

# Biosynthesis of Lysosomal Hydrolases: Their Synthesis in Bound Polysomes and the Role of Co- and Post-translational Processing in Determining Their Subcellular Distribution

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**ABSTRACT** By in vitro translation of mRNA's isolated from free and membrane-bound polysomes, direct evidence was obtained for the synthesis of two lysosomal hydrolases,  $\beta$ -glucuronidase of the rat preputial gland and cathepsin D of mouse spleen, on polysomes bound to rough endoplasmic reticulum (ER) membranes. When the mRNA's for these two proteins were translated in the presence of microsomal membranes, the in vitro synthesized polypeptides were cotranslationally glycosylated and transferred into the microsomal lumen. Polypeptides synthesized in the absence of microsomal membranes were ~2,000 daltons larger than the respective unglycosylated microsomal polypeptides found after short times of labeling in cultured rat liver cells treated with tunicamycin. This strongly suggests that nascent chains of the lysosomal enzymes bear transient amino terminal signals which determine synthesis on bound polysomes and are removed during the cotranslational insertion of the polypeptides into the ER membranes.

In the line of cultured rat liver cells used for this work, newly synthesized lysosomal hydrolases showed a dual destination; ~60% of the microsomal polypeptides detected after short times of labeling were subsequently processed proteolytically to lower molecular weight forms characteristic of the mature enzymes. The remainder was secreted from the cells without further proteolytic processing. As previously observed by other investigations in cultured fibroblasts (A. Gonzalez-Noriega, J. H. Grubbs, V. Talkad, and W. S. Sly, 1980, *J. Cell Biol.* 85: 839-852; A. Hasilik and E. F. Neufeld, 1980, *J. Biol. Chem.*, 255:4937-4945.) the lysosomotropic amine chloroquine prevented the proteolytic maturation of newly synthesized hydrolases and enhanced their secretion. In addition, unglycosylated hydrolases synthesized in cells treated with tunicamycin were exclusively exported from the cells without undergoing proteolytic processing. These results support the notions that modified sugar residues serve as sorting out signals which address the hydrolases to their lysosomal destination and that final proteolytic cleavage of hydrolase precursors takes place within the lysosome itself.

Structural differences in the carbohydrate chains of intracellular and secreted precursors of cathepsin D were detected from their differential sensitivity to digestion with endoglycosidases H and D. These observations suggest that the hydrolases exported into the medium follow the normal secretory route and that some of their oligosaccharides are subject to modifications known to affect many secretory glycoproteins during their passage through the Golgi apparatus.

The study of lysosomal biogenesis promises to cast light on the discriminating mechanisms which direct the subcellular traffic of proteins. In several respects, lysosomal hydrolases resemble secretory proteins and indeed in many cell lines a substantial proportion of these proteins are exported into the extracellular medium (23). It may be inferred from this fact that lysosomal enzymes, like genuine secretory proteins, are synthesized in membrane bound ribosomes and at least initially follow the secretory route (9, 52).

This notion was originally based on cytochemical observations (3, 38) demonstrating the presence of hydrolases in the lumina of the endoplasmic reticulum (ER), Golgi complex, and GERL membrane systems. It is also supported by the glycoprotein character common to lysosomal enzymes and many secretory proteins, as well as by the functional analogy which can be made between the secretory discharge which takes place on the surface of the cell and the release of lysosomal content into phagocytic vacuoles. Yet, a direct demonstration of the synthesis of lysosomal enzymes on membrane-bound polysomes has not been provided, although in cell-free systems programmed with total mRNA preparations the cotranslational insertion of cathepsin D into microsomal vesicles has been demonstrated (11).

Here we show that mRNA's for the lysosomal enzymes cathepsin D and  $\beta$ -glucuronidase are segregated intracellularly in membrane-bound polysomes and provide evidence indicating that the nascent lysosomal polypeptides contain amino-terminal insertion signals which may be functionally analogous to those determining the cotranslational insertion of secretory polypeptides into the ER membranes.

Considerable evidence suggests (19, 20, 23, 25, 48, 54) that mannosyl phosphate groups on the oligosaccharides of newly synthesized lysosomal hydrolases serve as sorting-out signals that determine that these proteins are sequestered within lysosomes. We also present experiments which strongly support the hypothesis that carbohydrate moieties play a role in diverting lysosomal hydrolases from the secretory pathway to their functional destination within lysosomes. Using tunicamycin to prevent the glycosylation of nascent polypeptides synthesized in cultured hepatocytes, we found that unglycosylated lysosomal enzymes are completely secreted from cultured cells without undergoing proteolytic cleavages that normally seem to accompany their incorporation into the organelles.

## MATERIALS AND METHODS

Most reagents were purchased from Sigma Chemical Co. (St. Louis, MO) or from Fisher Scientific Co. (Pittsburgh, PA).  $^{35}\text{S}$ -Methionine (specific activity 1,000 Ci/mmol) was purchased from New England Nuclear (Boston, MA), Trasylol from Mobay Chemical Corp. (New York, NY), oligo(dT)-cellulose from Collaborative Research Inc. (Waltham, MA). RPMI 1640 medium and horse serum were purchased from Gibco Laboratories (Grand Island Biological Co., Grand Island, NY). Wheat germ was a gift of the General Mills Corporation (Minneapolis, MI).

### Animals

Sprague-Dawley female rats (150 g) and adult New Zealand white rabbits were obtained from Taconic Farms (Germantown, NY) and Swiss female mice from the Jackson Laboratory (Bar Harbor, ME).

### Preparation of Antiserum

$\beta$ -Glucuronidase was purified from rat preputial glands (24) and cathepsin D from rat spleen (61). The proteins (1–2 mg each) emulsified in complete Freund's adjuvant were injected intradermally at 10–15 sites on the back of rabbits. A booster injection (0.5 mg) was given 3 wk later and after another week the rabbits

were bled from the marginal ear vein. Antiserum was assayed by Ouchterlony double-diffusion analysis (40), and specific antibodies were isolated by affinity chromatography (33).

### Cell Culture

A rat hepatocyte line (clone 9) was kindly provided by Dr. M. Kaighn of the Pasadena Foundation for Medical Research. Cells were cultured at 37°C in an air 5% CO<sub>2</sub> atmosphere in RPMI 1640 medium supplemented with 10% horse serum and antibiotics.

### Labeling and Preparation of Cell Extracts

Hepatocyte monolayers were grown in 35-mm dishes to a density of  $\sim 5 \times 10^6$  cells/dish. Before labeling, the cultures were rinsed with RPMI free of methionine and incubated in this medium for 1 h to allow for the depletion of the intracellular methionine pool. Cells were pulse-labeled for 1 h in methionine-free medium supplemented with 10% dialyzed horse serum and  $^{35}\text{S}$ -methionine (100  $\mu\text{Ci}/\text{ml}$ ). For pulse-chase experiments, the labeling was terminated by adding ten times the normal concentration of L-methionine in the RPMI medium.

In experiments employing tunicamycin (2  $\mu\text{g}/\text{ml}$ ) or chloroquine (25  $\mu\text{M}$ ), confluent cultures were first incubated for 1 h in complete medium with the respective drug and then placed in methionine-free media for 1 h before the pulse-chase. The drugs were present throughout the incubation and pulse-chase periods.

At different time-points medium was collected and centrifuged for 2 min in an Eppendorf Model 5412 table-top centrifuge (Brinkmann Instruments, Inc., Westbury, NY) (15,000 g). After adding 0.8% SDS and 1% Triton X-100 the sample was boiled for 2 min and then diluted with an equal volume of 50 mM Tris-HCl 7.4, 190 mM NaCl, 6 mM EDTA, 2.5% Triton X-100, 0.02% Na<sub>3</sub>N, and 100 U/ml Trasylol (solution A).

Monolayers were washed twice with Moscona's phosphate-buffered saline (36), and cells were scraped off the dish into an SDS solution (20 mM Tris-HCl, 7.4, 2% SDS) and sonicated for 10–30 s in a sonifier with a microtip (Heat System Ultrasonics, Plainview, NY). The suspension was then adjusted to 1% Triton X-100, boiled for 2 min and centrifuged (2 min, 15,000 g) in an Eppendorf 5412 to remove insolubilized material. The supernatant was diluted fourfold with solution A.

### RNA Extraction

Mouse spleens, hepatocytes, or rat preputial glands which were frozen in liquid N<sub>2</sub> and pulverized in a mortar were homogenized in a solution containing 6 M guanidine-HCl (GuHCl), 10 mM dithiothreitol, and 25 mM NaAcetate at pH 5.0. RNA was separated by centrifugation through 0.2 volume of 5.7 M CsCl<sub>2</sub> as described by Liu et al. (32).

Free and membrane-bound polysomes were prepared from mouse spleen or rat preputial gland by the procedure of Ramsey and Steele (43). Polysome pellets were rinsed with sterile water and the RNA was extracted with 6 M guanidine-HCl as described above. Poly A(+) RNA was prepared by chromatography on oligo(dT)-cellulose (2).

### Cell Free Protein Synthesis

Wheat germ extracts (34) containing 12.5  $\mu\text{Ci}$  of  $^{35}\text{S}$ -methionine and 0.03 A<sub>260</sub> U of mRNA in 0.025 ml were incubated at 25°C for 90 min.

We added dog pancreas microsomal membranes (49) at a final concentration of 2 A<sub>260</sub> U/ml. To probe for the localization of polypeptides synthesized in the presence of membranes, samples were incubated with a mixture of trypsin and chymotrypsin (80  $\mu\text{g}/\text{ml}$  of each) for 3 h at 0°C. Proteolysis was terminated by the addition of 30 U of Trasylol.

### Immunoprecipitation

Samples from translation mixtures (0.2 ml) were boiled in 2% SDS, centrifuged (2 min at 15,000 g), and diluted fourfold with solution A (16). Supernatants were incubated with affinity-purified immunoglobulins (100  $\mu\text{g}/\text{ml}$ ) at room temperature for 1 h and at 4°C for 14 h before addition of protein-A-sepharose beads. After incubation for 3 h at room temperature, the beads were sedimented, washed three times with solution A containing 0.2% SDS, and boiled for 2 min in a solution of 10% SDS, 1 M DTT, 2 mM EDTA.

### Treatment with Endoglycosidase H and Endoglycosidase D N-Acetyl Glucosaminidases

Hepatocyte monolayers ( $5 \times 10^6$  cells) in 35-mm dishes were labeled for 30 min with  $^{35}\text{S}$ -methionine. Media were collected and cellular debris were removed

by centrifugation. Monolayers were washed and cell extracts were prepared by sonication in 2% SDS as described above. After removing insoluble material by centrifugation, supernatants of cell extracts and media were diluted with 1 M citrate-phosphate buffer (pH 5.0 or pH 6.4) to a final concentration of 50 mM and incubated with endoglycosidase (endo) H ( $8 \times 10^{-4}$  U/ml) or a crude preparation of endo D (obtained from Dr. P. Atkinson, Albert Einstein College of Medicine) containing  $\beta$ -galactosidase,  $\beta$ -N-acetylglucosaminidase and trace amounts of neuraminidase (37), for 15 h at 37°C at pH 5 or 6.4, respectively. Additional neuraminidase (10 U/ml) was added to endo D-treated and control samples. Samples were then adjusted to 0.8% SDS-1% Triton X-100, boiled for 2 min, diluted with an equal volume of solution A, and processed for immunoprecipitation to be followed by gel electrophoresis and fluorography.

## RESULTS

In vitro translation experiments with mRNA's from free and membrane-bound polysomes obtained from the rat preputial gland and the mouse spleen, two rich sources of  $\beta$ -glucuronidase and cathepsin D respectively (30, 61), were carried out to determine the subcellular site of synthesis of these lysosomal hydrolases. Fig. 1 shows that only mRNA's samples extracted from bound polysomes directed the synthesis of polypeptides which were immunoprecipitated with specific antibodies against each of the lysosomal enzymes. In both cases the immunoprecipitates contained <0.1% of the total in vitro incorporated radioactivity. The polypeptide brought down with anti- $\beta$ -glucuronidase IgG from translation mixtures programmed by preputial gland mRNA was slightly smaller ( $M_r$ , 70,000; pre-pro- $\beta$ -glucuronidase, Fig. 1a) than the mature polypeptide purified from the preputial gland or the in vivo labeled rat hepatocyte enzyme ( $M_r$ , 72,000; pro- $\beta$ -glucuronidase, Fig. 1c), from which it could, however, be clearly resolved in the same gels. On the other hand, the polypeptide immunoprecipitated with antibodies against cathepsin D from systems programmed with mRNA from spleen bound polysomes was much larger ( $M_r$ , 48,000; pre-pro-cathepsin D, Fig. 1d) than the mature protein ( $M_r$ , 31,000, Fig. 1f). A similar large size-difference between mature porcine cathepsin D ( $M_r$ , 30,000) and the corresponding polypeptide ( $M_r$ , 43,000) synthesized in a system programmed with total cytoplasmic porcine spleen mRNA was observed by Erickson and Blobel (11).

The segregation of translatable mRNA's for  $\beta$ -glucuronidase and cathepsin D in bound polysomes implies that the corresponding polypeptides are cotranslationally inserted into ER membranes (1). A direct demonstration of this insertion was achieved in cell-free translation systems supplemented with microsomal membranes stripped of ribosomes, which also allowed a study of the cotranslational modifications which affect these lysosomal polypeptides (Figs. 2 and 3). When translation was carried out in the presence of membranes (Figs. 2b and 3b), but not when these were added after translation was completed (Figs. 2c and 3c), polypeptides which migrated with slightly lower mobility (corresponding to  $M_r$ , 72,000 for pro- $\beta$ -glucuronidase and 50,000 for pro-cathepsin D) than the products synthesized in the absence of membranes were generated. Since lysosomal hydrolases are glycoproteins with asparagine-linked carbohydrate (23, 54), the increase in apparent  $M_r$  observed when synthesis was carried out in the presence of microsomal membranes most likely reflects the cotranslational addition of oligosaccharide chains to the nascent peptides emerging from bound polysomes. It should be noted that, as is the case for several secretory and membrane glycoproteins (10, 31), this addition could mask a concurrent shortening of the polypeptide chains caused by removal of an amino terminal signal peptide during insertion into the ER membranes.

To establish whether the cotranslationally modified lyso-

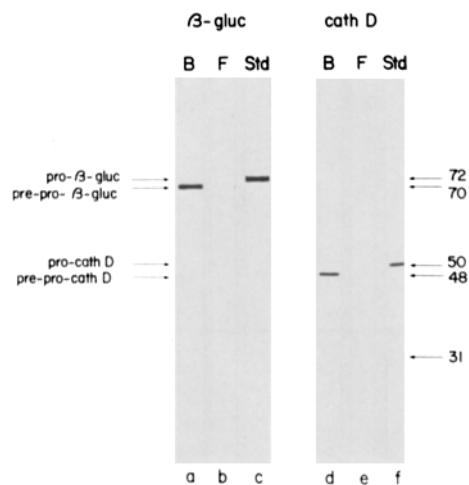


FIGURE 1 In vitro biosynthesis of lysosomal enzymes. Messenger RNA samples isolated from free (b and e) or membrane-bound polysomes (a and d) prepared from the mouse spleen (cathepsin D) or rat preputial gland ( $\beta$ -glucuronidase) were used to program wheat germ cell-free translation systems (0.06 A260 of mRNA in 50  $\mu$ l). Samples (0.2 ml) containing  $\sim 10^7$  cpm of incorporated  $^{35}$ S-methionine were used for immunoprecipitation of in vitro synthesized polypeptides with specific antibodies against cathepsin D or  $\beta$ -glucuronidase (100  $\mu$ g of IgG for each). The immunoprecipitates were analyzed by SDS PAGE (10%) followed by fluorography (72 h) of the dried slab gel. Precursors of  $\beta$ -glucuronidase and cathepsin D (c and f), which are included as standards, were labeled in rat liver hepatocytes (C1-9) for 1 h with  $^{35}$ S-methionine, recovered by immunoprecipitation, and analyzed by electrophoresis (for details, see Materials and Methods). Measurements are  $\times 10^3$ .

somal polypeptides had been transferred into the microsomal lumen or incorporated into the membranes, their accessibility to exogenous trypsin and chymotrypsin was determined. It was found (Figs. 2e and 3e) that the modified products were completely resistant to the attack of the proteases, which were added after translation in the presence of membranes had been completed. On the other hand, the unmodified polypeptides present in the same mixtures, or in mixtures which received membranes posttranslationally, were completely digested (Figs. 2d and 2f, and 3d and 3f). Furthermore, it was determined that the size of the cotranslationally modified lysosomal polypeptides was unaffected by the proteases unless detergent was added. In this case digestion proceeded to yield acid-soluble fragments (not shown), indicating that the polypeptides had been completely transferred across the membrane and segregated into the microsomal lumen, as is the case with secretory proteins (6). These results are in agreement with those of Erickson and Blobel (11), who demonstrated the cotranslational glycosylation and segregation of in vitro synthesized porcine spleen cathepsin D into microsomal vesicles.

It remained to be determined whether the vectorial discharge of  $\beta$ -glucuronidase and cathepsin D across the membranes entails the proteolytic removal of amino terminal segments, which for many products of bound polysomes serve as signals that mediate the insertion of nascent chains into the membranes (cf. Kreibich et al. [27]). To this effect, the electrophoretic mobilities of the unmodified and cotranslationally modified in vitro synthesized polypeptides were compared with the mobilities of polypeptides synthesized in vivo, which were immunoprecipitated after a short time of labeling from control cultured cells and from cells treated with tunicamycin to prevent glycosylation (29). A tissue culture system of rat hepato-

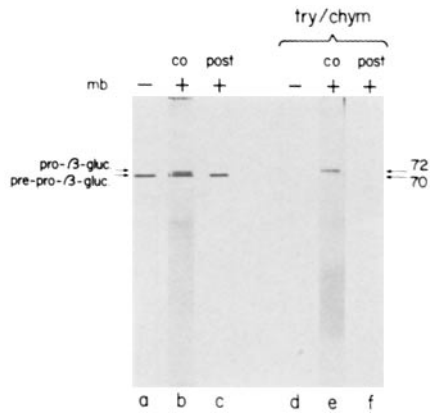


FIGURE 2 Cotranslational translocation and processing of  $\beta$ -glucuronidase by dog pancreas microsomal membranes. Messenger RNA samples isolated from the rat preputial gland were translated in wheat germ cell-free systems (0.06  $A_{260}$  RNA in 50  $\mu$ l final volume). The in vitro synthesized  $\beta$ -glucuronidase was purified by immunoprecipitation (100  $\mu$ g of anti- $\beta$ -glucuronidase IgG) from translation mixtures which contained no microsomal membranes (a and d) or to which dog pancreas microsomes (2  $A_{260}$ /ml) were added before (b and e) translation or after (c and f) translation was completed. After protein synthesis, aliquots (100  $\mu$ l) of the translation mixtures were subjected to protease treatment (d, e, and f; 80  $\mu$ g/ml each of trypsin and chymotrypsin) for 3 h at 4°C. All samples were then treated for immunoprecipitation with specific antibody, and immunoprecipitates were analyzed by electrophoresis and fluorography. Measurements,  $\times 10^3$ .

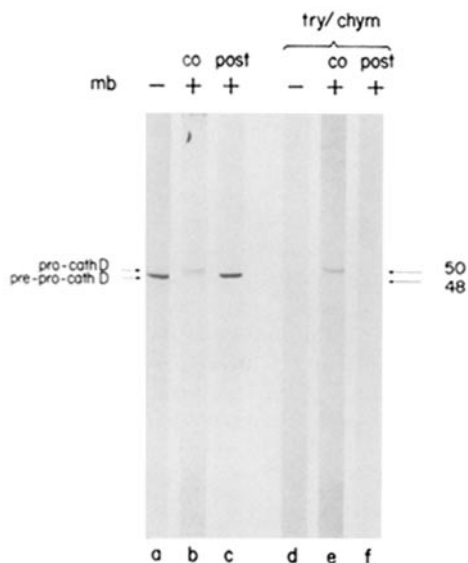


FIGURE 3 Cotranslational processing and vectorial discharge of cathepsin D. Messenger RNA samples isolated from the mouse spleen were translated in wheat germ cell-free systems (0.06  $A_{260}$  RNA in 50  $\mu$ l final volume). The in vitro synthesized cathepsin D was purified by immunoprecipitation (100  $\mu$ g of anti-cathepsin D IgG) from translation mixtures which contained no microsomal membranes (a and d) or to which dog pancreas microsomes (2  $A_{260}$ /ml) were added before (b and e) translation or after (c and f) translation was completed. After protein synthesis, aliquots (100  $\mu$ l) of the translation mixtures were subjected to protease treatment (d, e, and f; 80  $\mu$ g/ml each of trypsin and chymotrypsin) for 3 h at 4°C. All samples were then treated for immunoprecipitation with specific antibody, and immunoprecipitates were analyzed by electrophoresis and fluorography. Measurements,  $\times 10^3$ .

cytes (59) was chosen for these experiments, because in vivo synthesis of  $\beta$ -glucuronidase and cathepsin D was easily detectable in these cells by immunoprecipitation after 15 min of labeling, when the newly synthesized lysosomal enzymes should still be found within the ER. Moreover, total mRNA samples could be prepared from the cultured hepatocytes which directed the in vitro synthesis of both  $\beta$ -glucuronidase and cathepsin D. The size of the translation products could thus be directly compared with that of in vivo synthesized polypeptides produced under the direction of the same messenger. It was found (Fig. 4) that polypeptides immunoprecipitated from control cells labeled for 15 min (Fig. 4d and i) were slightly larger ( $M_r$ , 72,000 for pro- $\beta$ -glucuronidase and 50,000 for pro-cathepsin D) than the unmodified products of in vitro synthesis ( $M_r$ , 70,000 and 48,000; Fig. 4a and f). In fact, the in vivo products from control cells were indistinguishable in electrophoretic mobility from the in vitro products which were cotranslationally modified when synthesis was carried out in the presence of microsomal membranes (Fig. 4b and g). On the other hand, the unglycosylated polypeptides present in cells treated with tunicamycin (Fig. 4c and h) had a lower apparent  $M_r$  (68,000 for pro- $\beta$ -glucuronidase and 47,000 for pro-cathepsin D) than the unmodified in vitro synthesized polypeptides. These observations suggest that the primary translation products of  $\beta$ -glucuronidase and cathepsin D mRNA contain transient amino terminal insertion signals, which are cotranslationally removed from the nascent chains by a peptidase present in ER membranes. The decrease in polypeptide length caused by removal of the insertion signal is, however, normally masked by the increase in apparent molecular weight due to cotranslational glycosylation, which is prevented by tunicamycin.

#### Post-translational Events in the Synthesis of Cathepsin D and $\beta$ -Glucuronidase

Several investigators (7, 11, 18, 35) have demonstrated in different systems the existence of intracellular precursors for lysosomal enzymes, which are larger than the mature proteins and are processed proteolytically. We have followed post-translational events in the biosynthesis of cathepsin D and  $\beta$ -glucuronidase in confluent monolayers of rat hepatocytes which were labeled with  $^{35}$ S-methionine for 15 min (Fig. 4) or for 1 h (Fig. 5) and chased for different periods with an excess of unlabeled amino acid. Labeled polypeptides present in the cells or secreted in the medium during the chase were immunoprecipitated and characterized electrophoretically.

Figs. 4 and 5 show that the biosynthesis of cathepsin D in rat hepatocytes involves the production of a sequence of several intermediates of decreasing size, encompassing the product detected after 15 min of labeling ( $M_r$ , 50,000, pro-cathepsin D; Figs. 4 and 5a) and polypeptides with apparent  $M_r$  ranging from 48,000 to 31,000; Fig. 5b and e). Progression along this sequence is slow, and, even after 5 h of chase (Fig. 5e), the largest form of cathepsin D ( $M_r$ , 50,000) still represented a significant portion of the intracellular products. Derivatives smaller ( $M_r$ , 13,000–15,000) than the 31,000 polypeptide, which have been observed in other systems (18), were not detected during the first 5 h of chase although this may be due to the inability of our antibodies to precipitate such fragments. Secretion of cathepsin D was a fast process and began during the 1-h pulse (Fig. 5f). A considerable fraction of the cathepsin D polypeptide (50%) synthesized during the pulse ( $M_r$ , 50,000) was secreted in the medium during 5 h of chase. The electrophoretic mobility of the secreted polypeptide ( $M_r$ , 50,500)

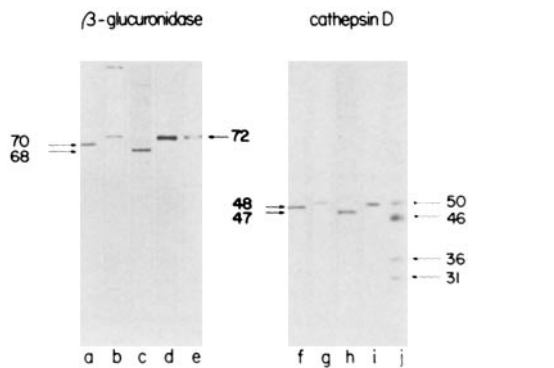


FIGURE 4 Effect of cotranslational cleavage and/or glycosylation on the apparent molecular weight of  $\beta$ -glucuronidase and cathepsin D. Messenger RNA, isolated from rat hepatocyte cultures (C1-9), was translated in a wheat germ cell-free system (0.06  $A_{260}$  RNA/50  $\mu$ l) in the presence (b and g) or absence (a and f) of dog pancreas microsomal membranes (2  $A_{260}$ /ml). The in vitro products are compared with polypeptides labeled in vivo in rat hepatocyte cultures which were incubated for 15 min with  $^{35}$ S-methionine in the presence (c and h) or absence (d, e, i, and j) of tunicamycin (2  $\mu$ g/ml) and were prepared for immunoprecipitation immediately after the pulse (c, h, d, and i) or after a 5-h chase (e and j). All samples were immunoprecipitated and analyzed by polyacrylamide gel (10%) electrophoresis followed by fluorography. Measurements,  $\times 10^3$ .

appeared, however, slightly though detectably slower than that of the corresponding intracellular form (Fig. 5f-j). It should be noted that only the largest detected cathepsin D polypeptide was exported from the hepatocytes. A similar secretion of the cathepsin D precursor has been observed with human fibroblast (18) and endothelial cells (21) although in one of these cases small amounts of the mature enzyme were detected in the medium (18).

In contrast to the relatively rapid intracellular processing of cathepsin D, labeled immunoprecipitable  $\beta$ -glucuronidase polypeptides found at the end of the pulse did not show an apparent change in electrophoretic mobility until the chase was extended beyond 5 h (Fig. 5k-o). After 8 h of incubation in cold medium a slightly smaller ( $M_r$ , 71,000) form of  $\beta$ -glucuronidase was detected, which accumulated slowly during the next 12 h. These observations confirm the reports of Swank and his associates (7), who first detected a small change in size during the maturation of  $\beta$ -glucuronidase in mouse kidney and fibroblasts. The change involved in the maturation of  $\beta$ -glucuronidase (1,000 daltons) in rat hepatocytes is, however, even smaller than the one reported with other systems (1,500-3,000 daltons). It should be noted (Fig. 5p-t) that, as was the case with cathepsin D, secretion of  $\beta$ -glucuronidase was much faster than intracellular maturation by proteolytic processing. A fraction of the newly synthesized enzyme detected at the end of the pulse began to be secreted into the medium without detectable processing as soon as 3 h after the pulse. The mature form of  $\beta$ -glucuronidase (71,000) which is seen intracellularly after 8 h of chase was not secreted even when incubation was prolonged to 20 h when up to 40% of the pulse-labeled product was found in the medium.

#### Enhancement of the Secretion of Cathepsin D and $\beta$ -Glucuronidase in Cells Treated with Chloroquine

Previous studies have shown that lysosomotropic amines such as chloroquine enhance the secretion of lysosomal en-

zymes by cultured fibroblasts (17, 18) and impair the intracellular processing of newly synthesized hydrolases (18). Figs. 6 and 7 demonstrate that chloroquine had a dramatic effect on the secretion of cathepsin D and  $\beta$ -glucuronidase by rat hepatocytes. When these cells were incubated with the drug throughout the pulse and chase periods, conversion of the labeled precursors ( $M_r$ , 50,000 and 72,000 for cathepsin D and  $\beta$ -glucuronidase, respectively) to lower molecular weight forms was abolished (Figs. 6 and 7i-l) and secretion of the precursors was accelerated (Figs. 6 and 7u-x). While control cells, even during prolonged chases (up to 20 h), exported to the medium no >50% of the newly synthesized enzymes, chloroquine-treated cells were almost totally devoid of labeled hydrolases after 5 h of chase. The complete secretion of unprocessed precursors caused by chloroquine reinforces the suggestion that final proteolytic processing of lysosomal enzymes takes place within the lysosomes (18).

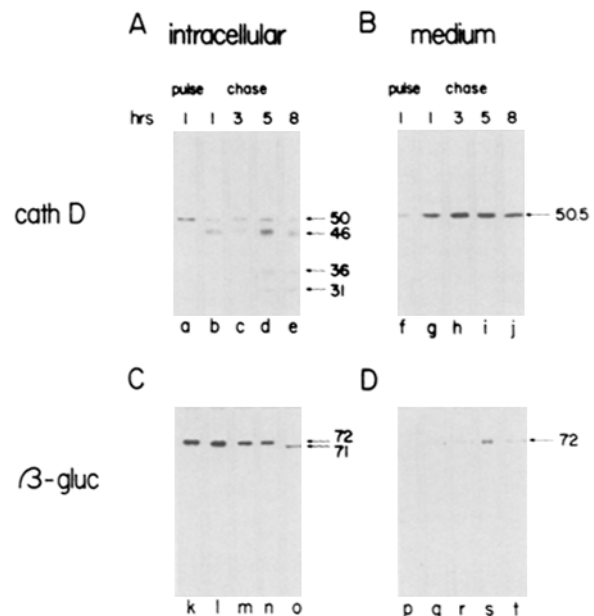
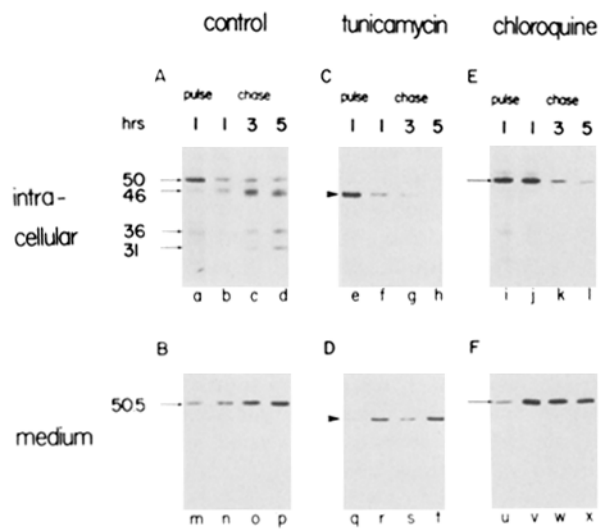


FIGURE 5 Post-translational events in the synthesis of cathepsin D and  $\beta$ -glucuronidase. Confluent monolayers of rat hepatocytes were labeled with  $^{35}$ S-methionine for 1 h and chased for different times with an excess of the unlabeled amino acid. Labeled polypeptides present in the cells or secreted in the medium during the chase were immunoprecipitated (100  $\mu$ g/ml IgG) and characterized electrophoretically. (A) Intracellular cathepsin D immunoprecipitated immediately following a 1-h pulse (a) or after a chase of 1, 3, 5, or 8 h (b, c, d, and e). Conversion of the pro-cathepsin D (a, 50,000 daltons) to the 31,000-dalton cathepsin D occurs through a number of intermediates (b, c, d, and e, 46,000 and 36,000 daltons) during  $\sim$ 5 h. (B) Immunoprecipitated cathepsin D secreted during a 1-h pulse (f) or during a chase of 1, 3, 5, or 8 h (g, h, i, and j). The secreted polypeptide (50,500 dalton) has an electrophoretic mobility slightly slower than that of the corresponding intracellular form (50,000 daltons). (C) Intracellular  $\beta$ -glucuronidase immunoprecipitated immediately following a 1-h pulse (k) or after a chase of 1, 3, 5, or 8 h (l, m, n, and o). In contrast to cathepsin D, an apparent change in electrophoretic mobility did not appear until the chase was extended beyond 5 h (lane o, 71,000 dalton). (D) Immunoprecipitated  $\beta$ -glucuronidase secreted during a 1-h pulse (p) or during a chase of 1, 3, 5, or 8 h (q, r, s, and t). A fraction of the newly synthesized polypeptide detected at the end of the pulse began to be secreted into the medium as soon as 3 h after the chase (lanes r, s, and t, 72,000 daltons). The mature form of  $\beta$ -glucuronidase was not secreted.

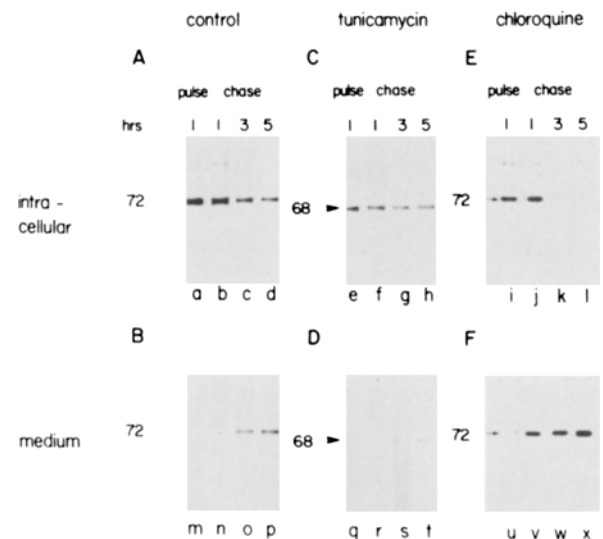


**FIGURE 6** Effect of tunicamycin and chloroquine on the kinetics of intracellular processing and secretion of cathepsin D. Confluent monolayers of rat hepatocytes were labeled for 1 h with  $^{35}\text{S}$ -methionine and chased for different periods with an excess of the unlabeled amino acid. Cultures were incubated with tunicamycin ( $2\ \mu\text{g}/\text{ml}$ ) or chloroquine ( $25\ \mu\text{M}$ ) for 1 h before the pulse, and the drugs were present throughout the pulse-chase periods. Labeled polypeptides present in the cells (A, C, and E) or secreted in the medium (B, D, and F) during the chase were immunoprecipitated ( $100\ \mu\text{g}/\text{ml}$  anti-cathepsin D IgG) and characterized electrophoretically. Intracellular (A) and secreted (B) cathepsin D immunoprecipitated following a 1-h pulse (a and m) and chased for 1, 3, or 5 h (b, c, d; n, o, p). Conversion of the pro-cathepsin D (a, 50,000 dalton) to the 31,000-dalton cathepsin D occurs through a number of intermediates (b, c, and d; 46,000 and 36,000 daltons) during  $\sim 5$  h. Only the unprocessed precursor is secreted (m, n, o, and p). Intracellular (C) and secreted (D) cathepsin D immunoprecipitated following a 1-h pulse (e and q) and chased for 1, 3, or 5 h (f, g, h; r, s, t) in the presence of tunicamycin. The unglycosylated form of pro-cathepsin D (e; 47,000 dalton) was not processed to any lower molecular weight derivatives (f, g, and h), and the intracellular concentration continuously decreased throughout the chase in parallel with an accumulation in the medium (r, s, t). Intracellular (E) and secreted (F) cathepsin D immunoprecipitated following a 1-h pulse (i and u) and chased for 1, 3, or 5 h (j, k, l; v, w, x) in the presence of chloroquine. Conversion of the pro-cathepsin D precursor (i, 50,000 daltons) to lower molecular weight forms was abolished, and the intracellular concentration of pro-cathepsin D decreased throughout the chase period with a concomitant increase in the concentration of pro-cathepsin D secreted in the medium (v, w, and x, 50,500 daltons).

### Sensitivity of Intracellular and Secreted Precursors of Lysosomal Hydrolases to Digestion by Endo H and D

An examination of the sensitivity to digestion by endo H and D provided a means to detect structural differences in the carbohydrate side chains of intracellular and secreted precursors of cathepsin D. The fact that the intracellular precursors of lysosomal hydrolases have high-mannose oligosaccharide chains (54) was corroborated by the complete sensitivity of the cathepsin D precursor to digestion with endo H. This enzyme reduced the apparent molecular weight of pro-cathepsin D from 50,000 to 48,500 (Fig. 8b). Furthermore, incubation with endo D (an enzyme which cleaves off oligosaccharides with five or less mannose residues from which terminal sugars have been removed) did not affect the size of the intracellular

cathepsin D precursor (Fig. 8f). On the other hand, digestion of the secreted precursor (50,500 dalton) with endo H showed that this material represents a mixture of polypeptides which differed in the extent and type of oligosaccharide processing. While some of the secreted cathepsin D chains behaved like the intracellular precursors in their sensitivity to endo H, decreasing in size to 48,500 dalton, others decreased only to 50,000 dalton (Fig. 8d). A likely explanation for these findings is that each secreted polypeptide molecule may contain more than one type of oligosaccharide chain and that, while in some polypeptides all chains are susceptible to endo H, in others some oligosaccharides are resistant to this glycosidase, which therefore only causes a smaller reduction in molecular size. In this case it may be expected that at least some of the oligosaccharides resistant to endo H may be sensitive to digestion with endo D. Indeed, incubation with this glycosidase showed that, in contrast to the polypeptides found intracellularly, which



**FIGURE 7** Effect of tunicamycin and chloroquine on the kinetics of intracellular processing and secretion of  $\beta$ -glucuronidase. Confluent monolayers of rat hepatocytes were labeled for 1 h with  $^{35}\text{S}$ -methionine and chased for different periods with an excess of the unlabeled amino acid. Cultures were incubated with tunicamycin ( $2\ \mu\text{g}/\text{ml}$ ) or chloroquine ( $25\ \mu\text{M}$ ) for 1 h before the chase, and the drugs were present throughout the pulse-chase periods. Labeled polypeptides present in the cells (A, C, and E) or secreted in the medium (B, D, and F) during the chase were immunoprecipitated ( $100\ \mu\text{g}/\text{ml}$  anti- $\beta$ -glucuronidase) and characterized electrophoretically. Intracellular (A) and secreted (B)  $\beta$ -glucuronidase immunoprecipitated after a 1-h pulse (a and m) and chased for 1, 3, or 5 h (b, c, d; n, o, p). No change in electrophoretic mobility was observed through 5 h of chase (72,000 daltons). Only when the chase was extended beyond 5 h (Fig. 5, lane o, 71,000 daltons) was the conversion to the mature form seen. In contrast to cathepsin D (Fig. 6 m, n, o, and p),  $\beta$ -glucuronidase secretion was slower and appeared after 3 h of chase (o). Only the unprocessed form of  $\beta$ -glucuronidase was secreted (72,000 daltons). Intracellular (C) and secreted (D)  $\beta$ -glucuronidase immunoprecipitated after a 1-h pulse (e and q) and chased for 1, 3, or 5 h (f, g, h; r, s, t) in the presence of tunicamycin. The unglycosylated form of pro- $\beta$ -glucuronidase (e, 68,000 daltons) decreased throughout the chase and began to accumulate in the medium (r, s, and t, 68,000 daltons). Intracellular (E) and secreted (F)  $\beta$ -glucuronidase immunoprecipitated after a 1-h pulse (i and u) and chased for 1, 3, or 5 h (j, k, l; v, w, x) in the presence of chloroquine. The intracellular concentration of pro- $\beta$ -glucuronidase decreased throughout the chase period with a concomitant increase in the concentration of pro- $\beta$ -glucuronidase secreted in the medium (v, w, and x, 72,000 daltons).

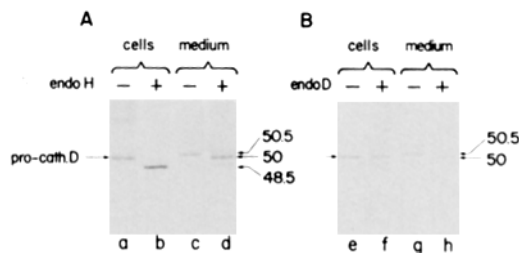


FIGURE 8 Sensitivity of cathepsin D to endoglycosidase H or endoglycosidase D. Confluent monolayers of rat hepatocytes were labeled during 30 min with  $^{35}\text{S}$ -methionine, and the cell extracts (a, b, e, and f) and medium (c, d, g, and h) were kept as controls (a, c, e, and g) or treated with endoglycosidase H ( $8 \times 10^{-4}$  U/ml; 15 h at  $37^\circ\text{C}$ ); b, d) or a crude preparation of endoglycosidase D (15 h  $37^\circ\text{C}$ ; f and h) before immunoprecipitation with anti-cathepsin D IgG (100  $\mu\text{g}/\text{ml}$ ) as described in Materials and Methods. Intracellular pro-cathepsin D incubated with endoglycosidase H (b) showed an increase in electrophoretic mobility (48,500 daltons). Incubation of secreted pro-cathepsin D (50,500 daltons) with endoglycosidase H gave rise to two polypeptides of  $M_r$ , 48,500 and to 50,000 daltons (d).

were all resistant to endo D, all those in the extracellular medium contained oligosaccharides susceptible to this glycosidase (Fig. 8h).

#### *Inhibition of Post-translational Processing and Secretion of Unglycosylated Lysosomal Hydrolases in Cells Treated with Tunicamycin*

Considerable evidence has accumulated (19, 20, 23, 25, 48, 54) suggesting that phosphomannosyl groups in oligosaccharide chains of newly synthesized lysosomal enzymes serve as recognition markers which mediate the transfer of the enzymes to lysosomes. It was therefore of interest to determine the fate of the unglycosylated lysosomal polypeptides, which were identified after short times of labeling in cells treated with tunicamycin. As seen in Figs. 6 and 7 the behavior of the unglycosylated forms of pro-cathepsin D ( $M_r$ , 47,000) and pro- $\beta$ -glucuronidase ( $M_r$ , 68,000) after a labeling pulse differed from that of the normal polypeptides synthesized in control cells in two respects: (a) the unglycosylated forms were not processed to any lower molecular weight derivatives (Figs. 6 and 7f-h). (b) their intracellular concentration continuously decreased throughout the chase, in parallel with their accumulation in the extracellular compartment, so that eventually complete (pro-cathepsin D), or almost complete (pro- $\beta$ -glucuronidase) secretion of the unglycosylated lysosomal polypeptides occurred (Figs. 6 and 7q-t). These observations are consistent with the operation of a mechanism in which carbohydrate chains bearing phosphomannosyl groups are necessary for the sorting-out of lysosomal hydrolases from other proteins contained in the ER (13, 18). The enhancement of secretion caused by tunicamycin stresses the considerable overlap which must exist between the early stages of the secretory pathway and the pathway leading to lysosomes.

#### DISCUSSION

The above results demonstrate directly that mRNA's for the lysosomal enzymes cathepsin D and  $\beta$ -glucuronidase are translated on polysomes bound to ER membranes. For cathepsin D this had already been inferred by Erickson and Blobel (11) from their finding that addition of microsomal vesicles to cell-free systems programmed with total porcine spleen mRNA

leads to the cotranslational modification of the in vitro synthesized polypeptide and to its sequestration within the microsomal lumen. In agreement with these observations we found that lysosomal polypeptides transferred into the microsomal lumen in vitro were electrophoretically indistinguishable from precursors of the mature enzymes which were labeled during short radioactive pulses in vivo and can therefore be presumed to be contained within the ER lumen. As would be expected if the lysosomal polypeptides undergo glycosylation during their transfer across the microsomal membrane, the putative microsomal forms of  $\beta$ -glucuronidase and cathepsin D immunoprecipitated from cultured hepatocytes were of larger apparent molecular weight than the primary translation products synthesized in vitro in the absence of membranes. On the other hand, the corresponding unglycosylated polypeptides immunoprecipitated from cells treated with tunicamycin were smaller than the unmodified products of in vitro translation. This strongly suggests that cotranslational processing of the lysosomal hydrolases includes, in addition to core glycosylation, a shortening of the nascent polypeptide, which can only occur by removal of an amino terminal segment. Similar observations using tunicamycin first revealed the presence of transient amino terminal insertion signals in membrane glycoproteins (10), for which the decrease in polypeptide length caused by signal removal is masked by an overall gain in size caused by core glycosylation. It is worth noting that removal of amino terminal segments which may serve as insertion signals is not an obligatory step during the synthesis of all polypeptides made on bound ribosomes. Indeed, original amino terminal segments have been found to be retained in secretory (42), ER (4), and plasma membrane proteins (8), and the possibility cannot be discarded that such segments may be conserved in some lysosomal proteins.

Taken together, however, the observations just discussed suggest that insertion of lysosomal hydrolases into the ER membrane is a process completely analogous to the vectorial discharge of secretory proteins into the microsomal lumen. Of course, amino acid sequence determinations are necessary to establish whether amino terminal signals in prelysosomal and presecretory proteins are structurally equivalent. This seems likely, since signals in secretory and membrane proteins show striking similarities and both classes of polypeptides appear to use the same microsomal translocation apparatus (14, 26, 27).

Several investigators (18, 35, 50) have reported that lysosomal enzymes are synthesized as larger molecular weight precursors which only after post-translational proteolytic processing yield the mature forms found in lysosomes. As reported for human placenta (5) and porcine spleen (11, 12), the biosynthesis of cathepsin D (31,000 daltons) in rat hepatocytes was followed by the production of a series of intermediates derived by successive cleavages from a large microsomal precursor (50,000 daltons). On the other hand, only a single post-translational proteolytic cleavage product was detected after the biosynthesis of  $\beta$ -glucuronidase. This appeared  $>5$  h after the polypeptide was synthesized and represented an even smaller change in molecular weight than those reported for  $\beta$ -glucuronidase in mouse kidney and macrophages (7, 51).

The functional significance of the proteolytic processing steps which follow the biosynthesis of lysosomal enzymes is yet unclear. Removal of peptide segments does not appear to be required for the activation of many hydrolases which are secreted as enzymatically active precursors (18), although processing may be required for the activation of cathepsin D.

Furthermore, trimming of precursors is a slow process which takes place several hours after synthesis; thus, even though segments removed by proteolysis may be important in guiding the newly synthesized hydrolases to their destination, it appears unlikely that their removal takes place along the pathway which leads to the lysosomes. Indeed, the fact that chloroquine, a drug which impairs intralysosomal digestion by raising the lysosomal pH (39, 60), completely prevented processing of the newly synthesized hydrolases supports the suggestion, made by Hasilik and Neufeld (18) on the basis of similar observations with human fibroblasts, that trimming of the precursors takes place only after their arrival in the lysosomes.

The fact that lysosomal hydrolases can be secreted has long been recognized (23, 48) and serves to emphasize the common origin of secretory and lysosomal polypeptides in the ER. In cultured hepatocytes, secretion of cathepsin D and  $\beta$ -glucuronidase was a quantitatively important process which involved ~30–40% of the newly synthesized enzymes and began well before any post-translational proteolytic cleavage was detectable. As was first noted for the enzymes of human fibroblasts (18), only uncleaved precursors of cathepsin D and  $\beta$ -glucuronidase were exported from the cultured hepatocytes. The dramatic effect of chloroquine in promoting a massive secretion of the unprocessed hydrolases is in accordance with the notion (17) that this drug eventually interferes with the transfer of newly synthesized hydrolases to lysosomes by impairing the recycling or re-using of membrane receptors which mediate this process.

It is currently thought (13, 19, 25) that phosphomannosyl groups which have been detected on the oligosaccharide chains of newly synthesized hydrolases (20, 54) serve as recognition markers which are necessary to ensure the correct destination of the polypeptides to lysosomes. In addition, membrane-bound receptors capable of binding lysosomal polypeptides carrying these markers have been identified on the cell surface (13, 21, 45, 56) and in intracellular membranes (13). The hypothesis that phosphomannosyl groups serve as sorting-out signals for lysosomal enzymes implies that unglycosylated polypeptides of hydrolases synthesized in the presence of tunicamycin should be unable to reach their lysosomal destination. It is notable that although it has been reported (57) that tunicamycin changes the distribution of hydrolytic activities in the intracellular and extracellular compartments, the newly synthesized unglycosylated polypeptides present in tunicamycin-treated cells have not been characterized and their fate has not been established. We found that unglycosylated cathepsin D and  $\beta$ -glucuronidase synthesized in tunicamycin-treated hepatocytes were quantitatively secreted into the medium as unprocessed precursors. These observations support the notion that modified sugar residues are necessary for addressing the polypeptides to lysosomes and suggest that in the absence of the phosphomannosyl marker the polypeptides are secreted. As is the case with other secretory products (53), the absence of sugars does not impair secretion of lysosomal hydrolases. Indeed, secretion became the fate for all newly synthesized unglycosylated hydrolases. Furthermore, in the presence of tunicamycin, proteolytic processing was completely abolished. These observations provide independent support for the notion that the cleavage steps which follow the biosynthesis of lysosomal hydrolases occur only after the polypeptides arrive in lysosomes.

The alternative possibility that unglycosylated polypeptides cannot serve as substrates for processing proteases is rendered

unlikely by the finding that proteolytic processing of viral envelope glycoproteins, which reach the plasma membrane (15), proceeds unimpaired on unglycosylated polypeptides synthesized in tunicamycin-treated cells.

Recent studies (22, 44) suggest that phosphorylation of newly synthesized hydrolases is a relatively early event which involves transfer of an GlcNAc phosphate residue from UDP-GlcNAc. The "covered" phosphate groups generated by this transfer do not appear, however, to be recognized by the specific membrane-bound receptors until the GlcNAc cover is removed by a phosphodiesterase which may be localized in the Golgi apparatus or GERL membrane system (58, but cf. reference 55). The differences in the sensitivity to endoglycosidases which we have detected between the intracellular and secreted forms of hydrolase precursors suggest that the secreted polypeptides indeed follow the normal secretory route which includes the Golgi apparatus and provides for trimming of oligosaccharide residues and possibly terminal glycosylation. It still remains to be determined at which point the secretory and lysosomal pathways diverge, in particular, whether enzymes which reach the lysosomes depart intracellularly from the secretory pathway (cf. references 28, 41, 47) or whether they first reach the cell surface but are later interiorized by endocytosis (21, 56).

The lack of sensitivity to endo D of the intracellular form of lysosomal enzymes may reflect the inability of Golgi complex enzymes to modify precursors bearing "uncovered" phosphate residues or more unlikely a bypass of these activities by lysosomal precursors.

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