichardt, L.F. (1981). Identification of a synaptic vesicle specific membrane protein with a wide distribution in neuronal and neurosecretory tissue. J. Cell Biol. 91, 257-269.

Maxfield, F.R. & Tycko, B. (1981). Rapid acidification of endocytotic vesicles containing α-2-macroglobulin. J. Cell Biol. 91, 212a.

McGrath, M., Witte, O., Pincus, T. & Weissman, I.L. (1978). Retrovirus purification: Method that conserves envelope glycoprotein and maximizes infectivity. J. Virol. 25, 923-927.

McMurray, W.C. & Magee, W.L. (1972). Phospholipid metabolism. Ann. Rev. Biochem. 41, 129-160.

Melcher, B. (1981). Membrane bound ribosomes of myeloma cells. IV. Initiation of immunoglobulin mRNA translation occurs on free ribosomes. J. Cell Biol. 88, 42-50.

Meldolesi, J. (1974). Dynamics of cytoplasmic membranes in guinea pig pancreatic acinar cells. I. Synthesis and turnover of membrane proteins. J. Cell Biol. 61, 1-13.

Morre, D.J., Kartenbeck, J. & Franke, W.W. (1979). Membrane flow and interconversions among endomembranes. Biochim. Biophys. Acta 559, 71-152.

Morre, D.J., Keenan, T.W. & Mollenhauer, H.H. (1971). Golgi apparatus function in membrane transformation and product compartmentation: Studies with cell fractions isolated from rat liver. In: Advances in Cytopharmacology, vol. 1 (eds. F. Clementi & B. Ceccarelli), Raven Press, N.Y. p. 159.

Mostov, K.E., Kraehenbuhl, J.P. & Blobel, G. (1980). Receptor-mediated transcellular transport of immunoglobulin: Synthesis of secretory component as multiple and larger transmembrane forms. Proc. Nat. Acad. Sci. USA 77, 7257-7261.

Muller, W.A., Steinman, R.M. & Cohn, Z.A. (1980). The membrane proteins of the vacuolar system. II. Bidirectional flow between secondary lysosomes and plasma membrane. J. Cell Biol. 86, 304-314.

Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A.C.Y., Cohen, S.N. & Numa, S. (1979). Nucleotide sequence of cloned cDNA for bovine corticotropin-β-lipotropin precursor. Nature 278, 423-427.

Neufeld, E.F. (1981). The receptor for lysosomal enzymes on the surface of cultured fibroblasts. In: Receptor-Mediated Binding and Internalization of Toxins and Hormones. (Eds. Middlebrook & Kohn), pp. 11-14.

Neufeld, E.F., Sando, G.N., Garvin, A.J. & Rome, L.H. (1977). The transport of lysosomal enzymes. J. Supramol. Struct. 6, 95-101.

Nichols, B.A., Bainton, D.F. & Farquhar, M.G. (1971). Differentiation of monocytes. Origin, nature and fate of their azurophil granules. J. Cell Biol. 50, 498-515.

Nicholson, G.L. & Blaustein, J. (1972). The interaction of Ricinus communis agglutinin with normal and tumor cell surfaces. Biochim. Biophys. Acta 266, 543-547.

Novick, P., Ferro, S. & Schekman, R. (1981). Order of events in the yeast secretory pathway. Cell 25, 461-470.

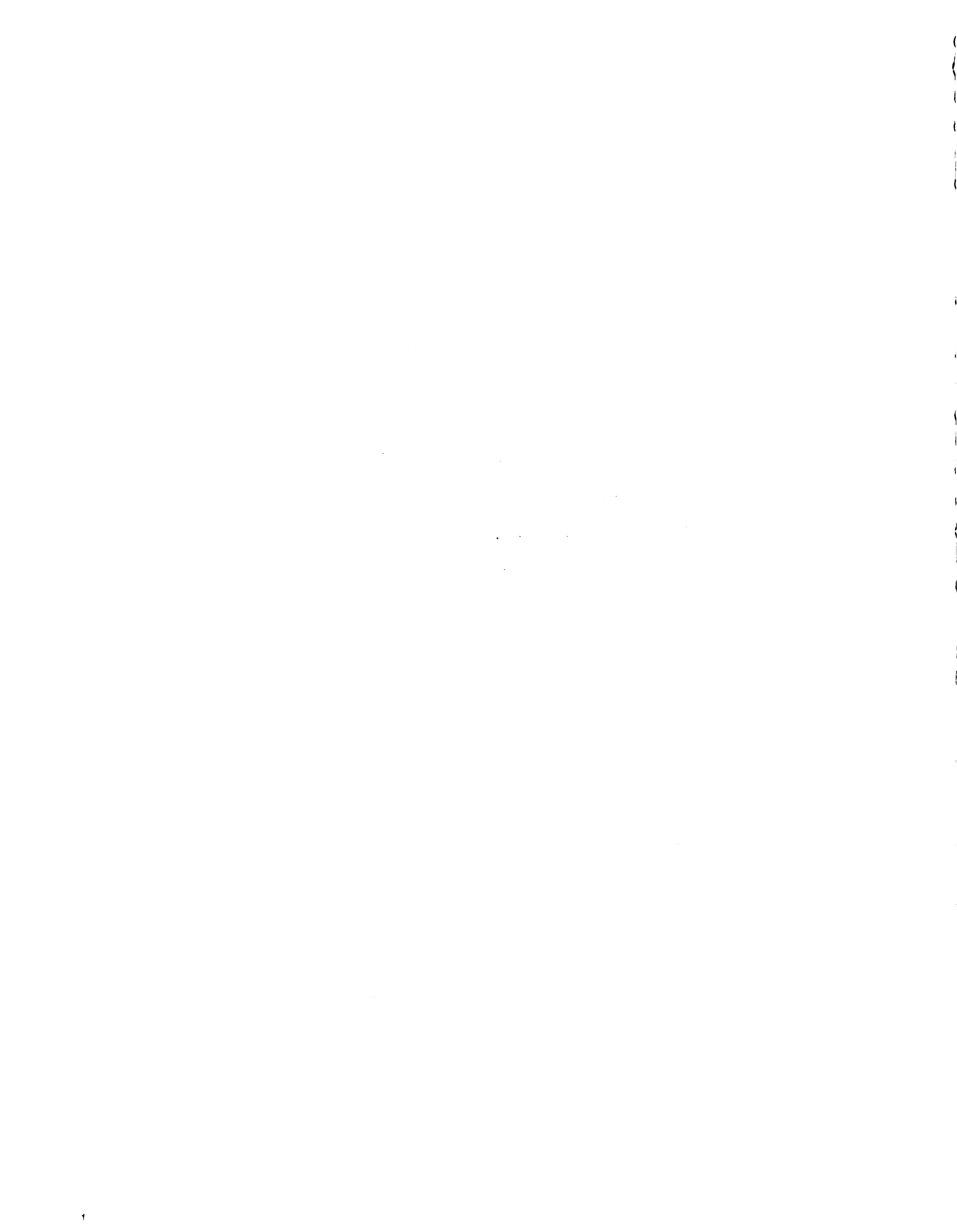
Novick, P., Field, C. & Schekman, R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell 21, 205-215.

Novikoff, A.B. (1976). The endoplasmic reticulum: A cytochemist's view (a review). Proc. Nat. Acad. Sci. USA 73, 2781-2787.

Novikoff, A.B., Mori, M., Quintara, N. & Yam, A. (1977). Studies of the secretory process in the mammalian exocrine pancreas. I. The condensing vacuole. J. Cell Biol. 75, 148-165.

Novikoff, P.M., Novikoff, A.B., Quintara, N. & Hauw, J.J. (1971). Golgi apparatus, GERL and lysosomes of neurons in rat dorsal root ganglion studied by thick section and thin section cytochemistry. J. Cell Biol. 50, 859-886.

Oliver, L., Vila-Porcile, E., Racadot, O., Peillon, F. & Racadot, J. (1975). Ultrastructure of pituitary tumor cells: A critical study. in The Anterior Pituitary (eds. A. Tixier-Vidal & M.G. Farquhar), Academic Press, N.Y., pp. 231-276.



Oliver, C. & Hand, A.R. (1978). Uptake and fate of luminally administered horseradish peroxidase in resting and isoproterenol-stimulated rat parotid acinar cells. J. Cell Biol. 76, 207-220.

Olsen, B.R., Berg, R.A., Kishida, Y. & Prockop, D.J. (1973). Collagen synthesis: Localization of prolyl hydroxylase in tendon cells detected with ferritin-labeled antibodies. Science 182, 825-827.

Orci, L., Montesana, R., Meda, P., Malaisse-Logae, F., Brown, D., Perrelet, A. & Vassalli, P. (1981). Heterogeneous distribution of filipin-cholesterol complexes across the cisternae of the Golgi apparatus. Proc. Nat. Acad. Sci. USA 78, 293-297.

Ottosen, P.D., Courtoy, P.J. & Farquhar, M.G. (1980). Pathways followed by membrane recovered from the surface of plasma cells and myeloma cells. J. Exptl. Med. 152, 1-19.

Paiement, J., Rachubinski, R.A., Ng Ying Kin, N.M.K., Sikstrom, R.A. & Bergeron, J.J.M. (1982). Membrane fusion and glycosylation in the rat hepatic Golgi apparatus. J. Cell Biol. 92, 147-154.

Palade, G. (1975). Intracellular aspects of the process of protein synthesis. Science 189, 347-358.

Palmiter, R.D., Gagnon, J. & Walsh, K.A. (1978). Ovalbumin: A secreted protein without a transient hydrophobic leader sequence. Proc. Nat. Acad. Sci. USA 75, 94-98.

Peters, T., Fleischer, B. & Fleischer, S. (1971). The biosynthesis of rat serum albumin. IV. Apparent passage through the Golgi apparatus. J. Biol. Chem. 246, 240-244.

Pfenninger, K.H. & Bunge, R.P. (1974). Freeze-fracturing of nerve growth cones and young fibers. A study of developing plasma membrane. J. Cell Biol. 63, 180-196.

Pfenninger, K.H. & Maylie-Pfenninger, M. (1981). Lectin labeling of sprouting neurons. II. Relative movement and appearance of glycoconjugates during plasmalemmal expansion. J. Cell Biol. 89, 547-559.

Phillips, M.A., Budarf, M.L. & Herbert, E. (1981). Glycosylation events in the processing and secretion of pro-ACTH-endorphin in mouse pituitary tumor cells. Biochemistry 20, 1166-1675

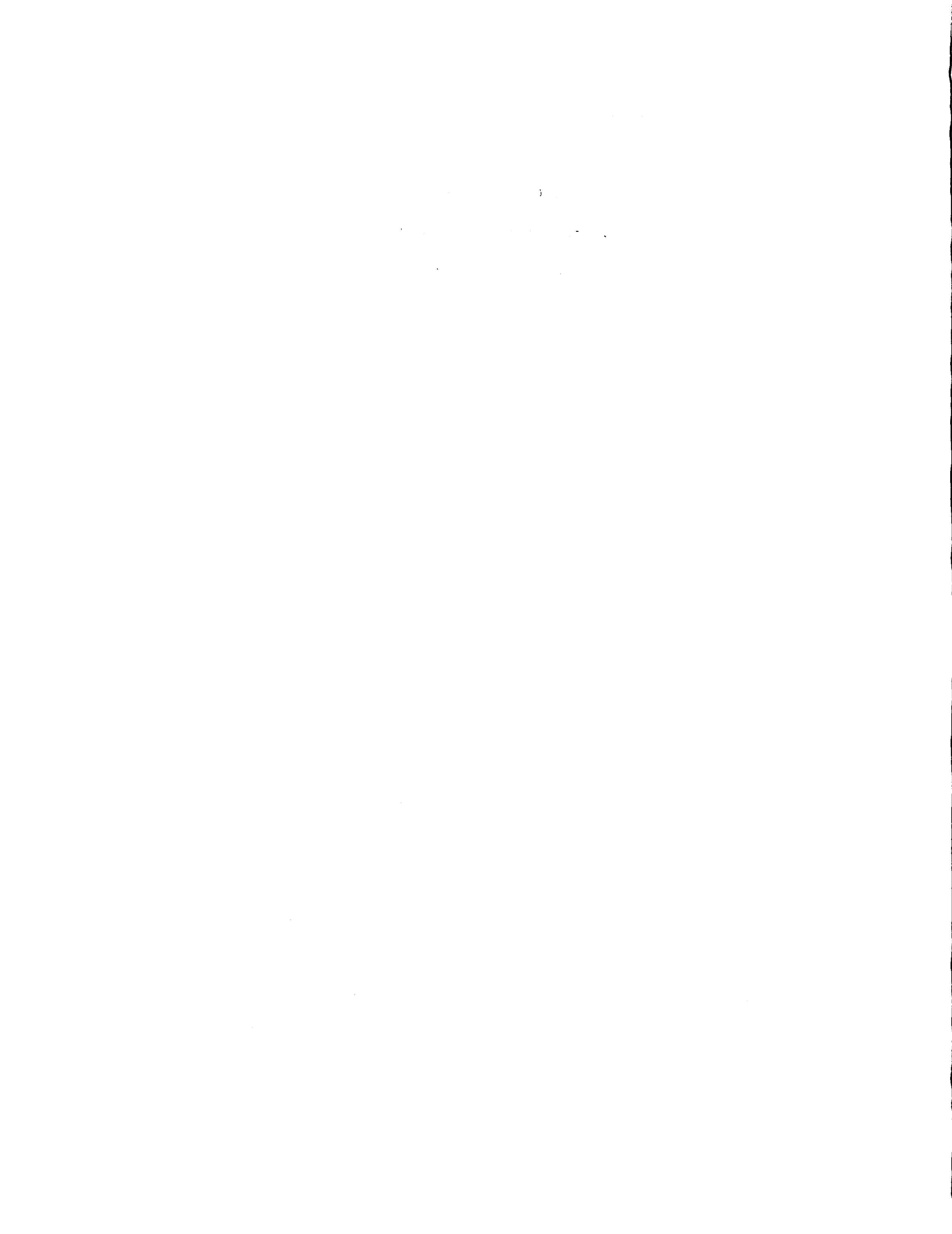
Pinto da Silva, P., Torrisi, M.R. & Kachar, B. (1981). Freeze-fracture cytochemistry: Localization of wheat germ agglutinin and concanavalin A binding sites on freeze-fractured pancreatic cells. J. Cell Biol. 91, 361-372.

Pless, D.D. & Lennarz, W.J. (1977). Enzymatic conversion of proteins to glycoproteins. Proc. Nat. Acad. Sci. USA 74, 134-138.

Porter, A.G., Barber, C., Carey, N.H., Hallewell, R.A., Threlfall, G. & Emtage, J.S. (1979). Complete nucleotide sequence of an influenza virus haemagglutinin gene from cloned DNA. Nature 282, 471-477.

Porter, C.W. & Bernacki, R.J. (1975). Ultrastructural evidence for ectoglycosyltransferase systems. Nature 256, 648-650.

Porter-Jordan, K., Johnson, R. & Fine, R.E. (1981). Role of coated vesicles in the intracellular transport of acetylcholine receptors and acetylcholinesterase. J. Cell Biol. 91, 383a.



Powell, J.T., Jarlfors, U. & Brew, K. (1977). Enzymic characteristics of fat globule membranes from bovine colostrum and bovine milk. J. Cell Biol. 72, 617-627.

Rambourg, A., Clermont, Y. & Marraud, A. (1974). Three-dimensional structure of the osmium-impregnated Golgi apparatus as seen in the high voltage electron microscope. Am. J. Anat. 140, 27-45.

Rees, L.H., Cook, D.M., Kendall, J.W., Allen, C.F., Kramer, R.M., Ratcliff, J.G. & Knight, R.A. (1971). A radioimmunoassay for rat plasma ACTH. Endocrinology 89, 254-261.

Reggio, H. & Dagorn, J.C. (1978). Ionic interactions between bovine chymotrypsinogen A and chondroitin sulfate ABC. J. Cell. Biol. 78, 951-957.

Reggio, H.A. & Palade, G.E. (1978). Sulfated compounds in the zymogen granules of the guinea pig pancreas. J. Cell Biol. 77, 288-314.

Reitman, M.L. & Kornfeld, S. (1981a). UDP-N-acetylglucosamine: Glycoprotein N-acetylglucosamine-1-phosphotransferase. J. Biol. Chem. 256, 4275-4281.

Reitman, M.L. & Kornfeld, S. (1981b). Lysosomal enzyme targeting. J. Biol. Chem. 256, 11977-11980.

Richter, J.D. & Smith, L.D. (1981). Differential capacity for translation and lack of competition between mRNAs that segregate to free and membrane-bound polysomes. Cell 27, 183-191.

Robbins, P.W., Hubbard, S.C., Turco, S.J. & Wirth, D.F. (1977). Proposal for a common oligosaccharide intermediate in the

synthesis of membrane glycoproteins. Cell 12, 893-900.

Roberts, J.L. & Herbert, E. (1977a). Characterization of a common precursor to corticotropin and β-lipotropin: Cell free synthesis of the precursor and identification of corticotropin peptides in the molecule. Proc. Nat. Acad. Sci. USA 74, 4826-4830.

Roberts, J.L. & Herbert, E. (1977b). Characterization of a common precursor to corticotropin and β-lipotropin: Identification of β-lipotropin peptides and their arrangement relative to corticotropin. Proc. Nat. Acad. Sci. USA 74, 5300-5304.

Roberts, J.L., Phillips, M., Rosa, P.A. & Herbert, E. (1978). Steps involved in the processing of common precursor forms of adreno-corticotropin and endorphin in cultures of mouse pituitary cells. Biochemistry 17, 3609-3618.

Roberts, J.L., Budarf, M.L., Baxter, J.D. & Herbert, E. (1979). Selective reduction of proadrenocorticotropin/endorphin proteins and mRNA activity in mouse pituitary tumor cells by glucocorticoids. Biochemistry 18, 4907-4915.

Rodriguez-Boulan, E. & Pendergast, M. (1980). Polarized distribution of viral envelope proteins in the plasma membrane of infected epithelial cells. Cell 20, 45-54.

Rodriguez-Boulan, E. & Sabatini, D.D. (1978). Asymmetric budding of viruses in epithelial monolayers: A model system for study of epithelial polarity. Proc. Nat. Acad. Sci. USA 75, 5071-5075.

Rome, L.H., Weissmann, B. & Neufeld, E.F. (1979). Direct demonstration of binding of a lysosomal enzyme, α-L-iduronidase, to receptors on cultured fibroblasts. Proc. Nat. Acad. Sci. USA

76, 2331-2334.

Ronzio, R.A. (1973). Glycoprotein synthesis in the adult rat pancreas. I. Subcellular distributions of UDP-galactose: glycoprotein galactosyltransferase and TPPase. Biochim. Biophys. Acta 313, 286-295.

Ronzio, R.A., Kronquist, K.E., Lewis, D.S., MacDonald, R.J., Mohrlak, S.H. & O'Donnell, J.J. (1978). Glycoprotein synthesis in the adult rat pancreas. IV. Subcellular distribution of membrane glycoproteins. Biochim. Biophys. Acta 508, 65-84.

Roseman, S. (1970). The synthesis of complex carbohydrates by multiglucosyltransferase systems and their potential function in intercellular adhesion. Chem. Phys. Lipids 5, 270-297.

Roth, S., McGuire, E.J. & Roseman, S. (1971). Evidence for cell surface glycosyltransferases. Their potential role in cellular recognition. J. Cell Biol. 51, 536-547.

Rothman, S.S. (1975). Proteins transport by the pancreas. Science 190, 747-753.

Rothman, J.E. (1981). The Golgi apparatus: two organelles in tandem. Science 213, 1212-1219.

Rothman, J.E., Bursztyn-Pettegrew, H. & Fine, R.E. (1980). Transport of the membrane glycoprotein of vesicular stomatitis virus to the cell surface in two stages by clathrin-coated vesicles. J. Cell Biol. 86, 162-171.

Rothman, J.E. & Fries, E. (1981). Transport of newly synthesized vesicular stomatitis viral glycoprotein to purified Golgi membranes. J. Cell Biol. 89, 162-168.

Rothman, J.E. & Lodish, H.F. (1977). Synchronized transmembrane insertion and glycosylation of a nascent membrane protein. Nature 269, 775-780.

Rotundo, R.L. & Fambrough, D.M. (1980). Secretion of acetylcholinesterase: Relation to acetylcholine receptor metabolism. Cell 22, 595-602.

Rubin, R.P. (1974). Calcium and The Secretory Process. Plenum Publishing Corporation, New York.

Russel, J.T. & Holz, R.W. (1981). Measurement of ΔpH and membrane potential in isolated neurosecretory vesicles from bovine neurohypophyses. J. Biol. Chem. 256, 5950-5953.

Sabatini, D.D., Kreibich, G., Morimoto, T. & Adesnik, M. (1982). Mechanism for the incorporation of proteins in membranes and organelles. J. Cell Biol. 92, 1-22.

Sabol, S.L., Ling, A. & Daniels, M.P. (1979). Endorphins of clonal pituitary tumor cells: Synthesis, storage, secretion and regulation by glucocorticoids. in Hormones and Cell Culture (eds. R. Ross & G. Sato), Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., pp. 843-853.

Salpeter, M.M. & Farquhar, M.G. (1981). High resolution analysis of the secretory pathway in mammotrophs of the rat anterior pituitary. J. Cell Biol. 91, 240-246.

Scarpa, A. & Johnson, R.G. (1976). Internal pH of isolated chromaffin vesicles. J. Biol. Chem. 251, 2189-2191.

Schaffner, W. & Weissmann, C. (1973). A rapid, sensitive and specific method for the determination of protein in dilute solution. Anal. Biochem. 56, 502-514.

Schechter, I., Burstein, Y., Zermell, R., Ziv, E., Kantor, F. & Papermaster, D.S. (1979). Messenger RNA of opsin from bovine retina: Isolation and partial sequence of the in vitro translation product. Proc. Nat. Acad. Sci. USA 76, 2654-2658.

Scheele, G., Jacoby, R. & Carner, T. (1980). Mechanism of compartmentation of secretory proteins: Transport of exocrine pancreatic proteins across the microsomal membrane. J. Cell Biol. 87, 611-628.

Scheele, G.A., Palade, G.E. & Tartakoff, A.M. (1978). Cell fractionation studies on the guinea pig pancreas. Redistribution of exocrine proteins during tissue homogenization. J. Cell Biol. 78, 110-130.

Schmidt, M. & Schlesinger, M.J. (1980). Relation of fatty acid attachment to the translation and maturation of vesicular stomatitis and Sindbis virus membrane glycoproteins. J. Biol. Chem. 255, 3334-3339.

Schneider, Y., Tulkens, P., deDuve, C. & Trouet, A. (1979). Fate of plasma membrane during endocytosis. II. Evidence for recycling (shuttle) of plasma membrane constituents. J. Cell Biol. 82, 466-474.

Schubart, U.K., Fleischer, N. & Erlichman, J. (1980). Ca^{2+}-dependent protein phosphorylation and insulin release in intact hamster insulinoma cells. J. Biol. Chem. 255, 11063-11066.

Shinnick, T.M., Lerner, R.A. & Sutcliffe, J.G. (1981). Nucleotide sequence of Moloney murine leukaemia virus. Nature 293, 543-548.

Silbert, J. & Freilich, L.S. (1980). Biosynthesis of chondroitin sulfate by a Golgi apparatus enriched preparation from cultures

of mouse mastocytoma cells. Biochem. J. 190, 307-313.

Simantov, R. (1978). Basal and potassium stimulated, calcium dependent release from pituitary cells. Life Sci. 23, 2503-2508.

Siperstein, E.R. & Miller, K.J. (1970). Further cytophysiologic evidence for the identity of the cells that produce adrenocorticotrophic hormone. Endocrinology 86, 451-486.

Sly, W.S. & Stahl, P. (1978). Receptor-mediated uptake of lysosomal enzymes. In: Transport of Macromolecules in Cellular Systems (ed. S.C. Silverstein), Dahlem Konferenzen, pp. 229-244.

Smith, R.E. & Farquhar, M.G. (1966). Lysosome function in the regulation of the secretory process in cells of the anterior pituitary gland. J. Cell Biol. 31, 319-347.

Steiner, D.F. (1976). Peptide hormone precursors: Biosynthesis, processing and significance. In: Peptide Hormones (ed. J.A. Parsons), University Park Press.

Steinhardt, R.A. & Alderton, J.M. (1982). Calmodulin confers calcium sensitivity on secretory exocytosis. Nature 295, 154-155.

Strand, M. & August, J.T. (1976). Oncornavirus envelope glycoprotein in the serum of mice. Virology 75, 130-144.

Strous, Ger J.A.M. & Lodish, H.F. (1980). Intracellular transport of secretory and membrane proteins in hepatoma cells infected by vesicular stomatitis virus. Cell 22, 709-717.

Tartakoff, A.M. (1982). Perturbation of the structure and function of the Golgi complex by monovalent carboxylic ionophores. Meth. Enzymo., in press.

Tartakoff, A. & Vassalli, P. (1977). Plasma cell immunoglobulin secretion. Arrest is accompanied by alterations of the Golgi complex. J. Exptl. Med. 146, 1332-1345.

Tartakoff, A. & Vassalli, P. (1979). Plasma cell immunoglobulin M molecules; their biosynthesis, assembly and intracellular transport. J. Cell Biol. 83, 284-299.

Tartakoff, A., Vassalli, P. & Detraz, M. (1978). Comparative studies of intracellular transport of secretory proteins. J. Cell Biol. 79, 694-707.

Tartakoff, A., Hoessli, D. & Vassalli, P. (1981). Intracellular transport of lymphoid surface glycoproteins: Role of the Golgi complex. J. Mol. Biol. 150, 525-535.

Tabas, I. & Kornfeld, S. (1978). The synthesis of complex type oligosaccharides III. J. Biol. Chem. 253, 7779-7786.

Tabas, I. & Kornfeld, S. (1979). Purification and characterization of a rat liver Golgi α-mannosidase capable of processing asparagine-linked oligosaccharides. J. Biol. Chem. 254, 11655-11663.

Tabas, I. & Kornfeld, S. (1980). Biosynthetic intermediates of β-glucuronidase contain high mannose oligosaccharides with blocked phosphate residues. J. Biol. Chem. 255, 6633-6639.

Tabas, I., Schlesinger, S. & Kornfeld, S. (1978). Processing of high mannose oligosaccharides to form complex type oligosaccharides on the newly synthesized polypeptides of the vesicular stomatitis virus G protein and the IgG heavy chain. J. Biol. Chem. 253, 716-722.

Turco, S.J., Stetson, B. & Robbins, P.W. (1977). Comparative rates of transfer of lipid-linked oligosaccharides to endogenous glycoprotein acceptors in vitro. Proc. Nat. Acad. Sci. USA 74, 4411-4414.

Vassalli, P., Lisowska-Berstein, B. & Lamm, M. (1971). Cell free synthesis of rat Ig. J. Mol. Biol. 56, 1-15.

Vila-Porcile, E. & Oliver, L. (1980). Exocytosis and related membrane events. In: Synthesis and Release of Adenohypophyseal Hormones (eds. M. Jutisz & K.W. McKerns), pp. 67-103.

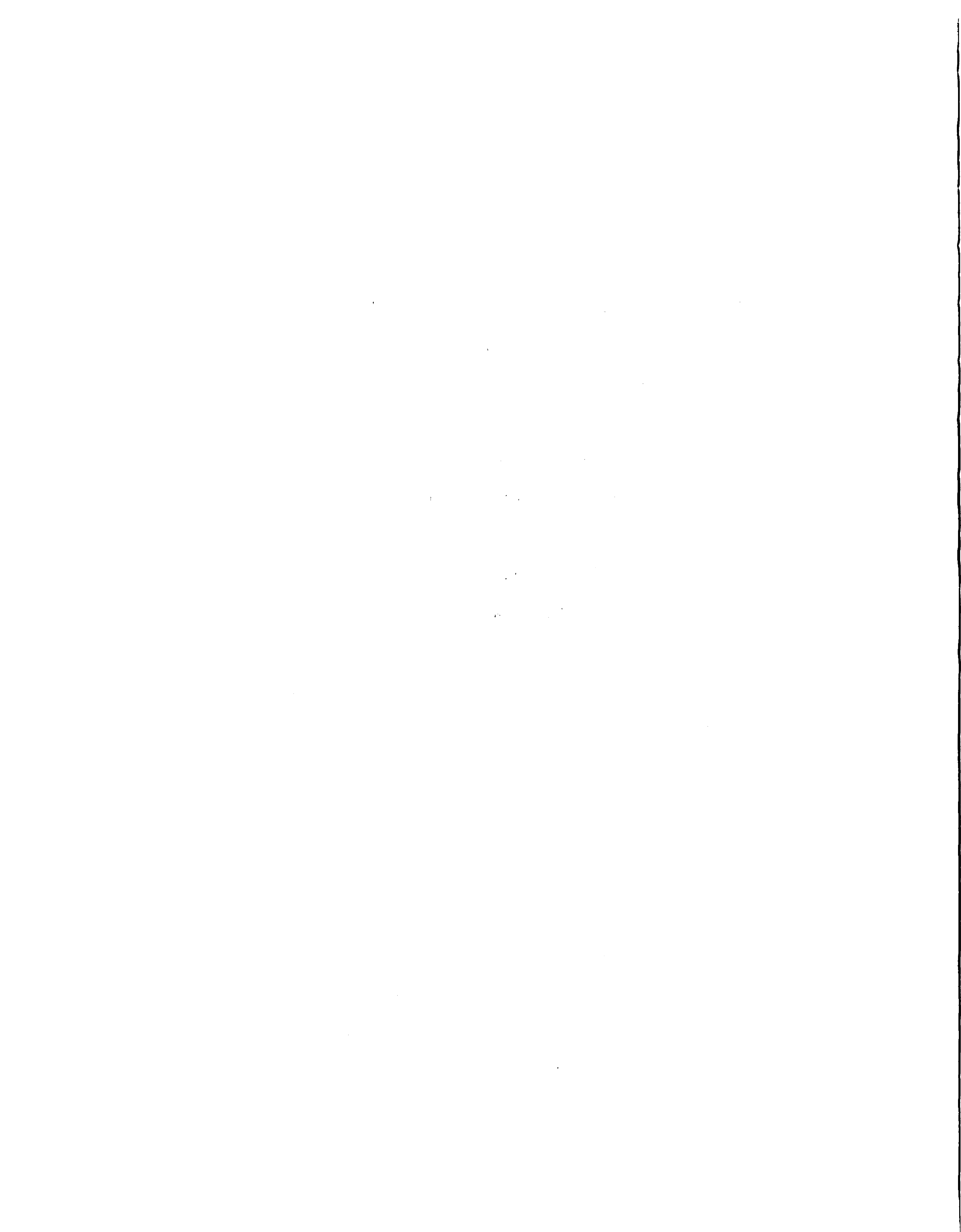
Wagner, J.A., Carlson, S.S. & Kelly, R.B. (1978). Chemical and physical characterization of cholinergic synaptic vesicles. Biochemistry 17, 1199-1206.

Waheed, A., Hasilik, A. & von Figura, K. (1981a). Processing of the phosphorylated recognition marker in lysosomal enzymes. J. Biol. Chem. 256, 5717-5721.

Waheed, A., Pohlmann, R., Hasilik, A. & von Figura, K. (1981b). Subcellular location of two enzymes involved in the synthesis of phosphorylated recognition markers in lysosomal enzymes. J. Biol. Chem. 256, 4150-4152.

Wallach, D., Kirshner, N. & Schramm, M. (1975). Non-parallel transport of membrane proteins and content proteins during assembly of the secretory granule in rat parotid gland. Biochim. Biophys. Acta 375, 87-105.

Walter, P. & Blobel, G. (1980). Purification of a membrane-associated protein complex required for protein translocation across the endoplasmic reticulum. Proc. Nat. Acad. Sci. USA 77, 7112-7116.



Walter, P. & Blobel, G. (1981). Translocation of proteins across the endoplasmic reticulum II and III. J. Cell Biol. 91, 551-561.

Walter, P., Ibrahimi, I. & Blobel, G. (1981). Translocation of proteins across the endoplasmic reticulum I. J. Cell Biol. 91, 545-550.

Willingham, M.C., Pastan, I.H., Sahagian, G.G., Jourdian, G.W. & Neufeld, E.F. (1981). Morphological study of the internalization of a lysosomal enzyme by the mannose-6-phosphate receptor in cultured Chinese hamster ovary cells. Proc. Nat. Acad. Sci. USA 78, 6967-6971.

Wilson, S.P. & Kirshner, N. (1976). Isolation and characterization of plasma membranes from the adrenal medulla. J. Neurochem. 27, 1289-1298.

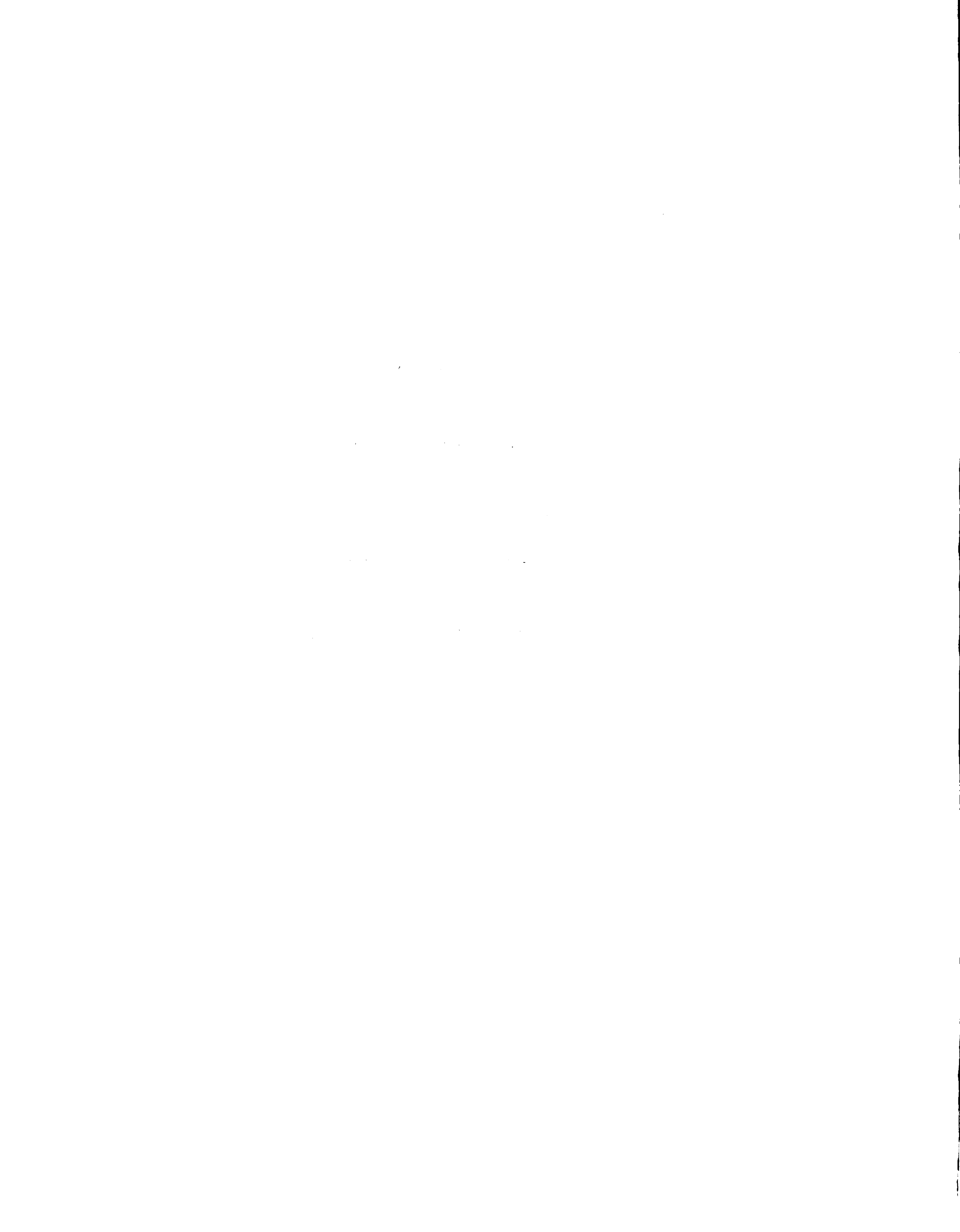
Winkler, H. (1976). The composition of adrenal chromaffin granules (review). Neuroscience 1, 65-80.

Winkler, H. (1977). The biogenesis of adrenal chromaffin granules. Neuroscience 2, 657-683.

Wirtz, K.W.A. (1974). Transfer of phospholipids between membranes. Biochim. Biophys. Acta 344, 95-117.

Witte, O.N., Tsukamoto-Adey, A. & Weissman, I.L. (1977). Cellular maturation of oncornavirus glycoproteins: Topological arrangement of precursor and product forms in cellular membranes. Virology 76, 539-553.

Witte, O.N. & Wirth, D.F. (1979). Structure of the murine leukemia virus envelope glycoprotein precursor. J. Virol. 29, 735-743.



Yamazaki, M. & Hayaishi, O. (1968). Allosteric properties of nucleoside diphosphatase and its identity with thiamine pyrophosphatase. J. Biol. Chem. 243, 2934-2942.

Yokota, S. & Fahimi, H.D. (1981). Immunocytochemical localization of albumin in the secretory apparatus of rat liver parenchymal cells. Proc. Nat. Acad. Sci. USA 78, 4970-4974.

Young, R.W. (1973). The role of the Golgi complex in sulfate metabolism. J. Cell Biol. 57, 175-189.

Zanini, A., Giannattasio, G., Nussdorfer, G., Margolis, R.K., Margolis, R.U. & Meldolesi, J. (1980). Molecular organization of prolactin granules. II. Glycosaminoglycans and glycoproteins of the matrix (and III). J. Cell Biol. 86, 260-278.

Ziemiecki, A., Garoff, H. & Simons, K. (1980). Formation of the Semliki Forest virus membrane glycoprotein complexes in the infected cell. J. Gen. Virol. 50, 111-123.

Zilberstein, A., Snider, M.D., Porter, M. & Lodish, H.F. (1980). Mutants of vesicular stomatitis virus blocked at different stages in maturation of the viral glycoprotein. Cell 21, 417-427.

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