Glucose Removal from *N*-linked Oligosaccharides Is Required for Efficient Maturation of Certain Secretory Glycoproteins from the Rough Endoplasmic Reticulum to the Golgi Complex

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ABSTRACT 1-Deoxynojirimycin is a specific inhibitor of glucosidases I and II, the first enzymes that process *N*-linked oligosaccharides after their transfer to polypeptides in the rough endoplasmic reticulum. In a pulse-chase experiment, 1-deoxynojirimycin greatly reduced the rate of secretion of α1-antitrypsin and α1-antichymotrypsin by human hepatoma HepG2 cells, but had marginal effects on secretion of the glycoproteins C3 and transferrin, or of albumin. As judged by equilibrium gradient centrifugation, 1-deoxynojirimycin caused α1-antitrypsin and α1-antichymotrypsin to accumulate in the rough endoplasmic reticulum. The oligosaccharides on cell-associated α1-antitrypsin and α1-antichymotrypsin synthesized in the presence of 1-deoxynojirimycin, remained sensitive to Endoglycosidase H and most likely had the structure $Glu_{1-3}Man_9GlcNAc_2$. Tunicamycin, an antibiotic that inhibits addition of *N*-linked oligosaccharide units to glycoproteins, had a similar differential effect on secretion of these proteins. Swainsonine, an inhibitor of the Golgi enzyme α-mannosidase II, had no effect on the rates of protein secretion, although the proteins were in this case secreted with an abnormal *N*-linked, partially complex, oligosaccharide. We conclude that the movement of α1-antitrypsin and α1-antichymotrypsin from the rough endoplasmic reticulum to the Golgi requires that the *N*-linked oligosaccharides be processed to at least the $Man_9GlcNAc_2$ form; possibly this oligosaccharide forms part of the recognition site of a transport receptor for certain secretory proteins.

Secretory proteins and cell surface glycoproteins are synthesized on ribosomes attached to the rough endoplasmic reticulum membrane. Cotranslationally, the polypeptides are transported across or inserted into the rough endoplasmic reticulum membrane (1–3), and one or more oligosaccharides, of structure $Glc_3Man_9GlcNAc_2$, are transferred to specific asn residues (4). By a series of membrane vesicles these proteins are transported first to the Golgi complex of membranes, then to the cell surface (1–3, 5, 6).

How proteins move from the rough endoplasmic reticulum to the Golgi is not known. A common hypothesis is that small vesicles bud off from a specialized region of the rough endoplasmic reticulum, containing a sample of the luminal contents and membrane proteins. These vesicles subsequently fuse with vesicles of the *cis*- (or proximal) region of the Golgi cisternae (2, 3). In hepatic parenchymal cells albumin and lipoprotein particles are localized to different terminal dilations of the rough endoplasmic reticulum and *cis*-Golgi (7). Thus it is possible that different vesicles transport different secretory products to the Golgi.

It is generally believed that all secretory and plasma membrane glycoprotein proteins migrate through the same Golgi vesicles. A combination of cell-fractionation and immunoelectronmicroscopic approaches has shown that albumin, transferrin, and the vesicular stomatitis viral glycoprotein and other glycoproteins are found in the same rough endoplasmic reticulum and Golgi cisternae (8–12). All of the predominant proteins secreted by the exocrine pancreas, likewise, are found in the same series of intracellular vesicles (13). Additionally, the high mannose oligosaccharides added to all of these proteins undergo the same intracellular modifications (4): Three glucose residues and probably one or more mannose residues

THE JOURNAL OF CELL BIOLOGY · VOLUME 98 MAY 1984 1720-1729

are removed in the rough endoplasmic reticulum by three enzymes, two glucosidases and an α-mannosidase, that are localized to this organelle (14–16). The remaining processing enzymes are localized to *cis*- or *trans*-Golgi vesicles (4, 6, 14, 17, 18).

Different secretory and membrane proteins mature through this intracellular pathway at very different rates (19–22). In HepG2 cells, at 32°C only 30 min are required for newly-made albumin and α1-antitrypsin to mature to the Golgi. About 50 min are required for C3 complement and α1-antichymotrypsin, and ~150 min for transferrin. The rate-limiting and distinctive step in intracellular maturation of these proteins is in movement from the rough endoplasmic reticulum to the Golgi, as secretion of all proteins from the Golgi requires about the same period of time—20 min (20). We (19, 20) and others (21) have postulated that one or more receptor proteins in the endoplasmic reticulum membrane regulates the selective transport of secretory proteins or membrane glycoproteins into transport vesicles en route to the Golgi.

The function of the *N*-linked oligosaccharides in intracellular maturation has been probed most often with tunicamycin, an inhibitor of synthesis of the oligosaccharide precursor (23). Many, but not all, glycoproteins fail to be secreted or to be expressed on the cell surface if normal glycosylation is inhibited (24–29). Interpretation of these results is difficult, however, since in specific cases unglycosylated proteins have been shown to be more sensitive to proteases (28, 30), less soluble (27, 31), or abnormally folded (32).

In this paper, we have studied the effect of 1-deoxynojirimycin and two other inhibitors of oligosaccharide processing on intracellular maturation and secretion of five proteins by human hepatoma HepG2 cells. 1-deoxynojirimycin inhibits glucosidases I and II, the first enzymes that process *N*-linked oligosaccharides after their attachment to proteins (33). We show that efficient maturation of some, but not all, secreted glycoproteins from the rough endoplasmic reticulum to the Golgi requires that the glucose residues on *N*-linked oligosaccharides be removed. One interpretation of our results is that a $Man_9GlcNAc_2$ oligosaccharide might be required for certain secretory proteins to bind to a putative transport receptor.

After this manuscript was submitted, a paper by Gross et al. (34) appeared that showed that 1-deoxynojirimycin inhibits both oligosaccharide processing and secretion of α1-proteinase inhibitor (α1-antitrypsin), one of the proteins we have studied, in primary hepatocytes.

MATERIALS AND METHODS

Materials: Swainsonine was a generous gift of Drs. D. Tulsiani and O. Touster, Vanderbilt University, and 1-deoxynojirimycin was generously provided by Drs. E. Truscheit and D. Schmidt, Bayer A. G., Elberfeld, Federal Republic of Germany. Rabbit antisera directed toward human albumin, transferrin, α1-antitrypsin, α1-antichymotrypsin, and C3 complement were purchased from DAKO Immunoglobulins, a/s. Fixed *Staphylococcus aureus* cells were purchased from the New England Enzyme Center, Inc. (Boston, MA), and [^{35}S]methionine from the Radiochemical Center, Amersham Corp., Arlington Heights, IL.

Growth and Labeling of HepG2 Cells: Culture dishes of 60-mm diam were seeded with 1.5×10^6 HepG2 cells, and incubated at 37°C in Eagle's minimal essential medium supplemented with 10% fetal calf serum (35). Cultures were generally fed on the second day after seeding and used on the fourth, at which time the cells had approximately doubled. Similar to our previous study (20) the cells were washed twice in complete PBS, placed in 1.0 ml methionine-free growth medium (MEM medium, Flow Laboratories, Inc., containing 10% dialyzed fetal calf serum), and incubated at 30.5 or 32°C. After 20 min, 40 μCi [^{35}S]methionine was added, and the culture incubated for a further 10 min (pulse). The plates were washed twice with PBS, and 2 ml chase medium (growth medium plus 10% fetal calf serum and 1 mM methionine) was added to each. After incubation at 32°C for the appropriate time (chase) the cultures were placed at room temperature. The medium was saved, and clarified by centrifugation at 10,000 *g* for 10 min at 4°C. Control studies indicated that the total amount of acid precipitable [^{35}S]radioactivity (cells plus medium) did not change more than 10% after chase periods up to 300 min. Additionally, immunoprecipitation studies (below) showed that there was <10% increase in radioactivity in any of the five proteins studied during the chase period, indicating that the chase was effective.

In the kinetic study (Figs. 1 and 2) medium was removed at 30 min of chase, and replaced with fresh prewarmed medium, and incubation was continued at 32°C. The process was repeated every 30 or 60 min until 240 min. The cells were washed thrice in complete PBS, and lysed by addition of 1.4 ml lysis buffer (0.14 M NaCl, 0.004 M KCl, 0.001 M $NaPO_4$; 0.02 M Tris, pH 7.4; 1% sodium deoxycholate; 1% NP40). The lysate was clarified by centrifugation for 10 min at top speed in an Eppendorf microcentrifuge, and used for quantitative immunoprecipitation.

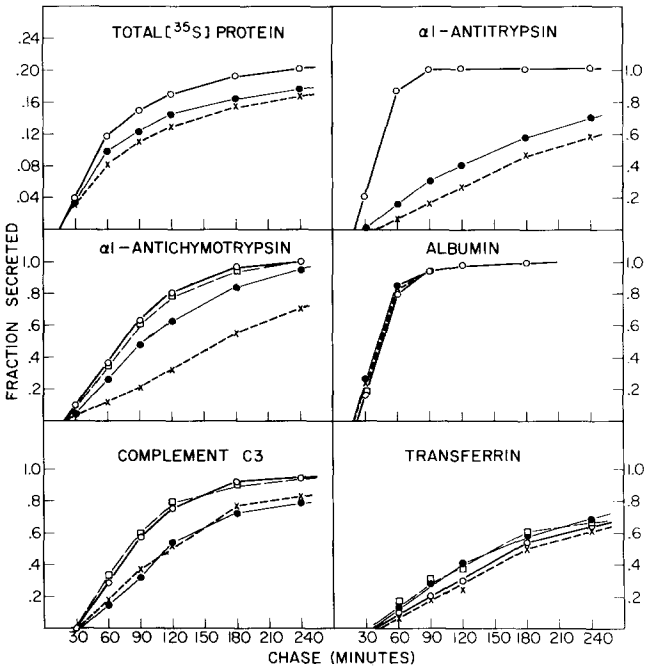


FIGURE 1 Effects of tunicamycin, 1-deoxynojirimycin, and swainsonine on secretion of hepatoma proteins. Four 60-mm culture dishes of HepG2 cells were placed in methionine-free medium containing either 10 μg/ml tunicamycin, 10 mM 1-deoxynojirimycin, 2 μg/ml swainsonine, or no drug (control). As detailed in Materials and Methods, the cells were labeled with [^{35}S]methionine and then chased, all in the continuous presence of the drugs. At 30, 60, 90, 120, 180, and 240 min of chase the medium was removed from all cultures, and replaced with fresh medium containing drugs, and the cells were replaced in the 32°C incubator. At 240 min the cells were lysed, as detailed in Materials and Methods. An equal aliquot of the lysate and all media was subjected, in duplicate, to precipitation with trichloracetic acid. From the amounts of radioactive protein released into the medium during the six intervals sampled, cumulative release of radioactive proteins into the medium can be calculated (*upper left*). Similarly, each of the media and the lysates were subjected to quantitative immunoprecipitates with each of the five antibodies noted, and the immunoprecipitation analyzed by gel electrophoresis and fluorography. The gels were scanned with a microdensitometer under conditions where the response of the film and the instrument was in the linear range. The areas under the relevant peaks(s) of the scan was a measure of the amount of radioactive protein released into the medium during the interval sampled, or remaining in the cells at 240 min. From these data (Fig. 2), one can calculate the cumulative amount of each protein released into the medium as function of the time of chase. O, control; □, swainsonine; ×, 1-deoxynojirimycin; ●, tunicamycin.

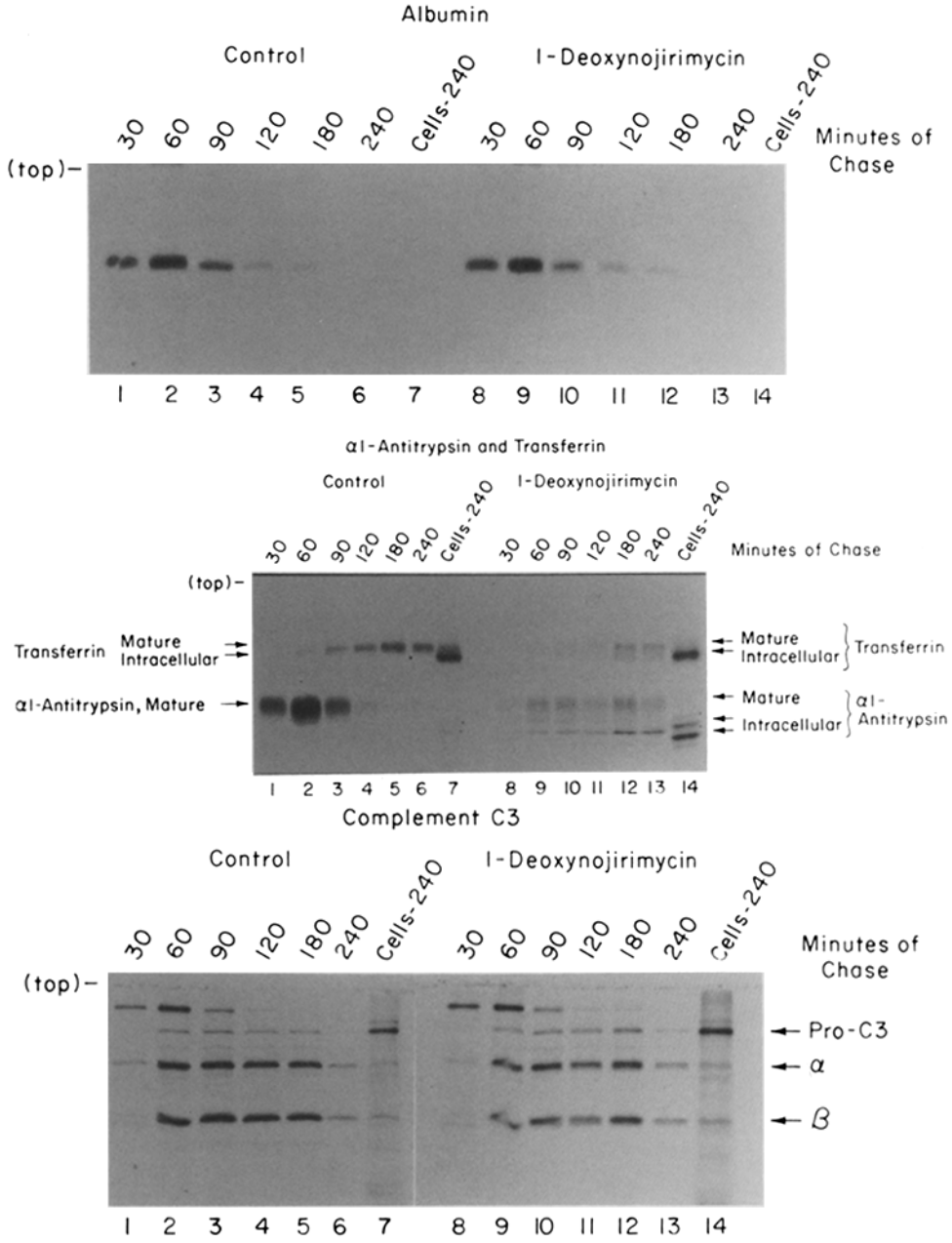


FIGURE 2 Effect of 1-deoxynojirimycin on secretion of albumin (*a*), α1-antitrypsin and transferrin (*b*), and C3 complement (*c*). As detailed in the legend to Fig. 1, HepG2 cells were pulse-chased labeled in the presence (lanes *8–14*) or absence (control, lanes *1–7*) of 1-deoxynojirimycin. Equal aliquots of medium and lysate were subjected to quantitative immunoprecipitation with antialbumin (*a*), anti-α1-antitrypsin, and antitransferrin together (*b*), and anti-C3 complement (*c*). Lanes *1* and *8*, protein secreted between 0 and 30 min of chase; lanes *2* and *9*, protein secreted between 30 and 60 min of chase; lanes *3* and *10*, protein secreted between 60 and 90 min of chase; lanes *4* and *11*, protein secreted between 90 and 120 min of chase; lanes *5* and *12*, protein secreted between 120 and 180 min of chase; lanes *6* and *13*, protein secreted between 180 and 240 min of chase; lanes *7* and *14*, protein remaining in the cells after 240 min of chase. In *b*, arrows indicate (top down) the mature secreted form of transferrin; the intracellular form that is sensitive to Endo H; the mature secreted form of α1-antitrypsin; and the two intracellular forms of α1-antitrypsin that are generated in the presence of 1-deoxynojirimycin. In *c*, the arrows indicate proC3; and the two cleaved products C3α and C3β.

Immunoprecipitations: Reactions (20) contained 200 μl lysis buffer, 100 μl lysate or medium, and 10 μl of appropriate rabbit antiserum. After incubation at 4°C for 1 h, with intermittent shaking, 50 μl of a 10% suspension of fixed, washed, *Staphylococcus aureus* cells (29) was added. Immunoprecipitates were recovered and washed as before (29) except that no SDS was used in the buffers. These were dissolved in SDS-containing buffer and analyzed by electrophoresis through 10% polyacrylamide gels.

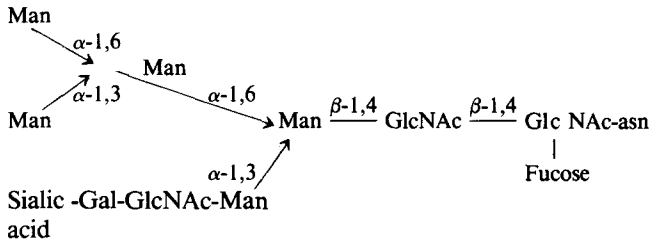
Controls established that these conditions were sufficient to recover >85% of the labeled protein in question. In particular, <10% additional labeled protein was recovered if the first supernatant from the immunoprecipitation was reacted with additional antiserum. In some cases, the immunoprecipitate were split into two portions; one was digested with Endoglycosidase H (Endo H) and the other mock-digested, as detailed previously (36).

Subcellular Fractionation of HepG2 Cells Pulse-chase Labeled with [^{35}S]Methionine: This protocol is similar to the one we used previously (20). For each sample, two 100-mm-diam culture dishes of HepG2 cells were labeled for 15 min with 150 μCi [^{35}S]methionine per plate,

then chased for 55 min at 32°C with unlabeled methionine. All subsequent procedures were done at 0°–4°C. The cells were washed twice in complete PBS, scraped from the dish, and resuspended in 2.5 ml buffer A (0.025 M HEPES, 0.05 M KCl, 0.002 M Mg acetate$_2$, pH 7.2, 1 mM dithiothreitol) containing 10% (wt/vol) sucrose. After swelling for 10 min, the cells were disrupted with 80 strokes in a tight-fitting Dounce homogenizer. Nuclei were removed by centrifugation at 1,000 *g* for 10 min. 2.5 ml of cytoplasm was layered on 1.0 ml 65% (wt/vol) sucrose and 2.0 ml 15% sucrose, all in buffer A, in a tube for the SW50.1 Beckman ultracentrifuge rotor (Beckman Instruments, Inc.). Following centrifugation for 75 min and 40,000 rpm, the membranes were recovered with a syringe at the 15–65% sucrose interphase. These were diluted to 15% sucrose, with buffer A, containing no sucrose, and layered upon a sucrose gradient (1.1 ml each of 65, 55, 50, 45, 40, 35, 30, and 20% wt/vol, all in buffer A) in a tube for the SW41 Beckman ultracentrifuge rotor. This was centrifuged for 2 h and 40,000 rpm. Nineteen equal 0.6 ml fractions were collected.

RESULTS

To probe the role of asn-linked oligosaccharides in the secretion of serum proteins by HepG2 cells, we have utilized three inhibitors of oligosaccharide synthesis or processing. Tunicamycin blocks the first step in the biosynthesis of $Glc_3Man_9GlcNAc_2$ pyrophosphoryldolichol; in its presence no asn-linked oligosaccharide units are added to glycoproteins (reviewed in reference 23). 1-deoxynojirimycin is an inhibitor of the oligosaccharide processing enzymes glucosidase I and II. This compound blocks the formation of complex oligosaccharides, and causes the accumulation of glucosylated, high-mannose oligosaccharides of the structure $Glc_{1-3}Man_9GlcNAc_2$-asn (33, 34). Swainsonine is a specific inhibitor of the processing enzyme α-mannosidase II (37) and causes the accumulation of the processing intermediate $Man_5GlcNAc_2$-asn. A hybrid oligosaccharide of the following structure:



is found on secreted α1-antitrypsin and on viral glycoproteins synthesized in the presence of Swainsonine (38–40).

Swainsonine Does Not Affect the Rate of Protein Secretion

Swainsonine has no effect on the rate or extent of secretion of the five proteins studied: the glycoproteins α1-antitrypsin, α1-antichymotrypsin, C3 complement, transferrin, and the nonglycosylated protein albumin (Fig. 1 and Table I). The glycoproteins secreted in the presence of this drug are, however, abnormal. On sodium dodecyl sulfate gels they migrate faster than do the normal secreted forms (Figs. 3 and 4, lanes *7*, compare with lanes *5*), yet slower than do the pulse labeled intracellular forms (compare lanes *7* and *3*). Whereas the normal secreted forms of transferrin, α1-antitrypsin, and α1-antichymotrypsin are resistant to Endo H (20; cf. Fig. 5, lanes *11* and *12*) those secreted in the presence of swainsonine are sensitive. Endo H generates a species that co-migrates with the pulse-labeled cellular protein that has been treated with Endo H (data not shown). These results are compatible with the conclusion that the oligosaccharides on proteins secreted in the presence of swainsonine have the hybrid structure noted above; this hybrid structure is sensitive to Endo H (38–40).

TABLE I

Effect of Inhibitors on Protein Secretion

Protein	Control	Tunicamycin*	1-deoxynojirimycin‡	Swainsonine§
Albumin	47	42	44	47
α1-antitrypsin	44	157	207	44
C3	82	114	121	79
α1-antichymotrypsin	75	94	166	79
transferrin	170	154	180	153

The data in Fig. 1 were used to determine the time (minutes) required for 50% of each protein to be secreted.
* 10 μg/ml.
‡ 5 mM.
§ 2 μg/ml.

1-Deoxynojirimycin Slows the Secretion of Certain HepG2 Proteins

Figs. 1 and 2 and Table I show that 1-deoxynojirimycin markedly slows the rate of secretion of newly-made α1-antitrypsin. Whereas half of the newly-made protein is normally secreted in <50 min, in the presence of 1-deoxynojirimycin over 200 min are required for secretion of 50% of α1-antitrypsin. This drug has a less dramatic effect on secretion of C3 complement (half-time increased from 80 to 120 min) and α1-antichymotrypsin (75 to 165 min). There is no effect on secretion of albumin or transferrin, results that indicate that the drug is not a general "poison" of cellular metabolism, and that inhibition of secretion is highly selective. Similar results (not shown) were obtained with concentrations of 1-deoxynojirimycin from 5 to 25 mM.

Inhibition of Processing by 1-Deoxynojirimycin Is Incomplete

Most of the small amount of α1-antitrypsin and α1-antichymotrypsin secreted in the presence of 1-deoxynojirimycin co-migrates with authentic secretory proteins (Fig. 2*b*; Fig. 3, lanes *5* and *6*; Fig. 4, lanes *5* and *6*) and are resistant to Endo H (Fig. 5, lanes *11–16*). These proteins could contain the normally processed "complex" oligosaccharides; thus the inhibition of glucosidases I and II would not be total under the conditions we used. Alternatively, the branch of the *N*-linked oligosaccharides that contains the three glucose residues (that attached to the $\alpha 1 \rightarrow 3$ linked core mannose) could remain unprocessed, while the other two branches, derived from the $\alpha 1 \rightarrow 6$ linked core mannose, could be processed to the complex form. The oligosaccharides would then have the composition (sialic acid gal glcNAc)$_2$($Glc_{1-3}Man_2$)Man_3 $GlcNAc_2$. To resolve this point, we took advantage of the finding that swainsonine blocks the conversion of the branches attached to the $\alpha 1 \rightarrow 6$ linked core mannose to the complex form (37–40). Thus we added swainsonine and 1-deoxynojirimycin together to HepG2 cells. Figs. 3 and 4 (lanes *8*) show that the proteins secreted co-migrate with the form synthesized in the presence of swainsonine alone (compare with lanes *7*). They are also sensitive to Endo H (not shown). If 1-deoxynojirimycin were to cause the secretion of the hybrid oligosaccharide noted above, then the combination of 1-deoxynojirimycin and swainsonine would block all of the

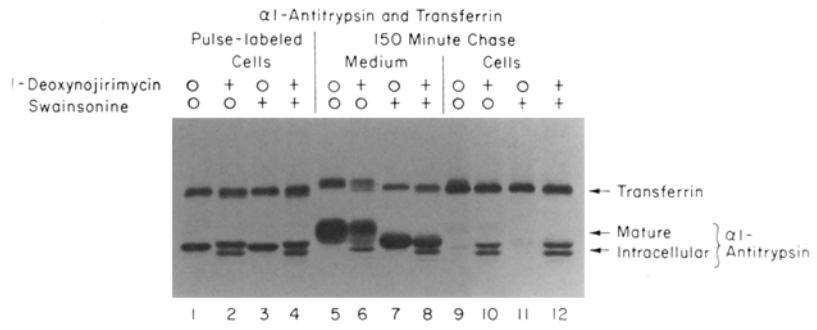


FIGURE 3 Effect of swainsonine and 1-deoxynojirimycin on secretion and gel motility of α1-antitrypsin and transferrin. One set of cells (lanes *1–4*) was pulse-labeled for 10 min with [^{35}S]methionine, lysed, and immunoprecipitated with a mixture of antitransferrin and anti-α1-antitrypsin. Another (lanes *5–12*) was pulse-labeled for 10 min and then chased for 150 min; the lysate (lanes *9–12*) and medium (lanes *5–8*) were immunoprecipitated. Lanes *1*, *5*, and *9*, control; lanes *2*, *6*, and *10*, 10 mM 1-deoxynojirimycin; lanes *3*, *7*, and *11*, 2 μg/ml swainsonine; lanes *4*, *8*, and *12*, 10 mM 1-deoxynojirimycin and 2 μg/ml swainsonine. Shown is an autoradiograph of the fixed, dried gel.

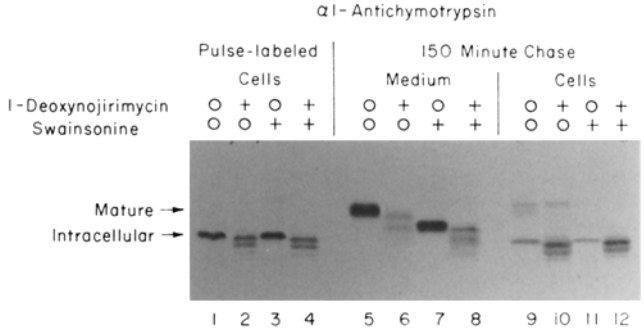


FIGURE 4 Effect of swainsonine and 1-deoxynojirimycin on secretion and gel mobility of α1-antichymotrypsin. The protocol is identical to that of Fig. 3, except immunoprecipitates using anti-α1-antichymotrypsin were analyzed.

branches of the oligosaccharide from conversion to a complex oligosaccharide. The expected secretory product would have the composition $Glc_{1-3}Man_7GlcNAc_2$; proteins with this oligosaccharide would migrate faster than the pulse labeled intracellular form, not slower, as was found (Figs. 3 and 4).

Most likely, therefore, 1-deoxynojirimycin inhibition of glucosidases was incomplete and some protein with normal complex oligosaccharides are produced. In the presence of both 1-deoxynojirimycin and swainsonine, the hybrid structure that swainsonine generates is found on most of the secreted glycoproteins.

1-Deoxynojirimycin Causes Accumulation of Inhibited Secretory Proteins in the Rough Endoplasmic Reticulum

The first set of experiments that established this point made use of Endo H. Endo H cuts the GlcNAc β1 → 4 GlcNAc bond in asn-linked oligosaccharides, leaving only a single GlcNAc residue attached to the protein. Substrates for Endo H include the $Glc_3Man_9GlcNAc_2$ oligosaccharide transferred in the rough endoplasmic reticulum to asn residues of glycoproteins, and processing intermediates such as $Man_9GlcNAc_2$, $Man_8GlcNAc_2$, and $Man_5GlcNAc_2$. Later intermediates in the processing to complex oligosaccharides are resistant to Endo H (41–43). Fig. 5 (lanes *1* and *2*) shows that in control cells, pulse labeled α1-antitrypsin, and transferrin are sensitive to Endo H. The slower migrating mature form secreted into the medium after a chase period is resistant to Endo H (lanes *11* and *12*) as is the co-migrating form that accumulates transiently in the cells during the chase period (lanes *3* and *4*).

In the presence of 1-deoxynojirimycin, pulse-labeled transferrin migrates identically to its normal counterpart, and is sensitive to Endo H (Fig. 5, lanes *5* and *6*). α1-antitrypsin synthesized in the presence of 1-deoxynojirimycin migrates as two discrete polypeptides, the slowest migrating of which migrates a bit slower than does its normal counterpart (compare lanes *1* and *6*). Mammalian α1-antitrypsin normally contains three asn-linked oligosaccharides (44, 45). We suggest that the slowest intracellular migrating species of α1-antitrypsin, synthesized in the presence of 1-deoxynojirimycin, contains three oligosaccharides of the structure $Glc_{1-3}Man_9GlcNAc_2$. The recent paper by Gross et al. (34) establishes this structure directly. Such a protein would migrate on SDS gels slower than would its normal counterpart which contains three oligosaccharides of structure $Man_9GlcNAc_2$; the three glucose residues are cleaved from normal proteins very quickly (4). The faster migrating species, synthesized in the presence of 1-deoxynojirimycin, would contain only two such oligosaccharides. As evidence for this point, Endo H converts all forms of pulse labeled α1-antitrypsin to the same nonglycosylated species (Fig. 5, lanes *2* and *5*).

Half of the pulse labeled α1-antitrypsin is normally converted to an Endo H resistant form after a 30-min chase (Fig. 5, lanes *3* and *4*; see reference 20). In the presence of 1-deoxynojirimycin, however, all forms of the cell-associated α1-antitrypsin remained Endo H sensitive, even after chase periods of 60 min (lanes *7–10*) or longer (not shown). Similarly, in 1-deoxynojirimycin-treated cells, over 90% of the pulse labeled α1-antichymotrypsin, associated with the cells after a chase period of up to 120 min, remained sensitive to Endo H (data not shown). Since the enzymes that process asn-linked oligosaccharides to an Endo H-resistant form are localized to the Golgi, these results are consistent with the notion that 1-deoxynojirimycin selectively blocks the maturation of α1-antitrypsin and α1-antichymotrypsin from the rough endoplasmic reticulum to the Golgi complex.

To confirm that 1-deoxynojirimycin selectively blocks the maturation of secretory proteins at the level of the rough endoplasmic reticulum, we employed subcellular fractionation experiments. Cells were either pulse labeled with [^{35}S] methionine, or pulse labeled and then chased for 55 min at 32°C. A postnuclear membrane fraction was prepared and centrifuged to equilibrium through a sucrose density gradient. Neither the rough endoplasmic reticulum or Golgi vesicles have been purified to homogeneity from any tissue culture cell. Thus, we must assume that a labeled secretory protein which bands at 1.10–1.16 g/cc is in Golgi vesicles, and not in

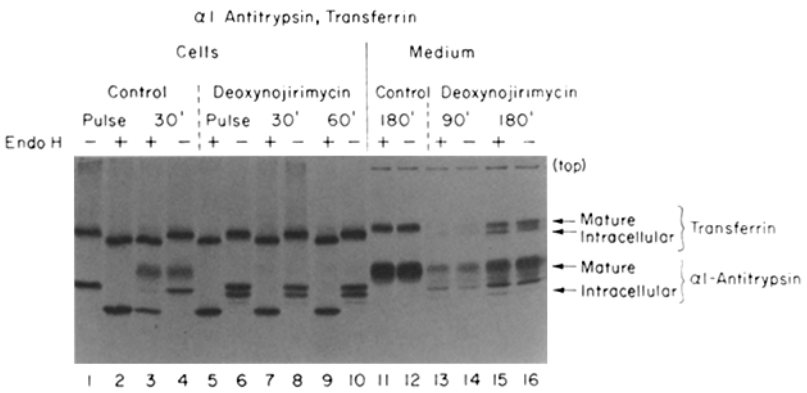


FIGURE 5 Digestion of transferrin and α1-antitrypsin, synthesized in the presence of 1-deoxynojirimycin, with Endo H. Immunoprecipitates of α1-antitrypsin and transferrin were split into equal aliquots; one was digested with Endo H (lanes *2, 3, 5, 7, 9, 11, 13,* and *15*) and the others undigested. Lanes *1* and *2*, control cells, pulse labeled; lanes *3* and *4*, control cells, chased 30 min; lanes *5* and *6*, 1-deoxynojirimycin-treated cells, pulse-labeled; lanes *7* and *8*, 1-deoxynojirimycin-treated cells, chased 30 min; lanes *9* and *10*, 1-deoxynojirimycin-treated cells, chased 60 min; lanes *11* and *12*, medium from control cells chased 180 min; lanes *13* and *14*, medium from 1-deoxynojirimycin-treated cells, chased 90 min; lanes *15* and *16*, medium from 1-deoxynojirimycin-treated cells, chased 180 min.

some other smooth-surfaced vesicles, and that secretory proteins banding at 1.18 g/cc are in rough endoplasmic reticulum vesicles. Indeed, over 85–90% of pulse labeled α1-antitrypsin, albumin, C3, α1-antichymotrypsin, and transferrin bands in membranes at a density of 1.16–1.20 g/cc, the density of rough endoplasmic reticulum (data not shown). Confirming our previous result (20), all of the mature, sialic-acid bearing α1-antitrypsin and α1-antichymotrypsin that accumulates intracellularly during the chase period bands in the 1.10–1.16 g/cc region (Fig. 6, fractions *9–14*). We recognize, of course, that other smooth cellular membranes such as the smooth endoplasmic reticulum and plasma membrane will bind in this region of the gradient, but we feel it unlikely that secretory proteins would be associated with these organelles, and not with the Golgi vesicles.

In control cells, after the 55-min chase period 75% of labeled intracellular albumin bands at the density of smooth surfaced membranes such as the Golgi (fractions *8–16*, Fig. 6; cf. reference 20). About 77% of α1-antitrypsin, and all of the mature Endo H resistant α1-antitrypsin, likewise bands at the density of the Golgi (Fig. 6). 1-deoxynojirimycin has no effect on the subcellular distribution of albumin. Importantly, in the presence of 1-deoxynojirimycin 76% of labeled α1-antitrypsin bands at the density of the rough endoplasmic reticulum. Clearly 1-deoxynojirimycin selectively blocks the maturation of this protein from the rough endoplasmic reticulum.

Quantitatively, in the control cells by 55 min of chase ~80% of pulse-labeled α1-antitrypsin has been secreted, and, of the 20% that remains in the cell, 77% bands in the Golgi region. Thus, 95% (= 0.80 + (0.20)(0.77)) of α1-antitrypsin has moved to or through the Golgi within 55 min of synthesis. In 1-deoxynojirimycin treated cells at 55 min of chase, by contrast, only 6% of labeled α1-antitrypsin has been secreted, and of that remaining in the cell, 24% is in the Golgi fraction. Thus, only 28% (= 0.06 + (0.94)(0.24)) of the protein has moved to or through the Golgi vesicles within 55 min of its synthesis.

In control cells, after the chase period, proteins such as α1-antichymotrypsin, which are secreted at an intermediate rate, distribute on the density gradient in an intermediate fashion (20). In this study ~60% are in the rough endoplasmic reticulum, and all of the Endo H resistant α1-antichymotrypsin bands with the Golgi (Fig. 6). By contrast, in the presence of 1-deoxynojirimycin over 75% of cell associated labeled α1-antichymotrypsin is associated with the rough endoplasmic reticulum. Thus most if not all of the inhibitory effect of 1-deoxynojirimycin on α1-antichymotrypsin secretion is also due to a block in maturation at the level of the rough endoplasmic reticulum.

Transferrin requires the longest period of time to mature from the rough endoplasmic reticulum to the Golgi (19, 20). After 55 min of chase almost all of the pulse labeled transferrin fractionates with the rough endoplasmic reticulum, and 1-deoxynojirimycin has, as might be expected, no effect on this profile.

Inhibition of Secretion by 1-Deoxynojirimycin Is Reversible and Occurs Early in the Secretory Pathway

In the experiment summarized in Table II, 1-deoxynojirimycin was added to cells during the labeling period and the first 30 (sample 3) or 60 min (sample 4) of the chase period, then removed. During the next 180–210 min almost all of the pulse labeled α1-antitrypsin was secreted. In the continuous presence of 1-deoxynojirimycin, by contrast, only 38% of the protein was secreted by 240 min. Thus, the block in secretion of α1-antitrypsin, synthesized in the presence of 1-deoxynojirimycin, can be reversed if the drug is subsequently removed. The secreted protein co-migrates with authentic α1-antitrypsin (not shown).

The experiment with sample 5, Table II, confirms that inhibition by 1-deoxynojirimycin occurs early in the maturation pathway. If the drug is added at 20 min of the chase period there is no effect on either the extent (line 5) or rate (data not shown) of secretion of labeled α1-antitrypsin. By 20 min of chase only 60–70% of the labeled α1-antitrypsin still remains in the rough endoplasmic reticulum. It is expected that the glucose residues will have been removed already from that fraction of the α1-antitrypsin still in the rough endoplasmic reticulum and thus that 1-deoxynojirimycin will have no effect.

Tunicamycin

Figs. 1, 7, and 8 show that tunicamycin has a similar effect on protein secretion to that of 1-deoxynojirimycin. The inhibition of secretion of α1-antitrypsin and C3, as well as of total [^{35}S]methionine labeled secretory proteins, is the same for both drugs. Inexplicably, tunicamycin has, reproducibly, less of an inhibitory effect on secretion of α1-antichymotrypsin than does 1-deoxynojirimycin. Nonglycosylated α1-anti-

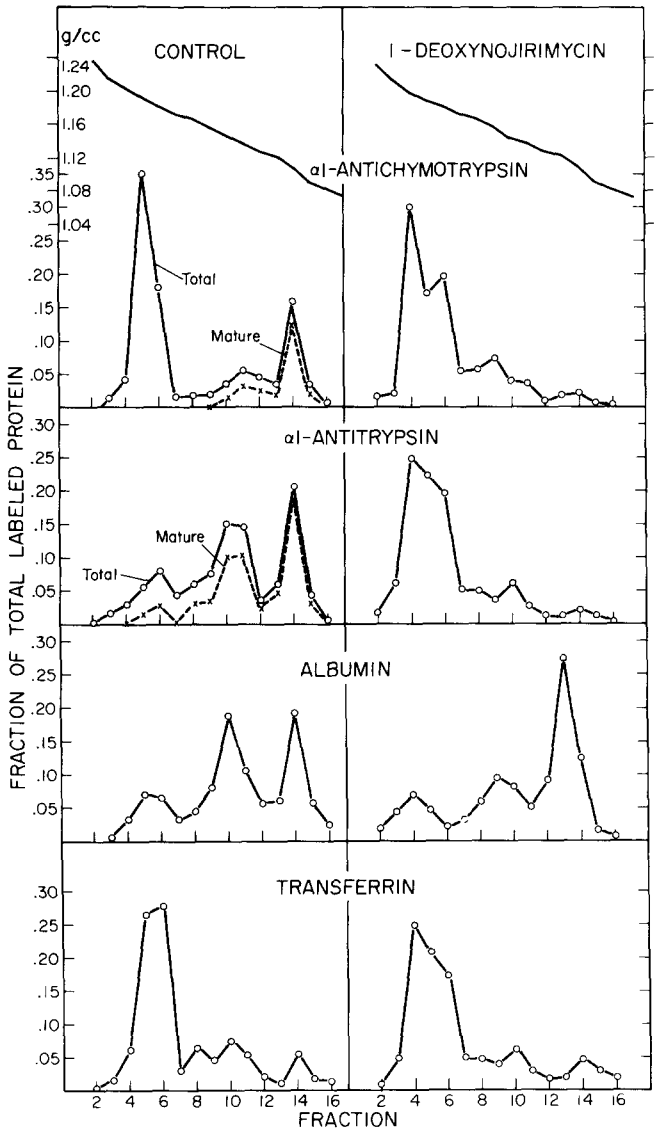


FIGURE 6 Subcellular distribution of labeled α1-antichymotrypsin, α1-antitrypsin, albumin, and transferrin in control cells (*left*) and 1-deoxynojirimycin-treated cells (*right*) after a 15-min pulse-label with [^{35}S]methionine and a 55-min chase. As is detailed in Materials and Methods, microsomes were isolated from control and 1-deoxynojirimycin-treated cells that had been pulse-labeled for 15 min and chased for 55 min. These were centrifuged to equilibrium on a sucrose gradient; the density of each fraction is plotted at the top of each figure. Of each fraction 100 μl was subjected to immunoprecipitation by each of the five rabbit anti-human serum proteins studied. For α1-antitrypsin, it was necessary to preclear the fractions first with normal rabbit serum and fixed *S. aureus* cells, followed by the immune serum and additional *S. aureus*. This was necessary to remove a minor nonspecific protein that migrated on the SDS gel near the Endo H-sensitive form of α1-antitrypsin. The immunoprecipitates were analyzed by SDS gel electrophoresis and fluorography. The autoradiogram was scanned, and the areas under all peaks determined. For α1-antitrypsin and α1-antichymotrypsin, in the control sample the radioactivity in the slow-migrating (Endo H-resistant) "mature" form (x) and the total radioactivity in both intracellular forms (O) is plotted as a percentage of the total radioactivity in this protein recovered from the gradient. The small amount of labeled protein at the top of the gradient (fractions 17–19) represents material that has leaked from vesicles, and is not depicted here nor included in calculations summarized in the text.

trypsin (Fig. 7) and C3 (Fig. 8) as well as the other proteins studied (not shown), are not degraded, but are secreted at a characteristic slow rate.

DISCUSSION

This study focuses on the factors that determine the rates at which individual secretory proteins are transported from the rough endoplasmic reticulum to the Golgi complex. Of the five serum proteins studied, albumin and α1-antitrypsin are normally transported the fastest, with half-times of ~30 min at 32°C. About 50 min are required for half of the α1-antichymotrypsin and C3 complement to be transported; transferrin, the slowest protein studied, takes 150 min. This is the rate-limiting and distinctive step in maturation of secretory proteins. Secretion of all proteins from the Golgi requires about 20 min (at 32°C) (20). Furthermore, swainsonine, an inhibitor of the Golgi enzyme α-mannosidase II (36), has no effect on the rate of secretion of any of the proteins studied (Fig. 1), even though the glycoproteins are released with abnormal oligosaccharides (38–40).

It is not known whether the rough endoplasmic reticulum pool sizes of the various secretory proteins are different. As movement of membrane (and presumably secretory) proteins out of an intracellular pool is a random process (reference 21), one might imagine that differences in pool sizes of individual protein species within the rough endoplasmic reticulum would cause differences in secretion rates. However, differences in the rough endoplasmic reticulum pool sizes are expected to arise as a consequence of differences in rates of transport of the different proteins to the Golgi, not as a cause of different transport rates. Consider a compartment (the rough endoplasmic reticulum) filled by two substances (proteins) at the same rate (synthesis), but in which the two substances exit at different rates (incorporation into transport vesicles). The substance that has the highest rate constant for exit will have the lowest pool size in the compartment. This conclusion is valid whether exit from the compartment is by a nonsaturable first order process, or by a saturable process involving binding of all of the substances to a common receptor (H. Lodish, calculations not shown).

HepG2 cells do have abundant cell surface receptors for transferrin (46). Addition of 50 μg/ml human transferrin to HepG2 cells had no effect on the rate of transferrin secretion (unpublished data), and in any case, there was no evidence that endocytosed transferrin was ever transported to the rough endoplasmic reticulum. Thus, it is unlikely that an intracellular pool of transferrin, generated by endocytosis, affects the transport of transferrin from the rough endoplasmic reticulum to the Golgi.

We have shown here that *N*-linked oligosaccharides play a key role in the transport of some proteins from the rough endoplasmic reticulum to the Golgi. The results are most dramatic for α1-antitrypsin. Nonglycosylated α1-antitrypsin is secreted only slowly and incompletely from HepG2 cells (Figs. 1 and 7*b*). Most of the labeled nonglycosylated α1-antitrypsin co-fractionates with rough endoplasmic reticulum membrane vesicles, even after a long period of chase (data not shown), a result indicating that at least one asn-linked oligosaccharide is required for movement from the endoplasmic reticulum.

However, the addition of an asn-linked oligosaccharide to α1-antitrypsin is not sufficient for normal maturation. Our

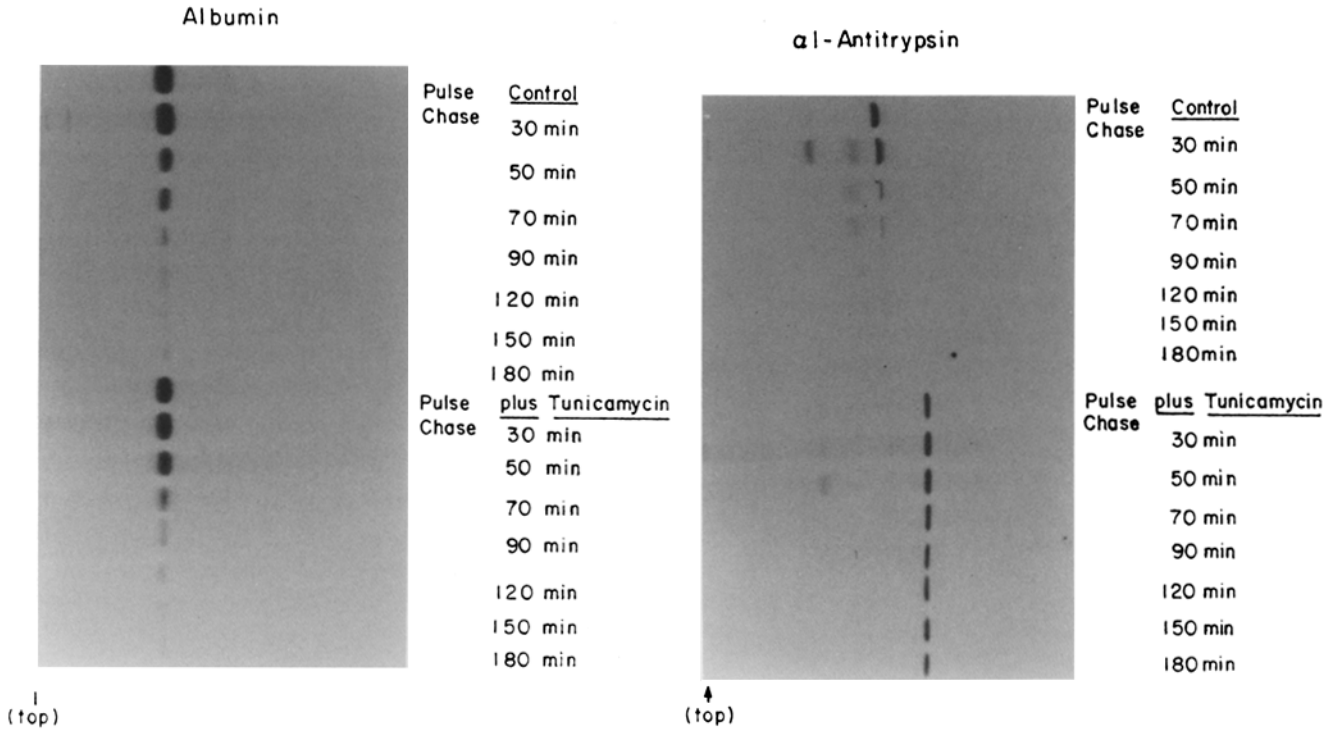


FIGURE 7 Effect of tunicamycin on secretion of albumin (*a*) and α1-antitrypsin (*b*). 16 plates were pulse-labeled with [^{35}S]-methionine, eight in the absence (lanes 1–8) and eight in the presence of tunicamycin. One set of cells was lysed after the pulse, and others after chase periods of the indicated length. From the lysates albumin (*a*) or α1-antitrypsin (*b*) were recovered by immunoprecipitation and analyzed by gel electrophoresis and autoradiography.

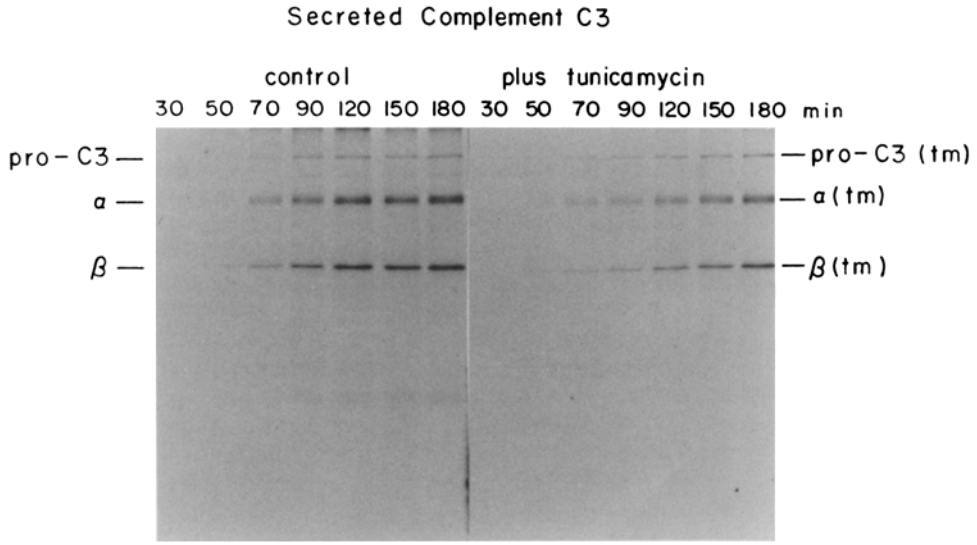


FIGURE 8 Effect of tunicamycin on secretion of C3 complement. The protocol was identical to that of Fig. 7, except the medium was recovered after the different periods of chase; C3 was recovered by quantitative immunoprecipitation and analyzed by gel electrophoresis and radioautography.

results with 1-deoxynojirimycin, an inhibitor of glucosidases I and II, indicate that removal of at least one glucose residue is essential for rapid movement of α1-antitrypsin from the rough endoplasmic reticulum. This drug causes the intracellular accumulation of high mannose oligosaccharides that contain one or more glucose residues, and blocks the conversion of high mannose to complex oligosaccharides (33, 34). 1-deoxynojirimycin inhibits the secretion of α1-antitrypsin (Fig. 1; see also reference 34) and causes the protein to accumulate in the rough endoplasmic reticulum (Fig. 6). As judged by sensitivity to Endo H, intracellular α1-antitrypsin maintains high mannose oligosaccharides (Fig. 5). Evidently, the reduced rate of formation of complex sugars on α1-antitrypsin is due to its slowness in reaching the Golgi membranes, wherein are localized the enzymes that convert high mannose oligosaccharides to a complex form (4, 18). Our studies on the gel mobility and Endo H-sensitivity of intracellular α1-antitrypsin, synthesized in the presence of 1-deoxynojirimycin, are consistent with an oligosaccharide composition of $Glc_{1-3}Man_9GlcNAc_2$ (Figs. 3–5); a recent paper has shown this directly (34).

Inhibition of maturation of α1-antitrypsin by 1-deoxynoji-

rimycin is highly specific. This drug has no effect on secretion of α1-antitrypsin synthesized before the drug is added, though it profoundly reduces secretion of the protein synthesized in its presence. Furthermore, α1-antitrypsin, synthesized in the presence of 1-deoxynojirimycin, will be secreted after the inhibitor is removed (Table II). Additionally, this drug has no effect on secretion of albumin nor of transferrin (Fig. 1). 1-deoxynojirimycin also reduces the rate of secretion of α1-antichymotrypsin. As with α1-antitrypsin, it causes the protein to accumulate in the rough endoplasmic reticulum (Fig. 6).

Precisely how asn-linked oligosaccharides influence maturation of proteins from the rough endoplasmic reticulum to the Golgi is not clear. Nonglycosylated proteins, synthesized in the presence of tunicamycin, frequently are less soluble than their normal counterparts or have an altered conformation that is reflected in enhanced sensitivity to proteases (see Introduction). Such abnormally folded or denatured proteins might precipitate on or in the endoplasmic reticulum membrane. Presumably these explanations do not apply to proteins synthesized in the presence of 1-deoxynojirimycin, since the drug causes a normal processing intermediate (apparently $Glc_{1-3}Man_9GlcNAc_2$) to accumulate within the endoplasmic reticulum.

It is possible that different vesicles are used to transport different secretory products from the endoplasmic reticulum. Implicit in this statement is the notion of a receptor protein that binds to specific secretory proteins in the endoplasmic reticulum membrane, and mediates their incorporation into the appropriate transport vesicle. Alternatively, all secretory proteins could be transported in the same vesicle. To explain the different rates of transport of proteins to the Golgi, we and others have postulated that one or more receptor proteins, localized to a specific region of the lumenal surface of the endoplasmic reticulum membrane, mediates the selective, rapid transport of certain secretory and membrane glycoproteins (19–21).

Albumin is normally transported to the Golgi very rapidly, and its transport is unaffected by any inhibitor of glycosylation. Clearly, asn-linked oligosaccharides play no role in the binding of albumin to any transport receptor.

The slow rate of transport of transferrin from the rough endoplasmic reticulum is similarly unaffected by tunicamycin or 1-deoxynojirimycin. We previously postulated that the slow migration of transferrin could be due to bulk phase (receptor-independent) movement of the lumenal contents of the rough endoplasmic reticulum, a process akin to pinocytosis of extracellular fluids into the cytoplasm.

Since a particular processing intermediate on the α1-antitrypsin oligosaccharides is essential for its rapid transport from the rough endoplasmic reticulum, we presume that this oligosaccharide forms part of the receptor-binding sites on at least this protein, and probably also α1-antichymotrypsin. The slow maturation of nonglycosylated α1-antitrypsin to the Golgi, or of α1-antitrypsin synthesized in the presence of 1-deoxynojirimycin, might not involve binding at all to a receptor, but rather bulk-phase fluid movement from the rough endoplasmic reticulum to the Golgi. It is noteworthy that both 1-deoxynojirimycin and tunicamycin cause α1-antitrypsin and α1-antichymotrypsin to be secreted with the same slow kinetics as is transferrin (Fig. 1).

One might imagine that HepG2 cells contain different glucosidase I or II enzymes that act on different secretory proteins, that these enzymes have different sensitivities to 1-deoxynojirimycin, and that removal of glucose residues is essential for all glycoproteins to mature to the Golgi. Although we cannot eliminate this possibility, we note that 1-deoxynojirimycin concentrations from 5 to 25 mM had the same differential effect on the secretion of the five proteins studied. Thus it is unlikely that we did not achieve the full effect of 1-deoxynojirimycin on protein secretion.

There are other explanations of our data that we cannot eliminate. Removal of the glucose residue on the α1-antitrypsin oligosaccharides could induce a major change in the protein conformation that, in turn, could be reflected in an enhanced solubility in the endoplasmic reticulum lumen, (and thus in a faster movement by bulk phase) or in an enhanced binding to the putative receptor. Or, a specific receptor might not be involved at all. Rather, removal of the three glucose residues might modify some bulk property of the protein, such as its aggregation or surface charge, that might affect its incorporation into transport vesicles.

Bromoconduritol, a specific inhibitor of glucosidase II, also causes intracellular accumulation of high mannose oligosaccharides. This drug inhibits generation of complex oligosaccharides, and causes an inhibition of release of influenza proteins into virions (43). However, it is not known where the maturation of the influenza haemagluttinin or neuraminidase is blocked.

The secretory proteins that accumulate in cells treated with 1-deoxynojirimycin can be used to generate defined substrates for use in experiments that attempt to identify such a transport receptor. These studies are in progress.

TABLE II

Inhibition of Secretion of α1-Antitrypsin by 1-Deoxynojirimycin Is Reversible

Sample	Presence of 1-deoxynojirimycin	Fraction of labeled α1-antitrypsin secreted
	min	
1	0	1.00
2	0–240	0.38
3	0–30	0.83
4	0–60	0.89
5	20–240	0.98

Five plates of HepG2 cells were placed in methionine-free medium in the presence (samples 2–4) or absence (samples 1 and 5) of 1-deoxynojirimycin. After a 20-min incubation, they were pulse-labeled for 10 min, then chased for 240 min. In sample 2, 1-deoxynojirimycin was present throughout the chase. In samples 3 and 4, the 1-deoxynojirimycin-containing chase medium was removed at 30 min (sample 3) or 60 min (sample 4) and replaced with fresh medium, without drugs. Incubation at 32°C was continued for a total of 240 min. In sample 5, 1-deoxynojirimycin was added only after 20 min of chase.

We thank M. Boucher for her patience typing the manuscript, and Drs. Tulsiani, Touster, Truscheit, and Schmidt for gifts of drugs.

This work was supported by National Institutes of Health grants AI-08814-15 and GM-27989-04.

Received for publication 26 September 1983, and in revised form 28 December 1983.

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