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The Use of Glycosylation Inhibitors to Study Glycoconjugate Function

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I. Introduction

Complex carbohydrates have been shown to be intricately involved in numerous biological systems including, but not limited to, fertilization (1), lysosomal enzyme targeting (2), uptake of or removal of glycoproteins from the blood (3), cell–cell comunication as well as cell adhesion (4,5), host–parasite interactions (6), and numerous other

physiological processes (7,8). The fact that these many different systems have complex carbohydrates as a common theme has given rise to the term "glycobiology," which has been defined as "a study of the structure and function of the carbohydrate modifications on different types of macromolecules" (9).

Complex carbohydrates are found attached both to proteins and to lipids. The glycoproteins produced by eucaryotic cells can have either N-linked oligosaccharides or O-linked oligosaccharides, and frequently both types of structures are found on the same protein (10). In addition, the number of oligosaccharide chains attached to the protein can vary from only one to many chains. In the N-linked types (described in more detail below), the carbohydrate is attached to the amide nitrogen of asparagine residues that occur in the tripeptide sequences, Asn–X–Ser (Thr)(11). However, this sequon is not sufficient for glycosylation to occur since many –Asn–X–Ser(Thr)– sequences that are found in proteins are not glycosylated. It appears that the conformation of the protein around this sequence and perhaps its exposure are critical factors in addition of carbohydrate. On the other hand, in the more common types of O-linked structures such as the mucins, the oligosaccharides are attached by O-glycosidic bonds that usually involve GalNAc linked to the OH groups of serine or threonine residues, but there is no known amino acid sequence necessary for glycosylation (12).

In terms of lipids, the best known and most common types of glycolipids are the sphingolipids, which contain oligosaccharide chains composed of some of the following sugars: glucose, galactose, GlcNAc, GalNAc, sialic acid, and fucose. These sugars may be present in various configurations and combinations and are linked to a ceramide that is made up of fatty acid and sphingosine. These compounds are of considerable importance to medicine since certain gangliosides have been shown to be receptors for diphtheria and cholera toxins, while some cerebrosides are involved in bacterial attachment to animal cells. Since a number of current reviews have covered these compounds in detail, they will not be considered here (13,14). A more recently described type of glycolipid is the glycosyl phosphatidylinositol (GPI) type, in which a glycan of mannose and GlcNAc (and sometimes other sugars such as galactose) is attached to the membrane phospholipid phosphatidylinositol. These GPI molecules appear to be important in anchoring many proteins to the membrane (15,16), and some of the inhibitors of N-linked oligosaccharide assembly also affect the synthesis of the glycan portion of these anchors. They will be considered in more detail in a later section.

Although a considerable amount of information is available concerning the structures of N-linked oligosaccharides and GPI glycans, there is relatively little data concerning the function of the carbohydrate portion of these molecules. It appears that protein (or lipid) glycosylation has two major roles: to modulate or change the biochemical properties of these molecules, including such parameters as bioactivity, folding, stability or turnover, immunoreactivity, and so on; and to serve as a determinant in molecular recognition events, such as lysosomal enzyme targeting, parasite or symbiote binding or adhesion to host cells, attachment of toxins or other biologically important ligands to cell surfaces, and so on (17).

In most cases, it has been somewhat difficult to demonstrate a functional role for the carbohydrate portion of the glycoprotein. One obvious way to do this would be to obtain the particular glycoprotein without its carbohydrate and then compare the biological and biochemical properties of the unglycosylated form with those of the normal glycoprotein. There are a number of ways to produce glycoproteins without their associated carbohydrate, especially in the case of the N-linked glycoproteins. For example, one can remove the N-linked oligosaccharides with endoglycosidases, such as endoglucosaminidase H (Endo H), which cleaves "high-mannose" oligosaccharides between the two internal GlcNAc residues (18), or peptide glycosidase F, which hydrolyzes most N-linked oligosaccharides at the GlcNAc to asparagine bond (19). The problem with this approach is that many N-linked oligosaccharides are resistant to cleavage by these enzymes when the protein is in its native state. Therefore, it is necessary to denature the protein in order to obtain complete removal of the N-linked oligosaccharides, making this method untenable for functional studies. Another problem with this approach is that removing the carbohydrate after the protein has been synthesized and has achieved its proper conformation may be quite different physiologically than having the protein synthesized in the cell in the absence of carbohydrate.

A second possibility is to produce the glycoprotein of interest in a cell system in the presence of the antibiotic tunicamycin (20). This compound inhibits the GlcNAc-1-P transferase that catalyzes the first step in the "dolichol cycle," thereby preventing the formation of lipid-linked oligosaccharides (21). This inhibition usually results in a complete blockage in N-linked glycosylation (22). The difficulty with this approach is that many glycoproteins may be improperly folded or become insoluble when they are synthesized without any N-linked carbohydrate. As a result, physiological studies again become of questionable value.

A third alternative for preventing glycosylation is to use site-directed mutagenesis to modify the Asn–X–Ser sequon(s) that is glycosylated to one that is a "non-acceptor" sequon (23,24). The beauty of this method is that one can modify one glycosylation site at a time (assuming that there is more than one) and determine how an alteration at that site affects function. Or one can modify any number of these sites at the same time and compare the various proteins. Two potential obstacles are (1) the gene coding for the protein of interest must be available and (2) one must make the assumption that the change in the amino acid sequence does not itself affect protein conformation. This methodology has provided some valuable insights into the role of asparagine-linked glycosylation. However, if all N-linked oligosaccharide sequons are modified, then the same problem of improper folding or solubility that one finds with tunicamycin would also apply here. Another possibility is to modify glycosylation rather than prevent it, so that the resulting N-linked glycoprotein contains an altered oligosaccharide structure. One can then determine if and how this alteration affects the biological activity of a given glycoprotein. There are several ways to modify N-linked oligosaccharide structures. For example, the protein of interest can be produced in a mutant cell line that has a defect in one of the biosynthetic enzymes. Cell lines that are missing various glycosyltransferases or processing glycosidases have been isolated, and several excellent reviews have described the various mutants and the techniques for their utilization (25,26). In this case also, one must have the gene for the glycoprotein of interest in order to introduce it into the mutant cell line for synthesis of the appropriate protein.

The carbohydrate structure can also be modified with selected inhibitors that block the activity of key enzymes in the processing pathway, so that synthesis is stopped at a premature step and the various types of complex chains cannot be formed. Such inhibitors and their sites of action are the main topics of this chapter. One disadvantage on the use of such inhibitors in *in vivo* studies is that all N-linked glycoproteins are affected, and therefore it may be difficult to relate changes in physiology to an effect on the specific glycoprotein of interest. In fact, one criticism that can be levied at the use of inhibitors in most biological systems (and also as chemotherapeutic agents) is that absolute specificity is seldom if ever achieved. Of course in the above case, if the altered glycoprotein can be isolated from the inhibited (or mutated) cell and its activity compared with the normal protein, either *in vitro* or in another system, then this criticism can be overcome.

One final method for altering carbohydrate structure is to disrupt or alter the normal transport of glycoproteins from the ER through the various Golgi stacks to their ultimate location. Since it is now clear

that certain of the oligosaccharide trimming reactions occur in the ER, whereas others occur in the cis-Golgi and still others in the medial Golgi, etc., the basis of this approach is to prevent the protein from being transported to that region where the enzymatic activity is located. If one can somehow prevent the glycoprotein from reaching the specific compartment that houses that activity, then that reaction should not occur. There are several valuable compounds that have such properties and these drugs will be discussed in a later section. These types of inhibitors have been extremely useful tools for helping us to understand the mechanism(s) of protein targeting.

In terms of the glycan portion of the GPI anchor, a number of recent studies have demonstrated that various inhibitors that act on the N-linked oligosaccharides also inhibit some of the reactions in anchor formation. These inhibitors are providing useful information on the biosynthetic pathway and should also be helpful for functional studies. However, at this stage, we still do not know enough about the synthesis of the glycan portion of the GPI anchors, nor do we really understand their function. This chapter will deal with various types of inhibitors that affect GPI anchor formation, as well as those that act on the N-linked glycoproteins.

II. Structure and Biosynthesis of N-Linked Oligosaccharides

The most common types of glycoproteins that are present in eucaryotic cells, as cell surface proteins, membrane proteins, and secretory proteins, are those having oligosaccharides attached to the amide nitrogen of asparagine (N-linked or asparagine-linked) (10). The oligosaccharide portion of these molecules may have a variety of different structures that fall into one of the general groups shown in Fig. 1. These are referred to as high-mannose, complex, or hybrid types of oligosaccharides. It can be seen from the figure that each of these structures contains the same basic core region (shown by the boxed-in area) composed of the oligosaccharide sequence Manα1,6(Manα1,3) Manβ1,4GlcNAcβ1,4GlcNAc. In the high-mannose structures, this core region is elongated by as many as six additional mannose residues (i.e., a total of nine mannose units) attached in α1,2, α1,3, and α1,6 linkages, as shown by the typical structure presented in Fig. 1. However, some high-mannose oligosaccharides may have fewer than six additional mannoses, since some trimming can occur during biosynthesis (see Section V).

On the other hand, in the complex types of oligosaccharides, the pentasaccharide core region can be extended with a number of

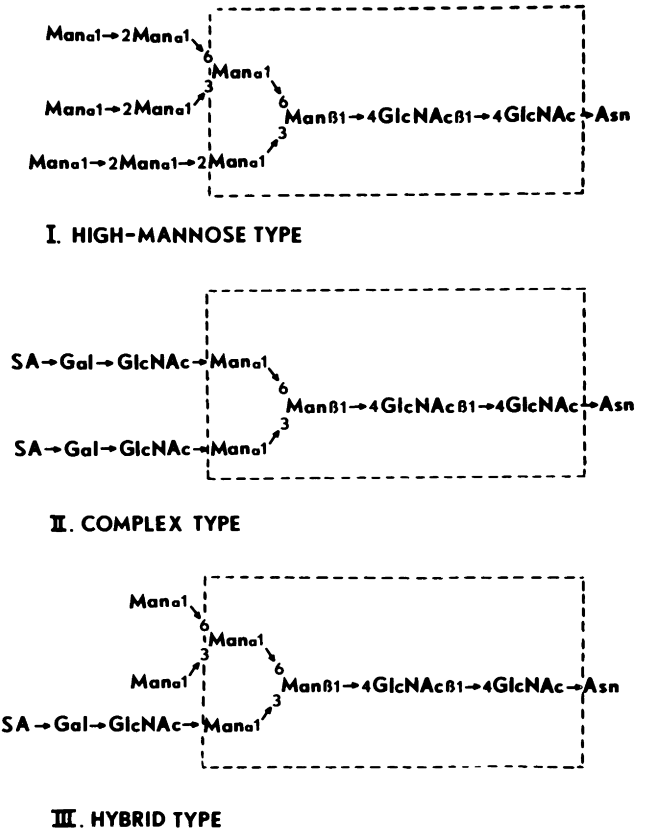


FIG. 1. Generalized structures of the various types of N- linked oligosaccharides. As indicated here, these structures are classified as high-mannose (top), complex (middle), and hybrid (bottom) structures.

different substitutions. Shown in the figure is a typical biantennary type of oligosaccharide, containing two of the trisaccharide sequences, NeuAcα2,6Galβ1,4GlcNAcβ1,2. On the other hand, some complex chains may have three (triantennary) or four (tetraantennary) of these trisaccharide sequences, as well as various other substitutions such as α1,6-linked L-fucose on the innermost GlcNAc or sulfate residues attached to this GlcNAc (10). Various other alterations may also occur, such as α1,3-linked fucose units on the outer GlcNAc residues, polylactosamine chains, a β1,4-bisecting GlcNAc residue attached to the β-linked mannose and so on, as well as various glycosidic linkages such as α2,3-linked sialic acid rather than α2,6-linked sialic acid, etc. Al-

though the exact number of different types of complex chains is not clear, it has been estimated that there are probably several hundred known structures (27).

The final type of N-linked oligosaccharide is the hybrid structure, which still contains a Manα1,6 (Manα1,3) branch on the α1,6-mannose arm, but also has a type of complex chain on the α1,3-branch. Most hybrid chains also apparantly have a bisecting GlcNAc attached in β1,4-linkage to the β-linked mannose, and this substitution may play a role in the control of further processing reactions.

The biosynthesis of all of these N-linked oligosaccharides involves two independent and sequential pathways that occur in the endoplasmic reticulum (ER) and Golgi apparatus of the cell. In the first series of reactions, membrane and secretory proteins that are undergoing synthesis on membrane-bound polysomes become glycosylated by transfer of a common oligosaccharide having the structure $Glc_3Man_9(GlcNAc)_2$ from its lipid-linked saccharide form, i.e., $Glc_3Man_9(GlcNAc)_2$-PP-dolichol, to specific –Asn–X–Ser (Thr)– sequences on the protein (28). This pathway is referred to as the "dolichol cycle" and is depicted in Fig. 2. A series of glycosyltransferases that are associated with the ER membrane sequentially add the sugars GlcNAc, mannose, and glucose to the lipid carrier dolichyl-P to give the above-mentioned lipid-linked oligosaccharide donor.

To study this pathway in animal cell culture systems, one can add one of the radioactive sugars, i.e., [^{3}H]glucosamine, [^{3}H]galactose (as a tag for the glucose residues), or [^{3}H]mannose, to the medium and examine the incorporation of the label into lipid-linked saccharides. These intermediates are easy to extract from the cells by a sequential extraction procedure, first with $CHCl_3$:CH_3OH:H_2O (1:1:1), which extracts the lipid-linked monosaccharides and smaller sized lipid-linked oligosaccharides, and then extraction of the remaining residue with $CHCl_3$:CH_3OH:H_2O (10:10:3) to obtain the lipid-linked oligosaccharides that contain the larger sized oligosaccharides. The intact lipid-linked monosaccharides can usually be identified by TLC, whereas the larger oligosaccharides from the lipid-linked oligosaccharides are usually released by mild acid hydrolysis and characterized by gel filtration on Bio-Gel P-4 or by HPLC (29,30).

Thus, the first enzyme in the biosynthesis of N-linked oligosaccharides transfers GlcNAc-1-P from UDP–GlcNAc to dolichyl-P to form dolichyl-PP-GlcNAc (31,32). This enzyme is the site of action of the very widely used glycosylation inhibitor tunicamycin (21) (discussed later). The GlcNAc-1-P transferase has been purified to homogeneity from bovine mammary glands (33), and the gene for this enzyme was recently cloned and expressed is COS cells (34). The next GlcNAc

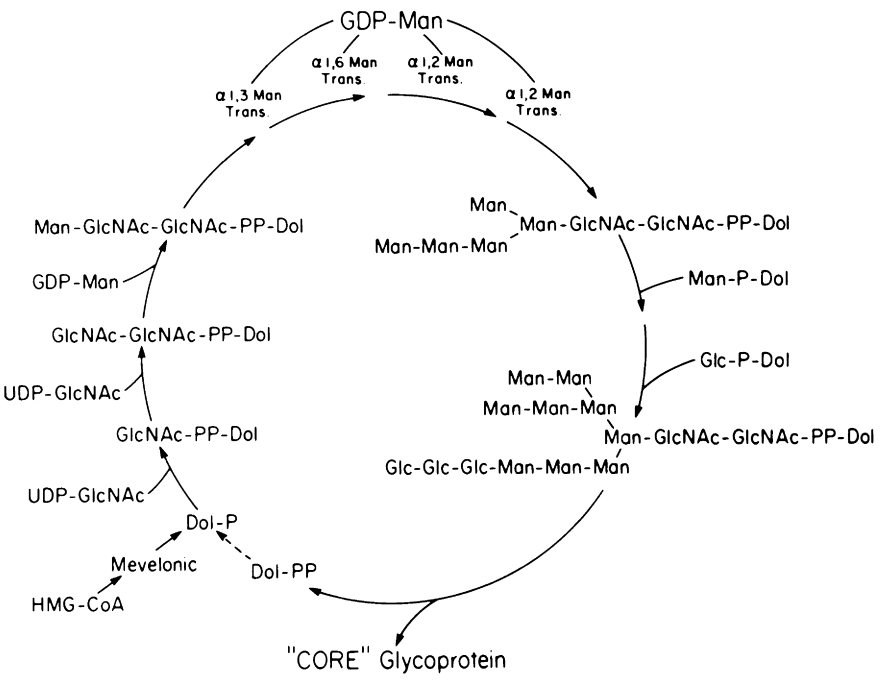


FIG. 2. Reactions involved in the assembly of the lipid-linked oligosaccharide that donates oligosaccharide to protein. This reaction sequence occurs in the ER of cells and involves the sequential transfer of sugars to dolichyl-P by a series of membrane-bound glycosyltransferases.

transferase adds the second GlcNAc, also from UDP–GlcNAc, to dolichyl-PP-GlcNAc to form GlcNAcβ1,4GlcNAc-PP-dolichol. This enzyme has recently been purified to homogeneity from pig aorta (35).

The next five sugars to be added are mannose residues and are donated via GDP–mannose. As indicated below, two lines of evidence have demonstrated that GDP–mannose, rather than dolichyl-P-mannose, is the mannosyl donor for these additions: (a) A mutant cell line that is missing the dolichyl-P-mannose synthase can still form the Manα1,2Manα1,2Manα1,3(Manα1,6)Manβ1,4GlcNAcβ1,4GlcNAc-PP-dolichol but is not able to further elongate it (36); and (b) amphomycin, an inhibitor of the *in vitro* formation of dolichyl-P-mannose (37), does not prevent the formation of the above $Man_5(GlcNAc)_2$-PP-dolichol, but it does block further elongation of this intermediate (38). The β-mannosyl transferase that adds the first mannose to the dolichol intermediates has recently been purified to homogeneity from pig aorta,

and an azido-labeled [^{32}P]GDP–mannose was used to identify the subunits of this protein (39). Several of the α-mannosyltransferases have also been partially purified from liver (40–43), and these enzymes require GDP–mannose rather than dolichyl-P-mannose as the mannosyl donor. However, these proteins have not yet been purified to homogeneity so that their detailed properties or their orientation in the ER membrane is not known.

Once the $Man_5(GlcNAc)_2$-PP-dolichol is formed, it can be further glycosylated by the addition of four more mannose residues and then three glucose units (44). These mannose residues all are believed to be donated via dolichyl-P-mannose (because of the topology of the oligosaccharide in the ER lumen and the results with mutants missing the dolichyl-P-mannose synthase) (36), whereas the glucosyl donor is dolichyl-P-glucose (45). Most of these enzymes have not been isolated or studied in any detail, and therefore little is known about their properties. However, the enzyme that adds the first mannose from dolichyl-P-mannose (i.e., the sixth mannose to the lipid) has been partially purified from aorta extracts and its substrate specificity was determined. For activity, this enzyme required dolichyl-P-mannose as the mannosyl donor and $Man_5(GlcNAc)_2$-PP-dolichol as the mannosyl acceptor (46). Likewise, the enzyme that adds the first glucose from dolichyl-P-glucose to $Man_9(GlcNAc)_2$-PP-dolichol was purified to near homogeneity from pig aorta and its substrate specificity and oligosaccharide acceptor specificity were determined. While a number of oligosaccharide structures linked to dolichol could serve as glucose acceptors, they all required the Manα1,2Manα1,2Manα1,3– branch for this activity (47). The enzyme(s) that adds the last two glucoses has not yet been purified.

The role of the glucose units in the biosynthesis of N-linked oligosaccharides is presumably to facilitate the transfer of oligosaccharide from lipid to protein. That is, the oligosaccharyltransferase that catalyzes this final step in the dolichol cycle recognizes or prefers glucose-containing lipid-linked oligosaccharides as the oligosaccharide donor (48,49). In fact, studies with microsomes showed that glucose-containing lipid-linked oligosaccharides were much more efficient oligosaccharide donors than were glucose-free lipid-linked oligosaccharides. Thus, this last step of the dolichol cycle occurs in the ER and the oligosaccharide is transferred to specific asparagine residues on the protein, as the polypeptide is being elongated on ER-bound polysomes (50). The oligosaccharyltransferase has recently been purified to apparant homogeneity from liver (51).

Once the oligosaccharide chain(s) is transferred to the protein, and while the protein is still undergoing synthesis in the ER, two

membrane-bound and highly specific glucosidases remove all three glucoses from the $Glc_3Man_9(GlcNAc)_2$. The processing pathway for N-linked oligosaccharides is outlined in Fig. 3. Thus, glucosidase I removes the outermost α1,2-linked glucose to give a $Glc_2Man_9(GlcNAc)_2$–protein (52), whereas glucosidase II removes the next two α1,3-linked glucoses to give the $Man_9(GlcNAc)_2$–protein (53). Glucosidase I has been purified from calf (54) and porcine (55) liver, from bovine mammary glands (56), from mung bean seedlings (57), and from *Saccharomyces cerevesiae* (58). This enzyme has a pH optimum in the neutral range (6.4–6.8), does not work with *p*-nitrophenyl-α-D-glucopyranoside, and is quite specific for α1,2-glucosidic linkages. The mammalian enzyme is a tetramer with subunits of about 85 kDa, whereas the yeast enzyme has subunits of about 95 kDa. In *S. cerevesiae*, the *gls1* mutation results in cells that lack glucosidase I and produce glycoproteins that maintain the $Glc_3Man_9(GlcNAc)_2$ structure (59). However, this alteration in structure has no effect on secretion of the glycoproteins, in contrast to mammalian cells in which such an alteration had profound effects (see section on glucosidase inhibitors). A CHO mutant cell line has been isolated by virtue of its resistance to L-PHA,

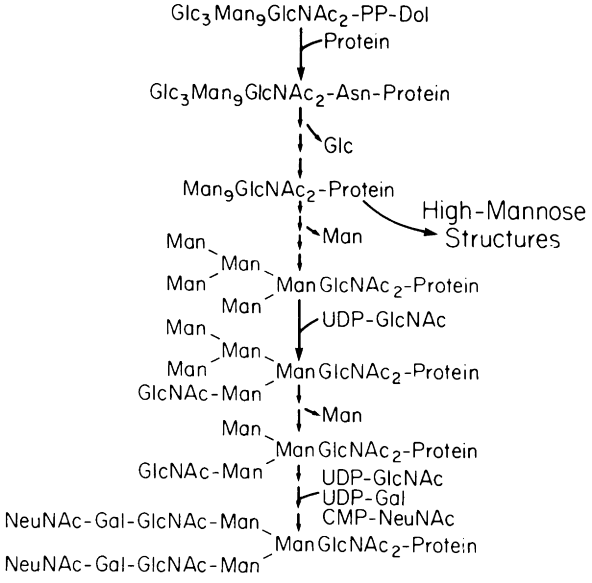


FIG. 3. Reactions involved in the processing of the protein- bound oligosaccharide. A series of glycosidases and glycosyltransferases, localized in the ER and Golgi, processes the oligosaccharide chains to give rise to the various types of N-linked structures seen in Fig. 1.

and this mutant cell, called Lec23, has been shown to lack glucosidase I (60). These cells were found to synthesize the G protein of VSV having hybrid and high-mannose oligosaccharides, as well as complex chains.

Glucosidase II was initially identified in microsomal fractions of rat liver (61), calf liver (62), and yeast (63) and was shown to remove both of the α1,3-linked glucoses from $Glc_2Man_9(GlcNAc)_2$ to form $Man_9(GlcNAc)_2$. However, removal of the outermost α1,3-linked glucose is quite rapid ($t_{1/2}$ = 5 min) as compared with cleavage of the innermost glucose ($t_{1/2}$ = 20–30 min). Although glucosidase II also can catalyze the cleavage of aryl-α-glucosides such as *p*-nitrophenyl-α-glucopyranoside, it appears to be fairly specific for α1,3-linked glucoses since its activity is inhibited by the α1,3-linked glucose disaccharide nigerose, but not by other α-linked glucose disaccharides (i.e., kojibiose, maltose, isomaltose, or trehalose) (64). Glucosidase II from pig kidney was reported to have a subunit molecular mass of 100 kDa and to be a high-mannose glycoprotein (65), whereas the enzyme from mung beans was composed of two identical glycoprotein subunits of 110 kDa, also with high mannose oligosaccharides (some of which probably also contain some glucose residues) (64). In some animal systems, however, the glucosidase II had quite different subunit molecular weights of around 65 kDa (56,66). The localization of the enzyme has also been studied and may vary, depending on the tissue, from being concentrated in rough ER (RER) and smooth ER (SER) in pig hepatocytes (67) to post-Golgi apparatus structures in pig kidney tubular cells (65).

The best substrates for assaying these processing glucosidases are radiolabeled $Glc_3Man_9GlcNAc$ and $Glc_2Man_9GlcNAc$. These substrates can be prepared by incubating animal cells (or virus-infected cells) in the presence of the processing inhibitors castanospermine (discussed later), to prevent the removal of glucose residues, and deoxymannojirimycin, to prevent the removal of α1,2-linked mannoses. [^{3}H]Galactose is then added to the cell cultures to label the glucose moieties in the newly synthesized glycoproteins. The glycopeptides resulting from pronase digestion of the glycoproteins are isolated by gel filtration on columns of Bio-Gel P-4, and these glycopeptides are then treated with Endo H to obtain the [^{3}H]glucose labeled $Glc_3Man_9GlcNAc$. This oligosaccharide can then be treated with a glucosidase I preparation that is free of glucosidase II and α-mannosidases to obtain [^{3}H]$Glc_2Man_9GlcNAc$. For assay of the processing glucosidase activities, the previously mentioned radiolabeled substrates are incubated with the appropriate enzyme preparations, and the release of labeled glucose is measured by a Concanavalin A–Sepharose binding assay (68).

The removal of three glucose residues from the $Glc_3Man_9(GlcNAc)_2$–protein results in the formation of a high-mannose Man_9

$(GlcNAc)_2$–protein, which can then be further processed to other high-mannose or complex types of oligosaccharides (as seen in Figs. 1 and 3). Early studies on the processing pathway indicated that there were two or three "neutral" α-mannosidase activities involved in trimming the N-linked oligosaccharide, and these activities were distinct from those of the lysosomal α-mannosidase (69). As a result of increasing interest in this area of research, a number of new α-mannosidase activities have been described and current work is aimed at trying to understand how these various enzymes function in the removal of α-linked mannoses from eucaryotic cell glycoproteins. In fact, one of the important reasons for having a number of specific mannosidase inhibitors (see Section V) is to be able to use them to distinguish among various mannosidase activities, as well as to determine what happens to processing and function of a given glycoprotein when a specific α-mannosidase is inhibited. A well-written review deals with the various α-mannosidases (70), so these enzymes will be only briefly covered here.

An ER α-mannosidase that removes a single α1,2-linked mannose from $Man_9(GlcNAc)_2$ to give a specific $Man_8(GlcNAc)_2$ isomer has been purified from yeast (71). This enzyme is inhibited by the processing inhibitor deoxymannojirimycin, requires Ca^{2+} for activity, and does not work with aryl-α-mannosides. The gene for the enzyme has been isolated and codes for a 63-kDa protein with three *N*-glycosylation sites, a Ca^{2+} binding consensus sequence, and a noncleavable signal sequence near the N terminus that acts as a transmembrane type II domain (72). A Ca^{2+} requiring α1,2-mannosidase was also purified from rabbit liver and this enzyme shows considerable homology (38% identity and 58% similarity) to the yeast enzyme (73). However, the rabbit liver enzyme can remove most if not all of the α1,2-linked mannoses from $Man_9(GlcNAc)_2$, and it requires either nonionic detergents or phospholipids for activity (74). Two other Ca^{2+} requiring α-mannosidases have been purified from calf (75) and pig (76) livers, and these enzymes differ from the rabbit enzyme in some of their properties. These enzymes also do not work with the aryl-α-mannopyranosides as substrates. The pig liver enzyme was localized by immunological techniques and found to be distributed in RER, SER, transitional elements of the RER, and vesicles that engage in transportation between the ER and the Golgi, but it was not in the Golgi (77).

Actually, the first neutral α-mannosidase to be reported was named mannosidase I and this enzyme was isolated from the Golgi apparatus of eucaryotic cells (78). This α-mannosidase has catalytic properties very similar to those of the Ca^{2+}-dependent mannosidases, but it apparantly does not need Ca^{2+}, although this is not certain because the requirement was not tested in the presence of EDTA (79). The mannosi-

dase I was resolved into two distinct proteins by chromatographic methods and these two forms were termed mannosidase IA and IB. IA was purified to homogeneity and shown to be a tetramer composed of 56 kDa glycoprotein subunits (80). Presumably IA and IB have similar substrate specificities, but it is not clear if they are derived from the same gene.

Once the four α1,2-linked mannose units are removed, a medial Golgi GlcNAc transferase (called GlcNAc transferase I) adds a GlcNAc from UDP–GlcNAc to the mannose that is attached to the β-linked mannose in α1,3-linkage (81). This GlcNAc transferase was purified over 100,000-fold to homogeneity from liver (82) and the enzyme has been sequenced and cloned (83). The GlcNAc is apparantly a recognition signal for another highly specific α-mannosidase, called mannosidase II, that cleaves the α1,3-linked and α1,6-linked mannoses from the GlcNAc–$Man_5(GlcNAc)_2$–protein to give a GlcNAcβ1,2Manα1, 3[Manα1,6]Manβ1,4GlcNAcβ1,4GlcNAc-protein (84). In spite of this substrate specificity, mannosidase II does act on the aryl-α-mannopyranosides such as *p*-nitrophenyl-α-D-mannopyranoside. Mannosidase II was purified to homogeneity from rat liver (85,86) and from mung bean seedlings (87). Both enzymes migrate on SDS gels as proteins of about 125 kDa, although the sequence for the murine enzyme predicts a MW of 132 kDa for the deglycosylated protein. Both enzymes are also glycoproteins and the murine enzyme has been shown to be a type II transmembrane glycoprotein with a single transmembrane region and a luminally oriented catalytic site (88).

There are several other α-mannosidases that are distinct from the above enzymes based on their sensitivity to processing inhibitors and to catalysis of high-mannose oligosaccharides. However, the relative role of these enzymes in the glycoprotein processing pathway remains to be established. One of these enzymes is a cytosolic and neutral α-mannosidase from rat liver (89). Although it was originally thought that this enzyme was a proteolytic product of an ER α-mannosidase, it now appears that both the cytosolic and the ER α-mannosidase are related to an α-mannosidase that was isolated from rat brain and that can cleave α1,2-, α1,3-, and α1,6-linked mannose residues (90). This last enzyme can cleave a $Man_9(GlcNAc)_2$ structure to a $Man_3(GlcNAc)_2$. The difference between the ER liver enzyme and the brain enzyme is that the former enzyme is also active with aryl-α-mannosides, whereas the latter is not. Two other α-mannosidases that can cleave α1,2-, α1,3-, and α1,6-linked mannoses have been purified from rat sperm (91) and rat liver (92).

Another type of hydrolytic enzyme that is also proposed to be involved in processing is an endo-α-mannosidase that can cleave a Glcα1,3Man

disaccharide from $Glc_1Man_9(GlcNAc)_2$ (or a Glc_3Man from $Glc_3Man_9(GlcNAc)_2$, etc.) (93). It is interesting to note that the $Man_8(GlcNAc)_2$ that results from the action of this enzyme is a different isomer from the $Man_8(GlcNAc)_2$ that is formed by the action of the ER α-mannosidase or the yeast α-mannosidase (94). This endomannosidase appears to be a resident of the Golgi apparatus and may function to trim oligosaccharides on glycoproteins that have escaped the ER still retaining one or more glucose residues.

As shown in Fig. 3, following the trimming of all of the glucose residues and a number of mannose units from the N-linked oligosaccharides, various glycosyltransferases act to add different sugars, such as GlcNAc, galactose, sialic acid, fucose, etc., to the core N-linked structure to form many different types of complex chains (95). Since there are no inhibitors available for these glycosyltransferases, they will not be considered further in this review.

III. Structure and Biosynthesis of Glycosyl Phosphatidylinositol Anchors

Glycosyl phosphatidylinositols are a recently discovered class of glycolipids that have been shown to anchor many proteins (and other molecules such as heparan sulfates) to biological membranes via covalent linkages. Recently, the chemical nature of these molecules has been deduced, but the complete structure is still elucidated only for a few of these anchors. The characterization of these glycolipids has involved compositional analyses using GC–MS in combination with specific chemical treatments and sequential digestion with exoglycosidases, analysis of glycosidic linkages by methylation analyses, and gel filtration and TLC of intact glycans and their degradation products (96). The best known and most thoroughly detailed GPI anchors are those of *Trypanosoma brucei* and rat Thy-1. Although these structures show some differences, they do have a conserved linear region shown in

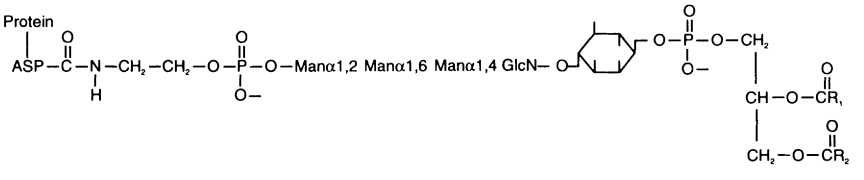


FIG. 4. General structure of the glycosylphosphatidylinositol that serves as a membrane anchor for many cell surface proteins.

Fig. 4 to be composed of 6-*O*-ethanolamine-P-6-Manα1,2Manα1, 6Manα1,4GlcNα1,6-inositol (97). In the case of the variable surface antigen (i.e., VSG) of trypanosomes, there are a number of galactose residues attached to the first mannose, whereas in Thy-1 there may be a GalNAc and/or an ethanolamine-P attached to this mannose (98). Also these structures may differ in the number of fatty acid residues attached to the inositol and also in the structure of the fatty acids.

Although some of the details of the biosynthesis of the glycan portion of these anchors are known, there are still considerable gaps in our understanding of the pathway. Nevertheless, the steps in biosynthesis are proposed to occur as outlined in Fig. 5. The sequence begins with the transfer of a GlcNAc from UDP–GlcNAc to phosphatidylinositol to form GlcNAc-PI, which then undergoes deacetylation to form GlcN-PI (99). These reactions have been demonstrated *in vivo* by labeling studies with ^{3}H-glucosamine and in cell-free extracts incubated with UDP–GlcNAc. Three mannose residues are then added to the GlcN-PI and these mannoses are all proposed to be derived from dolichyl-P-mannose, rather than from GDP–mannose (see Section IX for experimental evidence). None of these enzymes has been purified and virtually nothing is known about how other sugars such as Gal or GlcNAc are added. In addition, it is not clear when in the biosynthetic pathway the fatty acids are added to the PI, although there is now evidence that the GlcN-PI must be acylated to serve as an acceptor for the first mannose (100). Once the mannose units have been added, phosphatidylethanolamine serves as a donor of ethanolamine-P, which becomes attached via a phosphodiester bond to the 6 position of the terminal mannose. The signal for GPI addition to the nascent protein resides in a short hydrophobic carboxy-terminal peptide that is removed and replaced by the GPI anchor (101). This addition of protein to the GPI apparantly occurs on internal membranes, but the details of these biosynthetic steps are not known at this time.

IV. Inhibitors of Lipid-Linked Saccharide Synthesis

A. Tunicamycin

Probably the most widely used and most valuable inhibitor of N-linked glycosylation is tunicamycin. This compound is a nucleoside antibiotic that is produced by the bacterium *Streptomyces lysosuperificus* and was initially found to inhibit the replication of a number of enveloped viruses (102). The structure of tunicamycin was elegantly

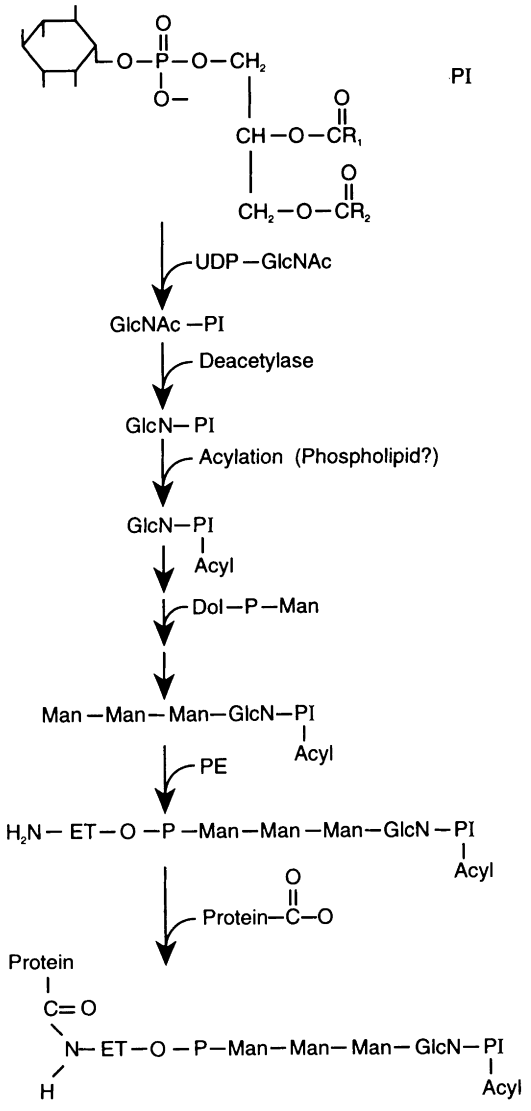
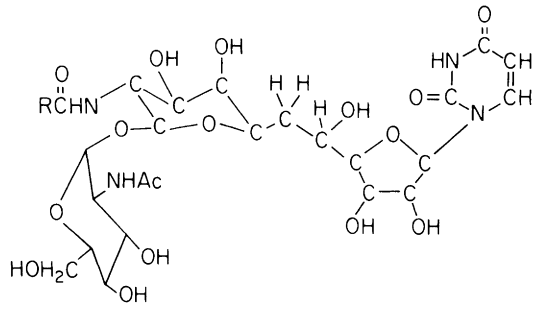


FIG. 5. Outline of the general pathway for biosynthesis of the glycan portion of the GPI anchors.

elucidated by Tamura and co-workers (103) and is shown in Fig. 6. The antibiotic contains a uracil moiety to which is attached, by an N-glycosidic bond, an 11 carbon aminodeoxydialdose called tunicamine. The tunicamine is itself substituted by having a GlcNAc attached to



I : R = $CH_3)_2CH(CH_2)_7CH{=}CH-$
II : $(CH_3)_2CH(CH_2)_8CH{=}CH-$
III : $CH_3(CH_2)_{10}CH{=}CH-$
IV : $CH_3(CH_2)_{11}CH{=}CH-$
V : $(CH_3)_2CH(CH_2)_9CH{=}CH-$
VI : R = $(CH_3)_2CH(CH_2)_{11}-$
VII : $(CH_3)_2CH(CH_2)_{10}CH{=}CH-$
VIII : $CH_3(CH_2)_{12}CH{=}CH-$
IX : $CH_3(CH_2)_{13}CH{=}CH-$
X : $(CH_3)_2CH(CH_2)_{11}CH{=}CH-$

FIG. 6. The structure(s) of the various tunicamycin components. *R* refers to the different fatty acids, designated I to X, that may be attached to the tunicamine portion of the molecule.

its anomeric carbon in an O-glycosidic bond and a fatty acid linked in amide bond to the amino group. The fatty acid may vary in structure from C_{12} to C_{16}, may be saturated or unsaturated, and may be branched or unbranched. In fact, tunicamycin is actually produced as a complex that can be separated into as many as 10 to 15 peaks by HPLC (22,104), and the basis of this separation is the fatty acid moiety. Interestingly enough, all of these peaks have biological activity, but those components with the longer chain fatty acids appear to be more effective inhibitors in cell culture (22), possibly because they are taken up by the cells at a faster rate.

The site of action of tunicamycin was initially demonstrated with a microsomal enzyme preparation from calf liver, and the antibiotic was shown to be a potent inhibitor of the enzyme that transfers GlcNAc-1-P from UDP–GlcNAc to dolichyl-P (32,105). This reaction produces dolichyl-PP-GlcNAc, which is the first intermediate in the dolichol pathway. Tunicamycin had the same site of action in plant microsomes (106) and in microsomes from chick oviduct (107). In addition, tunicamycin is a very potent inhibitor with partially purified preparations of the UDP–GlcNAc:dolichyl-P GlcNAc-1-P transferase from several animal tissues (22,32,108). This inhibitor also affects several microbial enzymes that are involved in the synthesis of peptidoglycan, i.e., the enzyme that forms undecaprenyl-PP-*N*-acetylmuramoyl-pentapeptide

(109), and the enzyme involved in production of undecaprenyl-PP-GlcNAc (110).

However, tunicamycin had no effect on other GlcNAc transferases such as the enzyme that adds the next GlcNAc to dolichyl-PP-GlcNAc (111), the enzymes that add terminal GlcNAc residues to the N-linked oligosaccharide core (107), the phosphotransferase that adds GlcNAc-1-P to high-mannose chains on lysosomal enzymes (112), and the transferase that adds GlcNAc to serine residues on nuclear proteins (113). It also had no effect on the formation of undecaprenyl-PP-galactose or undecaprenyl-PP-GalNAc (114), but at high concentrations it did inhibit formation of dolichyl-P-glucose (115). Summing up these data, it appears that tunicamycin inhibits phosphotransferases that catalyze the translocation of a GlcNAc-1-P from its UDP derivative to a polyprenyl-P, but the sugar must be chirally related to GlcNAc. Tunicamycin is probably a competitive inhibitor of the GlcNAc-1-P transferase, but it binds so tightly to this enzyme that it has not been possible to reverse the inhibition by adding high concentrations of UDP–GlcNAc (105). It was suggested that this compound was a tight-binding reversible inhibitor and might be a substrate-product transition-state analog (32).

Tunicamycin has been used in many cell culture systems to try and assess the role of N-linked glycosylation in the function of various glycoproteins. Probably the best test of whether tunicamycin is really effective in preventing glycosylation in cell culture is to determine whether it blocks the *in vivo* incorporation of labeled glucosamine into dolichyl-PP-GlcNAc or the incorporation of [2-^{3}H]mannose into lipid-linked oligosaccharides. To do this, one can incubate cell cultures in the presence of various concentrations of tunicamycin (usually 0.1 to 10 μg/ml) for 30 min to 1 hr and then add the labeled glucosamine or mannose to these cells. After an appropriate period of labeling (determined experimentally for each cell type), the cell pellet is extracted with $CHCl_3$:CH_3OH:H_2O (1:1:1) and then $CHCl_3$:CH_3OH:H_2O (10:10:3) to obtain the lipid-linked monosaccharides and the lipid-linked oligosaccharides (116). One important point here is that the effective concentration of inhibitor may vary widely depending on the cell system being used, and the optimum concentration needs to be determined in each case. In fact, it has been demonstrated that tunicamycin-resistant mutants can be isolated and these mutant cells have amplified levels of the GlcNAc-1-P transferase (117). Thus, it seems reasonable to assume that the level of this enzyme, and therefore the susceptibility of the cell to tunicamycin, may vary from cell to cell or from tissue to tissue.

Something else to consider when using tunicamycin for functional studies is that this compound may inhibit protein synthesis (118). For this reason, any effects on glycoprotein function should be thoroughly

examined to be certain that they result from the absence of carbohydrate, rather than from the loss of certain proteins. For example, in some of the various studies, at tunicamycin concentrations of 0.1 to 10 μg/ml, [^{3}H]leucine incorporation into protein was inhibited from 10 to 60%, while glycosylation was inhibited from 70 to 90%. But again, the important point is that some cell types are much more sensitive to inhibitors than are other cell types. Perhaps one control for some of these studies would be to use other inhibitors (i.e., cycloheximide or puromycin) that are known to block protein synthesis but do not affect carbohydrate synthesis or addition, or some other inhibitors that modify the structure of the carbohydrate chain, and compare the effects of these compounds with those of tunicamycin on the function of the particular glycoprotein.

The results of producing glycoproteins in cells in the presence of tunicamycin vary considerably depending on the glycoprotein in question. In terms of structural studies, tunicamycin at the appropriate concentration seems to prevent glycosylation of all N-linked glycoproteins, regardless of the biological system. However, in terms of the protein portion of the molecule, some glycoproteins are still synthesized at a normal rate and are still secreted even when they are not glycosylated (119). Thus, no effect of this inhibitor on the secretion of procollagen from chick embryo cells, secretion of a number of serum proteins from rat hepatocytes, interferon secretion by leukocytes or L cells, secretion of the enzyme β-*N*-acetylhexosaminidase by fibroblasts, and so on was observed (120). The conclusion here is that a number of different types of cells and glycoproteins do not require their carbohydrate for either folding or conformation and appear to function normally in the absence of added carbohydrate.

Some proteins, however, are greatly affected by the absence of carbohydrate. Thus, a considerable decrease in the rate of secretion of IgE and IgA by mouse and rat plasma cells was observed in the presence of tunicamycin, as was the secretion of IgM in hybridoma cells (121). One rather strange and interesting result that emerged from these studies was the observation that tunicamycin inhibited IgM secretion in hybridoma cells, but did not affect the secretion of IgG. These results led the authors to hypothesize that the differential effects of this inhibitor were due to factors intrinsic to the respective heavy-chain polypeptides themselves, rather than to some property of the cells (122). It would seem likely that such factors are related to the conformation of the respective proteins and to the influence of the carbohydrate on protein structure and/or folding.

Not surprisingly, tunicamycin also has dramatic effects on some membrane receptors, such as the acetylcholine receptor of muscle cells (123), the insulin receptor (124), the receptor for low-density

lipoproteins (LDL) (125), and the receptor for nerve growth factor (126), to cite only a few examples. In general, with all of these receptors, tunicamycin significantly decreased the number of receptor molecules at the cell surface, but did not change the total number of receptors in the cells or the binding affinity of those receptors that were present in the plasma membrane. In some of these studies, the effects of tunicamycin were reversible, and in one study with the insulin receptor, it appeared that the nonglycosylated receptor could become glycosylated after removal of the drug (127). That would suggest that in some cases, proteins might be glycosylated post-translationally. Since there is some precedence for surface glycoproteins being endocytosed and recycled back through the Golgi (and perhaps the ER) (128), it is conceivable that glycosylation could occur on some exposed asparagine sequons.

Probably one of the reasons for the great variation in tunicamycin effects on protein function is that some glycoproteins require their N-linked oligosaccharides for stability (or longevity), and in the absence of glycosylation these proteins are rapidly degraded. That is, the carbohydrate may protect or somehow prevent the degradative enzymes from attacking glycosylated proteins. Such has been suggested to be the case for fibronectin, acetylcholine receptor, influenza viral hemagglutinin, and various immunoglobulins, just to cite a few examples (129). In fact, in the influenza system, it was observed that when viral replication was examined in the presence of tunicamycin, the viral envelope glycoproteins could not be detected. However, when a protease inhibitor, such as TCPK or leupeptin, was added to the cells along with the tunicamycin, the viral proteins in their nonglycosylated form could be detected (130). These studies and others cited above all indicate that the influence of the N-linked oligosaccharides on the protein in question depends on its amino acid sequence, which in turn determines folding and conformation. Thus, the carbohydrate may play a much greater role in affecting or maintaining the conformation of some proteins than that of others.

One study that provides strong support for the hypothesis that the amino acid sequence of the protein determines what type of and how important a role the carbohydrate plays in the function of the glycoprotein was done using various temperature-sensitive mutants of vesicular stomatitis virus (VSV) that were grown in the presence of tunicamycin. With one strain of VSV (called the San Juan strain), tunicamycin prevented viral replication by more than 90% when the virus was grown at either 38 or 30°C. However, with another strain (called the Orsay strain), the drug inhibited viral replication by 90% when the virus was grown at 37°C, but there was only 30 to 50% inhibition at 30°C. Furthermore, when this virus was grown in tunicamycin at 30°C,

the nonglycosylated viral envelope protein (G protein) was detected at the host cell surface, indicating that it was synthesized and targeted in the normal fashion. However, at 37°C, this protein could not be detected at the cell surface. Finally, studies on the physical properties of these various nonglycosylated proteins also indicated that these proteins differed markedely in their solubility in detergents such as Triton X-114 at the different temperatures (131). The probable explanation for such results is that the carbohydrate has a significant role in providing a conformation that has increased solubility as well as stability.

B. Tunicamycin-Related Antibiotics

Five other antibiotics have been described that are now known to be structurally and functionally related to tunicamycin. These antibiotics are streptoviridin (132), mycospocidin (133), antibiotic 24101 (134), antibiotic MM 19290 (135), and corynetoxin (136). All of these compounds have the same general structure as tunicamycin, but they differ in the nature of the fatty acids attached to the tunicamine. Thus, in some cases, they may be identical to the various tunicamycin homologs discussed earlier. For example, the streptoviridin components have shorter chain fatty acids than the tunicamycins, as evidenced by the fact that their molecular weights range from 790 to 814, whereas those of tunicamycins are from 802 to 858 (22,132). On the other hand, HPLC comparison of the mycospocidin components with those from the tunicamycin complex showed a number of identical components (133). The corynetoxins are an interesting group of tunicamycin-related compounds that appear to be involved in or responsible for ryegrass toxicity. In this case, the developing seed heads of annual ryegrass may be invaded by a nematode which then causes a gall to be produced. Some of the nematodes carry the bacterium *Corynebacterium rathayi,* which produces the tunicamycin analog corynetoxin. The corynetoxins are apparantly toxic to sheep that eat the infected ryegrass (136).

All of these compounds appear to have the same action as tunicamycin, i.e., to inhibit the GlcNAc-1-P transferase. Detailed studies on the mechanism of action were done with streptoviridin and this inhibitor was compared with tunicamycin on the crude and partially purified preparations of the enzyme, as well as in cell culture systems (115). As indicated above for the various tunicamycin homologs, this group of analogs also showed differences in inhibitory activity, and those compounds with the longer chain fatty acids were more effective inhibitors in cell culture systems than those with shorter chain fatty acids. In addition, studies with cell-free extracts or partially purified

GlcNAc-1-P transferase preparations showed that the streptoviridins had a lower affinity for the enzyme than did the tunicamycin complex (115). In fact, with this inhibitor it was possible to demonstrate competitive inhibition with regard to UDP–GlcNAc, because the affinity of the GlcNAc-1-P transferase for streptoviridin was considerably lower than that for tunicamycin.

C. *Lipopeptide Antibiotics*

A number of other antibiotics and other compounds have been found to be useful inhibitors of some of the steps in the lipid-linked saccharide pathway. Many of these inhibitors, especially the antibiotics, were initially discovered because they block the formation of bacterial cell wall polymers such as peptidoglycan or lipopolysaccharide. Because the formation of these polymers also involves polyprenyl-linked saccharide intermediates, these compounds appeared to be likely candidates to test on the N-linked glycosylation pathway. For example, the antibiotics amphomycin and tsushimycin inhibit the transferase that adds phospho-*N*-acetylmuramoyl-pentapeptide to the growing peptidoglycan chains (137), whereas bacitracin inhibits the enzyme that catalyzes the dephosphorylation of undecaprenyl-pyrophosphate (138), the carrier lipid in cell wall biosynthesis. These antibiotics also inhibit the formation of N-linked oligosaccharide assembly as discussed later.

Amphomycin is a hydrophobic undecapeptide containing either 3-isododecanoic or 3-anteisododecanoic acids attached to the N-terminal aspartic acid in an amide linkage (139). The structure of amphomycin is presented in Fig. 7A. In cell-free extracts of aorta tissue, amphomycin blocked the formation of dolichyl-P-mannose from GDP–mannose, but it did not affect the transfer of mannose to lipid-linked oligosaccharides from either dolichyl-P-mannose or GDP–mannose (37,38). Thus, when microsomes were incubated with GDP–[^{14}C]mannose in the presence of enough amphomycin to completely inhibit the formation of dolichyl-P-[^{14}C]mannose, these extracts accumulated ^{14}C-$Man_5(GlcNAc)_2$-PP-dolichol, but they were not able to elongate this lipid to larger sized oligosaccharide-lipids. On the other hand, when dolichyl-P-[^{14}C]mannose was used as the substrate with these extracts, mannose was transferred to oligosaccharide-lipids that contained six or more mannose residues. These studies suggested that the first five mannose residues in the lipid-linked oligosaccharides were donated from GDP–mannose, whereas the next four mannoses came from dolichyl-P-mannose (37). Similar results were obtained with oviduct tissue (140) and embryonic liver (141). The isolation of a CHO mutant cell line missing the dolichyl-P-mannose synthase clearly established that doli-

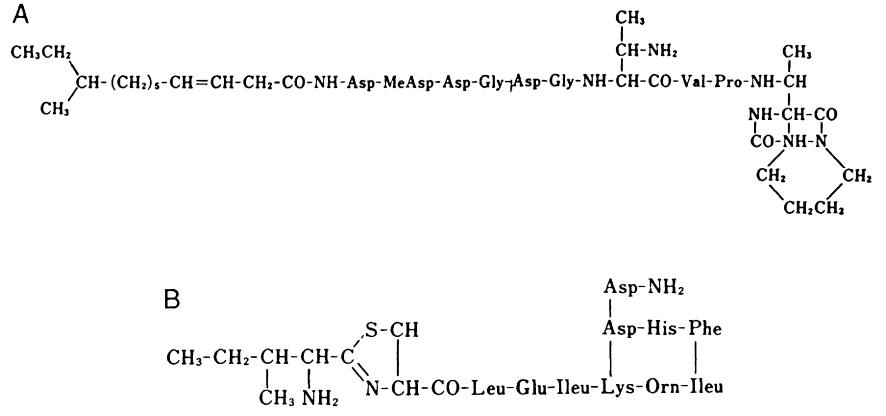


FIG. 7. Lipopeptide antibiotics that inhibit lipid-linked saccharide formation. (A) Structure of amphomycin. (B) Structure of bacitracin.

chyl-P-mannose was the mannosyl donor for the last four mannose residues (36).

In cell-free extracts of brain tissue, amphomycin inhibited the production of dolichyl-P-glucose, dolichyl-P-mannose, and dolichyl-PP-GlcNAc, leading these investigators to suggest that amphomycin might form a complex with dolichyl-P and dolichyl-P derivatives (142). Some evidence for such a complex between dolichyl-P and amphomycin has been obtained, although it is not clear how this relates to the inhibition of lipid-linked saccharide formation. Amphomycin has also been used in various other experiments, such as one study that demonstrated that dolichyl-P was involved in sugar translocation across the ER membrane (143), and in one case to show that dolichyl-P-mannose, rather than GDP–mannose, was involved in the elongation of $Man_7(GlcNAc)_2$-PP-dolichol (144). Another lipopeptide antibiotic, called tsushimycin, has a mechanism of action similar to that of amphomycin (145). Unfortunately, these peptide antibiotics do not appear to be effective in cell cultures of eucaryotic cells, probably because they do not cross the plasma membrane.

Bacitracin is another lipopeptide antibiotic that has been useful in studying both bacterial cell wall biosynthesis and the synthesis of lipid-linked saccharides. The structure of this compound is presented in Fig. 7B. Bacitracin was shown to inhibit the synthesis of bacterial cell wall peptidoglycan by inhibiting the dephosphorylation of undecaprenyl-pyrophosphate, which is necessary for the reutilization of this carrier molecule (138). In animal cells, the specific site of inhibition by this

antibiotic is not clear because it has been reported to act at several different points, including some of the mannosyltransferases that add mannose to the trisaccharide-lipid Manβ(GlcNAc)$_2$-PP-dolichol, the GlcNAc-1-P transferase that forms GlcNAc-PP-dolichol, and the GlcNAc transferase that forms GlcNAc-GlcNAc-PP-dolichol (146). It seems likely that the action of this inhibitor is similar to that of amphomycin; i.e., it may bind various dolichyl derivatives under the right conditions and therefore may interfere with a variety of reactions in the "dolichol cycle."

D. Other Inhibitors of Lipid-Linked Saccharide Formation

Several other antibiotics that affect the synthesis of bacterial cell walls are also effective on the reactions described here. Generally, these compounds have not been well defined structurally or functionally, but some of their inhibitory actions have been reported. The structure of diumycin is not known, but it was reported to inhibit the formation of dolichyl-P-mannose in a yeast membrane preparation, as well as the transfer of mannose from dolichyl-P-mannose to serine or threonine residues (147). In a membrane preparation from *Acanthamoeba*, diumycin blocked the transfer of mannose and GlcNAc to lipid-linked monosaccharides, but it did not affect the synthesis of dolichyl-P-glucose (148). In fact, diumycin (and another antibiotic, showdomycin) has been used to add support to the hypothesis that dolichyl-P-mannose is a positive regulator of the GlcNAc-1-P transferase that forms GlcNAc-PP-dolichol (143). Thus, when either of these drugs was added to a microsomal preparation, it blocked the formation of dolichyl-P-mannose from GDP–mannose and therefore prevented the stimulation of GlcNAc-PP-dolichol formation by GDP–mannose.

Flavomycin is an antibiotic of the moenomycin group of compounds. This drug interferes with the formation of lipid-linked saccharides in microsomes from pig brain (149). Unfortunately, no other studies have been reported with this compound, so additional studies will be needed before we can understand its mechanism of action. The same applies to diumycin.

Showdomycin is a nucleoside antibiotic whose structure is shown in Fig. 8. This compound has a maleimide group that reacts with sulfhydryls on proteins, and this reaction is probably the basis of the showdomycin inhibition of lipid-linked saccharide formation. In cell-free extracts of aorta, the synthesis of dolichyl-P-mannose and dolichyl-P-glucose were both inhibited by this drug, but the formation of dolichyl-PP-GlcNAc was not affected (150). However, in *Volvox* extracts,

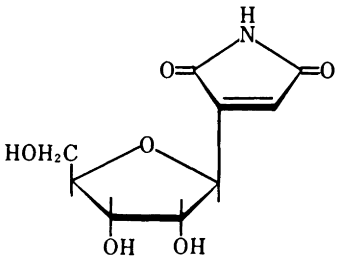


FIG. 8. Structure of showdomycin.

the syntheses of dolichyl-P-glucose and dolichyl-PP-GlcNAc were equally sensitive to showdomycin in the presence of Triton X-100, and this inhibition was reversed by the addition of dithiothreitol (151). As indicated earlier, showdomycin has also been used to study the regulation of lipid-linked saccharide formation by dolichyl-P-mannose.

E. Inhibition by Sugar Analogues

Probably two of the earliest inhibitors of viral envelope formation to be described were glucosamine (152) and 2-deoxyglucose (153). It is now known that glucosamine interferes with the formation of lipid-linked oligosaccharides and blocks protein glycosylation. This inhibition, however, requires intact cells and involves the metabolism of glucosamine probably to an intermediate such as UDP–glucosamine (154), which may compete with UDP–GlcNAc for various GlcNAc transferases. When MDCK cells were incubated in the presence of 1 m*M* glucosamine, there was a complete shift in the composition of the lipid-linked oligosaccharides from mostly $Glc_3Man_9(GlcNAc)_2$-PP-dolichol in normal cells to $Man_{6\text{-}8}(GlcNAc)_2$-PP-dolichol in inhibited cells. This shift to smaller sized oligosaccharide-lipids became even more pronounced when the concentration of glucosamine was raised to 10 m*M* (155). The glucosamine effect could be reversed by removal of this amino-sugar from the culture medium, but the exact site or mechanism of action of this compound is still unknown.

Interestingly enough, another amino-sugar, mannosamine, also inhibits the formation of lipid-linked oligosaccharides, but the mechanism of action of this compound is quite different from that of glucosamine. The inhibition by mannosamine was dose-dependent and resulted in a shift in the size of the oligosaccharide moieties of the lipid-linked oligosaccharides from $Glc_3Man_9(GlcNAc)_2$-PP-dolichol to lipids with $Man_3(GlcNAc)_2$ to $Man_9(GlcNAc)_2$. The major species were

oligosaccharides having five or six mannose residues. These studies demonstrated that mannosamine itself was incorporated into the lipid-linked oligosaccharides, mostly in place of the mannose that is in α1,3-linkage to the β-linked mannose. This substitution appears to prevent the addition of the α1,2-linked mannose to this α1,3-linked mannosamine (156). In the presence of mannosamine, a series of basic oligosaccharides linked to dolichol were formed, and at least some of these oligosaccharides were still transferred to protein. However, it is not clear whether these altered glycopeptides can be processed by the normal pathway or they accumulate in the cells as mannosamine and mannose containing structures.

In terms of glucose analogs, such as 2-deoxyglucose, it is now clear that this sugar is metabolized in cells and is converted to UDP–2-deoxyglucose and GDP–2-deoxyglucose, as well as to dolichyl-P-2-deoxyglucose. The inhibition of glycoprotein synthesis by this deoxysugar appears to be due to the GDP–2-deoxymannose (glucose), because mannose itself can reverse the inhibition (157). Probably when deoxyglucose is incorporated into the lipid-linked oligosaccharides instead of the mannose at the 3-branch, it acts like mannosamine and prevents further extension of this chain because additional mannoses are linked in α1,2-bonds. These 2-deoxyglucose-containing oligosaccharides appeared not to be transferred to protein in contrast to the situation with the mannosamine-containing oligosaccharides (from the lipid-linked oligosaccharides). A number of fluoro-sugars such as 2-deoxy-2-fluoro-D-mannose, and 4-deoxy-4-fluoro-D-mannose have also been shown to be inhibitors of glycosylation (158). It seems likely that the mechanism of action of these analogs is similar to that of 2-deoxyglucose.

F. Effects of Glucose Starvation and Alterations in Energy Charge

Some animal cells, especially Chinese hamster ovary (CHO) cells, show a phenomenon that has been called "glucose starvation." Thus, when glucose is removed from the medium, these cells can no longer produce the typical $Glc_3Man_9(GlcNAc)_2$-PP-dolichol that is the natural oligosaccharide donor for N-linked glycosylation. Instead, such cells produce a truncated lipid-linked oligosaccharide that is a $Man_5(GlcNAc)_2$-PP-dolichol (i.e., Manα1,2Manα1,2Manα1,3[Manα1,6]Manβ1,4GlcNAcβ1,4GlcNAc-) and then glucosylate this intermediate to produce a $Glc_3Man_5(GlcNAc)_2$-PP-dolichol (159–161). It appears that under glucose starvation conditions, the cells are not able to synthesize dolichyl-P-mannose, but the reason for this lesion is not clear because these cells still do make dolichyl-P-glucose and appear to glucosylate

the oligosaccharides. In several cell lines, the effect of glucose starvation was seen within 20 min after the removal of glucose, as long as the cells were at low to moderate density, but at high density no effect was seen (162). The glucose starvation phenomenon could be overcome by the addition of glucose, but not the addition of galactose, glutamine, pyruvate, inositol, or glycerol. In rat hepatoma cells that were starved for glucose, a lower molecular weight form of α_1-acid glycoprotein was produced and this decrease in molecular weight was due not only to the truncated oligosaccharides, but also to fewer oligosaccharide chains on the protein (163).

Carbonyl-cyanide *m*-chlorophenylhydrazone (CCCP) is an uncoupler of oxidative phosphorylation that causes cells to be depleted of energy. When this compound is given to various animal cells, it causes a similar situation to that seen with glucose starvartion; i.e., cells are unable to produce dolichyl-P-mannose, although they still synthesize dolichyl-PP-GlcNAc and dolichyl-P-glucose. These cells form a $Glc_3Man_5(GlcNAc)_2$-PP-dolichol and transfer this oligosaccharide to protein (164). On the other hand, in thyroid slices incubated with CCCP, $Man_{9\text{-}8}(GlcNAc)_2$-PP-dolichol intermediates were produced, and a decrease in N-linked glycosylation was observed (165). In this study, other inhibitors of respiration such as N_2 or antimycin A caused the same effects. Since some uncouplers of oxidative phosphorylation have been reported to disrupt the recycling of the glucose transporter, these compounds may limit the entry of glucose into the cells, thus mimicking the glucose starvation effect. The puzzle here is the same as that mentioned earlier; i.e., Why is the formation of dolichyl-P-mannose inhibited, but not that of dolichyl-P-glucose? Perhaps it could have something to do with the postulation that the dolichyl-P-mannose synthase is a regulatory enzyme and the protein itself undergoes phosphorylation under some conditions.

V. Inhibitors of N-Linked Glycoprotein Processing

A. Glucosidase Inhibitors

A number of naturally occurring, sugarlike compounds in which the ring oxygen is replaced by a nitrogen have been isolated, and these alkaloids have been found to be potent inhibitors of various glycosidases. Apparantly this nitrogen in the ring makes these compounds inactive from a metabolic standpoint, but they are still recognized by specific glycosidases and therefore function as useful enzyme inhibitors. Because the enzymes involved in the early glycoprotein processing

reactions are glycosidases, these inhibitors have played a significant role in studying the sequence of reactions of N-linked oligosaccharides, as well as the requirement for glycoprotein processing in many animal cell lines. Many of these inhibitors have been isolated from plant sources, but the characterization of their structures and the demonstration of their biological activity have stimulated the chemical synthesis of a number of other analogues. The plant inhibitors are all alkaloids and fall into various chemical classes designated piperidines, pyrrolidines, indolizidines, and pyrrolizidines (166).

Castanospermine is an indolizidine alkaloid that is found in the seeds of the Australian chestnut tree, *Castanospermum australe* (167). Its structure is shown in Fig. 9. This alkaloid is a potent inhibitor of β-glucosidase (168) and also inhibits the glycoprotein processing glucosidases (I and II), as well as various other α-glucosidases such as sucrase, maltase, and lysosomal α- glucosidase (169). Because this compound

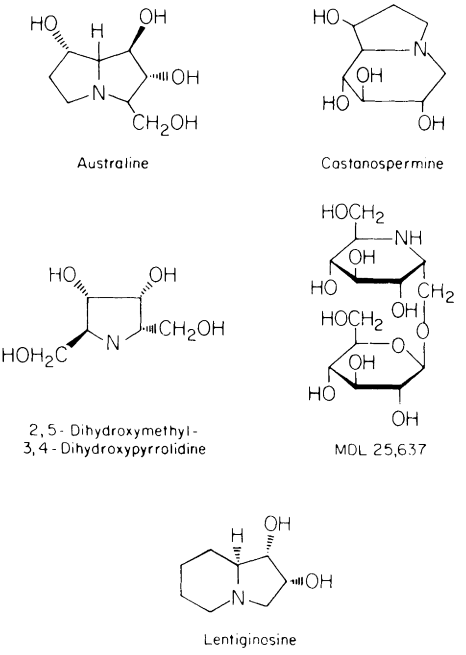


FIG. 9. Structures of various glucosidase inhibitors. Castanospermine and 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine are inhibitors of both glucosidase I and glucosidase II, whereas australine is a much better inhibitor of glucosidase I than glucosidase II and MDL is a more potent inhibitor of glucosidase II. Lentiginosine, shown for comparison, is an inhibitor of amyloglucosidase but is ineffective against the processing glucosidases.

is such a strong inhibitor of α-glucosidases (such as sucrase and maltase) and therefore prevents the normal digestion of starch and sucrose, the seeds of this plant are toxic to animals and cause severe diarrhea (170). When various cultured animal cells are grown or incubated in the presence of this alkaloid, the processing of N-linked glycoproteins is blocked at the first step in the pathway (see Fig. 3), and the asparagine-linked glycoproteins have oligosaccharides mostly of the $Glc_3Man_{7-9}(GlcNAc)_2$ structure. Another glucosidase inhibitor is the polyhydroxylated piperidine analog nojirimycin, which corresponds to glucose in the pyranose ring form (see Fig. 9). This compound was originally isolated from *Streptomyces* and was found to be a potent inhibitor of α- and β-glucosidases (171). Chemical reduction of nojirimycin gave the more stable deoxynojirimycin, which is also a glucosidase inhibitor and which has subsequently been isolated from bacteria and plants (172). Deoxynojirimycin causes the same general effects in cell culture as does castanospermine, although it is more active toward glucosidase II than toward glucosidase I, and it does cause other alterations such as changes in the structures of the lipid-linked saccharides (173). Another glucosidase I inhibitor that also is found in plants is the pyrrolidine alkaloid 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine (174). This compound, although considerably less effective than the six-membered ring structures described above (e.g., compare the structure in Fig. 9 with those of castanospermine and deoxynojirimycin), causes similar changes in the structures of the N-linked oligosaccharides.

The effect on glycoprotein targeting and function of preventing the removal of glucoses from the N-linked oligosaccharides is quite dramatic. For example, when hepatocytes (Hep-G2 cells) are incubated in the presence of deoxynojirimycin, the rate of secretion of α_1-antitrypsin was substantially diminished, whereas the rate of secretion of other N-linked glycoproteins such as ceruloplasmin and the C3 component of complement was only marginally altered. Based on some preliminary cell fractionation studies, it was suggested that the presence of glucose on the oligosaccharides of certain glycoproteins might retard the movement of these glycoproteins from the ER to the Golgi apparatus (175). Similar results were obtained with fibroblasts and smooth muscle cells when the effect of castanospermine on the LDL receptor was examined. In this case, there was a significant decrease in the binding and endocytosis of LDL when arterial smooth muscle cells were upregulated to produce LDL receptors in the presence of castanospermine. The reason for this decreased binding was shown to be due to a reduced number of receptors at the cell surface rather than to a change in the affinity of receptor for its ligand. Furthermore, the total number of LDL receptors in the cell did not change in the presence of castanospermine,

indicating that this drug did not inhibit the synthesis of the receptor, nor did it affect its degradation. Cell fractionation studies designed to isolate various membrane fractions demonstrated a shift in the distribution of LDL receptors within the cell compartments and indicated a decrease in receptors at the cell surface and a corresponding increase in receptors in the ER–Golgi membrane fractions (176).

In the case of IM-9 lymphocytes, castanospermine was used to study the biosynthesis and processing of the insulin receptor. When these cells were treated with this alkaloid, they showed a 50% decrease in cell-surface insulin receptors as demonstrated by the binding of ^{125}I-insulin, as well as by Na^{125}I-lactoperoxidase labeling and by cross-linking studies with ^{125}I-insulin. These studies demonstrated that processing, i.e., removal of glucose from the N-linked oligosaccharides, was not necessary for the cleavage of the insulin proreceptor, i.e., for protein maturation. However, as indicated above, the presence of glucose appears to slow down the transport of glycoproteins from the ER to the Golgi apparatus (177). These data suggest the presence of a glucose receptor in the ER that "holds up" or binds glucose-containing glycoproteins, or the data may indicate that some proteins undergo a conformational change when glucose is removed, and the newly altered protein is transferred from the ER to the Golgi at a more rapid rate.

In the case of synthesis of the E_2 glycoprotein of coronavirus, castanospermine or deoxynojirimycin caused a drop in virus formation by 2 logs and also caused a dramatic decrease in the appearance of E_2 at the cell surface. Interestingly enough, the E_2 that was produced in the presence of these drugs was still acylated with a fatty acid, as was the control E_2, but the glycoprotein accumulated intracellularly in a compartment that was distinct from the Golgi apparatus (178). Could this compartment be the ER?

In another case, post-translational addition of palmitic acid was also not affected by glucosidase I inhibitors. The Na channel of rat brain neurons is composed of α- and β-subunits that form a complex during maturation. The α-subunit is post-translationally modified by the addition of a palmitic acid, and the incorporation of labeled palmitate into this glycoprotein is blocked by the glycosylation inhibitor tunicamycin. However, the α-subunit formed in the presence of castanospermine is of lower molecular weight, does not contain sialic acid, and has a lower sulfate content, but it is similar to control α-subunit in its content of palmitic acid (179). The drug also did not prevent the covalent assembly of α- and β-subunits, nor did it affect the biological activity of the channel with regard to its binding of saxitoxin.

The external glycoprotein of the AIDS-associated virus HIV is called GP120 and is a heavily glycosylated protein that is involved in the

mechanism of attachment of HIV to the CD4 receptor on T lymphocytes and other susceptible cells. The GP120 interacts with target molecules on susceptible cells to cause the fusion of the cells, which is referred to as the formation of syncytia. Both glucosidase I inhibitors (i.e., castanospermine and deoxynojirimycin) caused a drastic inhibition of virus replication as well as of syncytium formation (180–182). Because of these interesting results, castanospermine and deoxynojirimycin, as well as other glucosidase I and II inhibitors and analogs of these various inhibitors, are being tested as potential anti-AIDS compounds.

As shown in Fig. 9, there are other compounds besides castanospermine and deoxynojirimycin, that also have activity against the α-glucosidases and that function as glycoprotein processing inhibitors. One of these compounds is the pyrrolidine alkaloid mentioned earlier and referred to as 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine (DMDP), which has been found in several plants of the *Leguminosae* family. In cell culture, DMDP gives the same type of oligosaccharide structure, i.e., $Glc_3Man_{7\text{-}9}(GlcNAc)_2$, as does castanospermine, although it is much less active as an inhibitor (i.e., higher concentrations are required) (174). Nevertheless, it is an interesting inhibitor because it demonstrates that a six-membered ring structure is not absolutely required for inhibitory activity, and knowledge of such structures helps us to define the particular structural requirements that are necessary for such biological activity. Along these lines, two other structures that are also interesting glucosidase inhibitors, partly because they are unusual and partly because they show more selectivity than those described thus far, are shown in Fig. 9.

Australine is a tetrahydroxypyrrolizidine alkaloid that was isolated from the seeds of the tree *C. australe* (as is castanospermine). This compound has a unique substitution pattern (i.e., (1*R*,2*R*,3*R*,7*S*,7α*S*)-3-hydroxymethyl-1,2,7-trihydroxypyrrolizidine). This bicyclic alkaloid is a reasonably good, competitive inhibitor of the fungal amyloglucosidase (an aryl-glucosidase) and it also inhibits the glycoprotein processing enzyme glucosidase I. However, in contrast to castanospermine and deoxynojirimycin, australine is a very poor inhibitor of glucosidase II (183). This compound is the first glucosidase inhibitor to distinguish between these two processing glucosidases. Thus, additional compounds such as australine that show increased specificity will be valuable to help us understand how these two glucosidases differ in their mechanism of catalysis and also to determine the chirality and spacial configurations that are necessary for activity of various glycosidases.

Another inhibitor whose structure is shown in Fig. 9 is 2,6-diamino-2,6-imino-7-O-(β-D-glucopyranosyl)-D-glycero-L-guloheptitol (MDL). This compound was chemically synthesized to mimic a disaccharide

that would act as a transition state analog of the intestinal enzyme sucrase (184). As anticipated, MDL inhibited rat intestinal maltase, sucrase, isomaltase, glucoamylase, and trehalase at micromolar concentrations. Interestingly enough, when tested on the glycoprotein processing glucosidases, it was the opposite of australine in its specificity. That is, MDL was a much more effective inhibitor of glucosidase II than it was of glucosidase I (185). In cell culture experiments, MDL caused the accumulation of glycoproteins having mostly $Glc_2Man_{7-9}(GlcNAc)_2$ structures. The finding that australine and MDL are more specific inhibitors than castanospermine and deoxynojirimycin will hopefully stimulate a more intense search for other naturally occurring inhibitors with improved selectivity for glucosidases and mannosidases, and will also entice chemists to use their imagination to synthesize more novel compounds.

B. Mannosidase Inhibitors

The first glycoprotein processing inhibitor to be reported was swainsonine (186), which is described in more detail below. This indolizidine alkaloid was initially found to be an inhibitor of lysosomal and aryl-mannosidases (187), and was later shown to specifically inhibit the processing mannosidase II (188). However, for ease of understanding, I will consider the various mannosidase inhibitors in the order in which they occur in the processing pathway rather than in the order in which they were first reported.

The discovery of deoxynojirimycin and castanospermine as glycoprotein processing inhibitors indicated that a good glycosidase inhibitor should possess the following properties: (a) a pyranose ring structure with a nitrogen in the ring replacing the oxygen and (b) the stereochemistry of the hydroxyl groups in keeping with those of the particular sugar that is recognized by the glycosidase of interest. Thus, it was reasonable to assume that a structure similar to deoxynojirimycin but with the mannose configuration (i.e., the 2-epimer) should be a good inhibitor of mannosidases. The 2-epimer of deoxynojirimycin was synthesized chemically and was indeed found to be a potent inhibitor of the glycoprotein processing enzyme mannosidase I (189). Surprisingly enough, deoxymannojirimycin did not inhibit jack bean or lysosomal α-mannosidase, nor did it inhibit the processing mannosidase II. In fact, this inhibitor demonstrates that it is dangerous to screen for new inhibitors using aryl-glycosidases as the source of enzymes or even using only a few of the processing enzymes, because the most useful and desirable processing inhibitors will be specific for a particular processing glycosidase and may not inhibit these other activities.

The structures of deoxymannojirimycin, swainsonine, and other known mannosidase inhibitors are shown in Fig. 10. Since the time when many of the studies on deoxymannojirimycin were done, several other neutral α-mannosidases that have different specificities and are enzymes distinct from mannosidase I or mannosidase II have been identified. Some of these enzymes have been found to be resistant to inhibition by deoxymannojirimycin, but there are not enough mannosidase inhibitors yet known to be able to specifically inhibit some of these activities. In addition, most of these other enzymes have not yet been purified, so they have not been tested against the various processing inhibitors. In animal cells, deoxymannojirimycin inhibited the Golgi mannosidase IA/B and caused the accumulation of glycoproteins having high-mannose oligosaccharides, mostly of the $Man_{8\text{-}9}(GlcNAc)_2$ structure (191). This inhibitor, however, did not prevent the secretion of IgD or IgM in cell culture, although the glucose analog deoxynojirimycin did (192). Thus the presence of glucose on the oligosaccharides does have a significant effect on the intracellular transport of at least some glycoproteins, but the presence of high-mannose oligosaccharides rather than the normal complex chains does not appear to be a problem

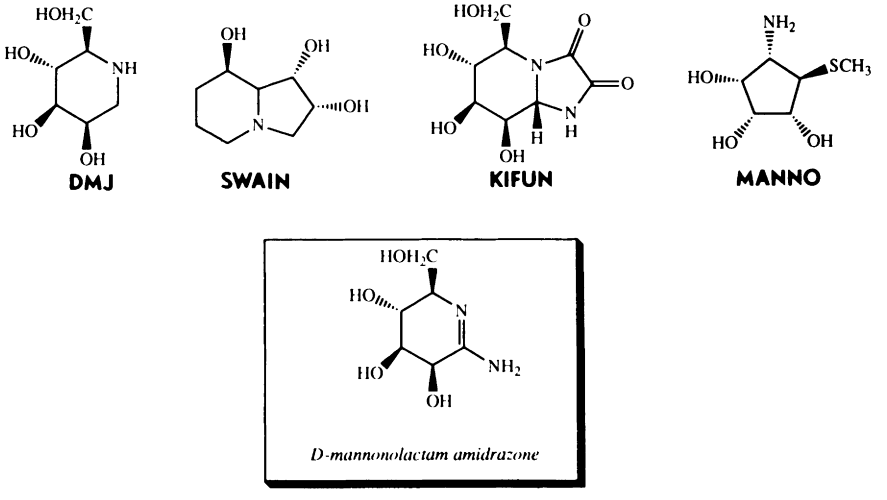


FIG. 10. Structures of various mannosidase inhibitors. Swainsonine (SWAIN) and mannostatin (MANNO) are potent inhibitors of mannosidase II while deoxymannojirimycin (DMJ) and kifunensine (KIFUN) are good inhibitors of mannosidase I. The mannonolactam amidrazone is a very potent general mannosidase inhibitor that acts on arylmannosidases, as well as mannosidase I and mannosidase II.

in terms of protein secretion. Deoxymannojirimycin was also without effect on the formation and appearance of the G protein of vesicular stomatitis virus, the hemagglutinin of influenza virus, and the HLA A,B, and C antigens, as well as on α_1-acid glycoprotein and α_1-protease inhibitor (192).

Deoxymannojirimycin was used in one study to determine if membrane glycoproteins were recycled through the Golgi apparatus during the endocytic process. In this experiment, membrane glycoproteins were synthesized and labeled with [^{3}H]mannose in the presence of deoxymannojirimycin so that the glycoproteins would all have high-mannose structures. Then the inhibitor and labeled sugar were removed, and the oligosaccharide structure of the transferrin receptor was determined at various times and in conditions under which this receptor would be endocytosed. The initial oligosaccharides of the transferrin receptor were high-mannose structures because of the presence of deoxymannojirimycin during their synthesis, but during the chase, a small percentage of the recycled receptor underwent processing and attained complex types of oligosaccharide structures, indicating that some of the endocytosed glycoprotein was recycled through the cis-Golgi compartments (193). However, the amount of glycoprotein that actually underwent modification during the recycling was quite small, indicating that, while some glycoprotein does recycle through the Golgi, this is probably not a major route.

The role of endoplasmic reticulum α-mannosidase was also examined in UT-1 cells, a cell line that overexpresses HMG CoA reductase. The HMG CoA reductase resides in the ER of cells and is a glycoprotein that has high mannose oligosaccharides mostly of the $Man_8(GlcNAc)_2$ and $Man_6(GlcNAc)_2$ structures. Previous studies indicated that "ER" α-mannosidase was not inhibited by deoxymannojirimycin. Thus, in the presence of deoxymannojirimycin, the initial trimming of the oligosaccharide chains of the reductase still occurred, but in this case, the major oligosaccharide structure on the protein was a $Man_8(GlcNAc)_2$ and the smaller sized oligosaccharides were not detected. These studies indicated that the ER α-mannosidase was involved in the removal of the first mannose, but that other α-mannosidases were necessary for the removal of other mannose residues (194).

Another mannosidase inhibitor is 1,4-dideoxy-1,4-imino-D-mannitol (DIM). This inhibitor has a furanose ring structure rather than the pyranose rings of most of the other inhibitors. DIM was synthesized chemically from benzyl-α-D-mannopyranose and was found to be a good inhibitor of jack bean α-mannosidase (195). It also inhibited the processing of N-linked glycoproteins in cell culture when it was added to the culture medium of MDCK cells. In those experiments, the major

oligosaccharides formed on the cell glycoproteins were $Man_9(GlcNAc)_2$ structures in keeping with the hypothesis that DIM inhibited the Golgi mannosidase I (196). However, it is not known whether DIM also inhibits the ER mannosidase, because this activity does not appear to be present in MDCK cells. Furthermore, there is no indication that this inhibitor has been tested against this ER mannosidase or other mannosidases, although it is active against mannosidase I. Thus, *in vitro* studies with DIM and a partially purified preparation of mannosidase I from rat liver showed that this compound inhibits the release of [^{3}H]mannose from [^{3}H]mannose-labeled $Man_9GlcNAc$. DIM is not nearly as effective as a mannosidase inhibitor as is swainsonine or kifunensine. However, it is much more readily available in large amounts because the chemical synthesis is quite simple. Therefore, DIM and its isomers should be valuable for animal studies, as well as for other experiments requiring large amounts of mannosidase inhibitor.

Kifunensine is an alkaloid, produced by the actinomycete *Kitasatosporia kifunense* and corresponding in structure to the cyclic oxamide derivative of 1-aminodeoxymannojirimycin (197). Kifunensine is a very weak inhibitor of jack bean α-mannosidase ($IC_{50} = 1.2 \times 10^{-4}\ M$), but it is a potent inhibitor of the glycoprotein processing mannosidase I ($IC_{50} = 2\text{–}5 \times 10^{-8}\ M$). Thus, this inhibitor is about 100 times more effective against this enzyme than is deoxymannojirimycin. On the other hand, kifunensine had no effect on mannosidase I (198). Using rat liver microsomes as a source of mannosidase activities, kifunensine strongly inhibited the Golgi mannosidase I but it was probably without effect on the proposed ER α-mannosidase. In cell culture with MDCK cells, kifunensine gave rise to glycoproteins having $Man_9(GlcNAc)_2$ structures just as did deoxymannojirimycin, but the amount of kifunensine needed for this effect was only about one-fiftieth as much as deoxymannojirimycin.

Recently, a broad-spectrum α-mannosidase inhibitor was synthesized chemically as a mimic of the mannopyranosyl cation, which is the putative intermediate in the hydrolysis of mannopyranosides (199). This compound, whose structure is shown in Fig. 10, is called mannonolactam amidrazone. This compound was found to be a more general mannosidase inhibitor than any of the other currently known compounds and those shown in Fig. 10. Thus, the amidrazone not only inhibits the Golgi mannosidase I ($IC_{50} = 4\ \mu M$) and mannosidase II ($IC_{50} = 90\text{–}100\ n M$), but also is the first compound that has been found to be a potent inhibitor of the soluble or ER α-mannosidase ($IC_{50} = 1\ \mu M$). In addition, the amidrazone also inhibited both the aryl-α-mannosidase and the aryl-β-mannosidase, although it was much more effective on the enzyme recognizing the α-linkage ($IC_{50} = 400\ n M$ for

jack bean α-mannosidase compared with 150 μM for β-mannosidase). In cell culture studies, the amidrazone completely prevented the formation of complex types of oligosaccharides and instead caused the formation of oligosaccharides having $Man_9(GlcNAc)_2$ and $Man_8(GlcNAc)_2$ structures. It is hypothesized that the reason that this compound is so effective against all of these mannosidases is that it is the first monosaccharide analog that mimics the true half-chair conformation of the cationic intermediate that is believed to be involved in the catalysis of the α-mannose glycosidic bond. This compound should serve as a useful model to synthesize other more specific mannosidase inhibitors.

As indicated above, the first glycoprotein processing inhibitor to be identified was swainsonine. The initial interest in this compound was due to the fact that it is found in plants that are toxic to grazing animals. In Australia, the major plant having this alkaloid is called *Swainsona canescens* (200), while in the United States, various plants of the genus *Astragalus* contain swainsonine (201, 202). These plants present a major hazard to the livestock industry and cause severe neurological and skeletal problems in animals that feed on them. Early studies on the inhibition of aryl-α-mannosidases by swainsonine suggested that its potent inhibitory activity ($IC_{50} = 1 \times 10^{-7}$ M) resulted from the structural similarity of the protonated form of swainsonine to that of the mannosyl cation, since the glycosyl ion intermediate is probably formed during natural hydrolysis by glycosidases (203).

Lymphnodes of sheep that had injested these toxic plants contained high levels of mannose-rich oligosaccharides (204), which led to the postulation that swainsonine causes a "lysosomal-like" storage disease that is similar to that of human α-mannosidosis, both morphologically and biochemically. That swainsonine is actually the causative agent of these symptoms was indicated by feeding pigs either the whole toxic plant (i.e., *Astragalus*) or swainsonine and showing that both sets of animals exhibited the same symptoms (205). In these animals, liver Golgi mannosidase II activity was significantly decreased, but strangely enough, a number of lysosomal hydrolases such as α-mannosidase, α-fucosidase, and β-hexosaminidase increased in activity. In addition, the activity of various hydrolases in the plasma was also elevated. In sheep fed locoweed, various high-mannose oligosaccharides were found in the urine and the two major oligosaccharides were identified as $Man_4(GlcNAc)_2$ and $Man_6(GlcNAc)_6$ (206). The relative abundance of the various oligosaccharides changed over the course of the feeding, but when the feeding was discontinued, the amount of urinary oligosaccharides declined rapidly and reached a baseline within about 12 days. Similar types of high-mannose oligosaccharides also accumulated in rats and guinea pigs fed swainsonine, but there were some

differences in the structures, especially the presence of one or two GlcNAc residues at and near the reducing terminus (207). These differences probably reflect the presence or absence of an endoglucosaminidase in some of these animals.

Swainsonine was the first compound that was shown to inhibit glycoprotein processing (186). In the initial studies, swainsonine was added to the culture medium of MDCK cells and was found to cause a great decrease in the amount of [2-^{3}H]mannose incorported into complex types of glycoproteins and a corresponding increase in incorporation of labeled sugar into oligosaccharides that were sensitive to digestion by endoglucosaminidase H. In influenza-virus-infected MDCK cells, swainsonine caused the viral hemagglutinin, an N-linked glycoprotein with several complex chains, to migrate more rapidly on SDS gels, and these changes were shown to be due to changes in the structure of the N-linked oligosaccharides (208). Later studies with purified processing enzymes showed that the site of action of swainsonine was the Golgi mannosidase II and that this alkaloid was inactive toward the processing mannosidase I (188). In keeping with this site of action, this indolizidine alkaloid also caused the formation of hybrid types of structures when it was placed in the culture medium of cells producing various glycoproteins such as the VSV G protein (209), fibronectin (210), and BHK cell surface glycoproteins (211). Swainsonine has been used with a number of cell culture systems to try and determine the role of specific oligosaccharide structures in glycoprotein function. In most of these studies, swainsonine was found to have little effect on the glycoprotein in question, indicating that a partial complex oligosaccharide (i.e., hybrid chain) is sufficient for functional activity, at least in tissue culture. For example, swainsonine did not impair the synthesis or export of thyroglobulin in porcine thyroid cells (212), nor did it affect surfactant glycoprotein A in Type II epithelial cells (213), H2-DK histocompatability antigen in macrophages (214), or von Willebrand protein in epithelial cells (215). It also did not affect the targeting or function of various membrane receptors, including insulin receptor (216), epidermal growth factor receptor (217), or the receptor for asialoglycoproteins (218). The inhibitor did, however, block the receptor-mediated uptake of mannose-terminated glycoproteins by macrophages. This blockage appeared to be due to the formation of hybrid types of glycoproteins on the macrophage surface, which then reacted with and bound the mannose receptors (219). Swainsonine proved to be useful in demonstrating the sequence of addition of the various sugars in the N-linked oligosaccharide assembly pathway. Thus, when fucosylation (220) and sulfation (221) of the influenza viral hemagglutinin was examined in cells grown in the presence of swainsonine or

castanospermine, it was found that the hemagglutinin did contain [^{3}H]-fucose and [^{35}S]sulfate when the virus was raised in the presence of swainsonine, but not when virus was produced in the presence of castanospermine (or deoxymannojirimycin). These studies suggest that the fucosyltransferase and the sulfotransferase recognize the GlcNAc–$Man_5(GlcNAc)_2$ structure as the appropriate acceptor substrate.

Some proteins do show a loss of function when they are produced in the presence of swainsonine. Thus, the glucocorticoid stimulation of resorptive cells, which appears to involve the attachment of osteoblasts and related cells to bone, is inhibited or blocked by swainsonine (222). This drug also reduced the interaction of *Tyrpanosoma cruzi* with peritoneal macrophages when either the host cells or the parasites were treated with swainsonine (223). Also a dramatic decline in the ability of B16 melanoma cells to colonize the lungs of experimental animals was observed when the cells were incubated in swainsonine (224). Treatment of lymphocytes with the mitogen concanavalin A stimulates them to proliferate, but this proliferation can be blocked by an immunosuppresive factor found in the serum of mice that contain the tumor sarcoma 180. The suppression caused by this factor is somehow overcome by swainsonine, indicating that this alkaloid might have some chemotherapeutic value in immunosuppressive diseases (225). The turnover or degradation of endocytosed glycoproteins is also inhibited by swainsonine and this leads to the accumulation of these glycoproteins in the lysosomes of tissue culture cells incubated with this drug. The block here appears to be due to the inhibition of lysosomal mannosidase (and perhaps other enzymes), indicating that the carbohydrate portion of these glycoproteins must be degraded before the protein (226). All of these studies suggest that swainsonine may have important effects on some cell systems and therefore it or some related compounds may have some chemotherapeutic value. However, additional studies will be required with these inhibitors.

Another inhibitor of mannosidase II that was recently isolated from a fungus is called mannostatin (227). This compound is interesting because it is the only one of these inhibitors presently known that has an exocyclic nitrogen rather than a nitrogen in the ring. In addition, as seen in Fig. 10, mannostatin has a thiomethyl group and a five-membered ring. Mannostatin was found to be a potent inhibitor of mannosidase II (IC_{50} = 100 n*M*) and jack bean mannosidase. In cell culture studies, mannostatin gave rise to the same types of hybrid structures that are observed in cells incubated in the presence of swasinsonine (228). However, the N-acetylated derivative of mannostatin was inactive against any of the mannosidases. Although mannostatin

is not any different in its site of action, or any more active than swainsonine, it is an interesting addition to the repertoire of inhibitors because it shows a heretofore unanticipated structure as a glycosidase inhibitor. This opens up a number of new possibilities for chemical synthesis of related structures or perhaps structures that combine some aspects of mannostatin and some aspects of other alkaloids.

A number of laboratories have been developing methods for the synthesis of the above alkaloids or their derivatives, as well as of other new inhibitors (229). So far, with the exception of mannostatin, all of the inhibitors have had a nitrogen in the ring in place of the oxygen, and at least three assymetric hydroxyl groups. The substitution of the ring oxygen by a nitrogen apparantly renders the compound metabolically inert but does not prevent the recognition of the compound by glycosidases or perhaps by other carbohydrate recognizing enzymes. Presumably the hydroxyl groups in these inhibitors have the same chirality as the sugar that is a substrate for the glycosidase to be inhibited. Interestingly enough, these inhibitors do not necessarily need to have a six-membered ring structure to mimic the catalytic intermediate that is involved in the substrate recognition or hydrolytic step. In fact, swainsonine best fits as a mannose analog when it is in a staggered or half-chair conformation (203), and other pyrrolidine or pyrrolizidine ring structures may also assume similar conformations. Molecular modeling studies with these compounds will help to understand the mechanism of inhibition by some of these compounds. Such modeling studies have been done with some of the mannosidase inhibitors compared with the mannopyranosyl cation as the active intermediate of jack bean mannosidase (230). However, the synthesis or isolation of other and different inhibitors of these enzymes will add to this information.

VI. Effects of Inhibitors of Protein Synthesis, or Compounds That Modify Protein Structure, on N-Linked Glycosylation

The synthesis of lipid-linked oligosaccharides in animal tissues or by cultured animal cells has been shown to be inhibited by several inhibitors of protein synthesis (231). For example, when MDCK cells were grown in the presence of cycloheximide or puromycin, both inhibitors of protein synthesis but by different mechanisms, there was a dose-dependent inhibition in the incorporation of [2-^{3}H]mannose into the lipid-linked oligosaccharides. However, these inhibitors did not affect mannose incorporation into dolichyl-P-mannose, nor did they block mannose incorporation from GDP–[^{14}C]mannose into lipid-linked

saccharides in cell-free extracts prepared from MDCK cells or from porcine aorta. These results could be explained by one of the following postulations: (a) limitation in the amount of dolichyl-P available to serve as a carrier of the sugars or (b) feedback inhibition in the synthesis of lipid-linked saccharides caused by the accumulation of lipid-linked oligosaccharide resulting from lack of acceptor protein. However, since the synthesis of dolichyl-P-mannose was not inhibited, the feedback mechanism seemed more likely (232).

Additional studies showed that the inhibition by cycloheximide could not be overcome by adding exogenous dolichyl-P, even though addition of dolichyl-P to normal cells did stimulate the incorporation of mannose into lipid-linked saccharides. However, the specific site of inhibition (or feedback inhibition) by these inhibitors is not known. Actinomycin, an inhibitor of RNA synthesis, was also found to inhibit the incorporation of mannose into lipid-linked oligosaccharides, but again the exact site of inhibition was not determined (233). Based on the above results, as well as additional studies on the levels of various intermediates in normal and inhibited cells, it was postulated that the mechanism of inhibition involved a feedback type of control that was caused by elevated levels of GTP in the cells (234).

In N-linked glycosylation, the consensus sequence that accepts the oligosaccharide chain is H_3N . . . Asn–X–Ser (Thr) . . . , where *X* can be any amino acid except proline or aspartic acid. This sequence is, however, not sufficient for glycosylation to occur, and the conformation of the protein around this glycosylation site is apparantly also critical. Statistical studies on a number of asparagine-linked glycoproteins have indicated that most of the asparagine residues that do become glycosylated are located in regions of the polypeptide chain that favor the formation of β-turns.

The importance of the threonine residue in the tripeptide sequence was examined by using a threonine analog, β-hydroxynorvaline, and determining its effects on cotranslational glycosylation in extracts of ascites cells. This analog inhibited the glycosylation of the α-subunit of human chorionic gonadotropin and the β subunit of bovine leutinizing hormone. This inhibition could be overcome by adding threonine, strongly suggesting that the analog was inhibiting by competing with threonine for incorporation into protein (235). In cultured fibroblasts, β-hydroxynorvaline caused the formation of a variety of cathepsin D molecules that differed from each other by having one, two, or no oligosaccharide chains on the protein. The nonglycosylated form of cathepsin was a minor component and it was rapidly degraded within 45 min of its synthesis. However, those forms having one or two oligo-

saccharide chains behaved normally, were segregated to the lysosomes, and underwent proteolytic maturation in the usual fashion (236). Similar types of results were observed with respect to the effects of this analog on the formation of α_1-acid glycoprotein (237).

Another amino acid analog that has interesting effects on glycosylation is threo-β-fluoroasparagine. This analog is toxic to asparagine-dependent cells when aspartic acid is included in the culture medium. The actual mechanism of toxicity has not been determined but it is believed to be due to the incorporation of the analog into protein. At 1 m*M* concentrations, this analog strongly inhibits glycosylation but this inhibition is blocked by adding asparagine, further strengthening the idea that the analog is incorporated. However, the erythro-analog was not an inhibitor of glycosylation (238). Interestingly enough, when the above two amino acid analogs (i.e., β-hydroxynorvaline and threo-β-fluoroasparagine) were used to inhibit protein synthesis, they also inhibited the incorporation of radioactive mannose into lipid-linked oligosaccharides as demonstrated with cycloheximide and puromycin (232). It seems likely that the toxicity of fluoroasparagine is due to its preventing the cells from glycosylating their N-linked glycoproteins in much the same manner as tunicamycin.

VII. Effects of Inhibitors of Polyprenol Synthesis on N-Linked Glycosylation

As indicated earlier, dolichyl-P serves as an obligatory carrier in the glycosylation of N-linked glycoproteins. Dolichyl-P is produced by the same initial pathway as that responsible for the synthesis of polyprenol units and, therefore, one would anticipate that inhibitors of polyprenol formation should also inhibit dolichyl-P formation. Furthermore, such inhibitors should also have a potent effect on the glycosylation of asparagine-linked glycoproteins. Since the formation of polyprenols utilizes the rate-limiting step catalyzed by the enzyme hydroxymethylglutaryl-Co A reductase (HMG CoA reductase), inhibitors of this enzyme such as 25-hydroxycholesterol and compactin (or mevanolin) should also inhibit glycosylation.

When smooth muscle cells, derived from aorta, are grown in the presence of 25-hydroxycholesterol, the incorporation of [^{14}C]acetate into cholesterol and into dolichyl-P is inhibited by up to 90%, as is N-linked glycosylation. However, mevalonate incorporation into these lipids is unaffected, as expected. Mevalonate, in fact, when added to inhibited cells, can overcome the inhibition with respect to N-linked glycosylation, presumably because it allows dolichyl-P

to be formed (239). On the other hand, in mouse L cells, hydroxycholesterol also affected the synthesis of dolichol and cholesterol, but in this system, there were conditions that gave great fluctuations in cholesterol synthesis but only slight effects on dolichyl-P formation. The authors postulated that there were other points in the pathway that must control the levels of these two lipids (i.e., cholesterol and dolichol) (240). Cholestyramineis a compound that when fed to animals causes an increase in the activity of HMG CoA reductase and a great stimulation in the incorporation of acetate into cholesterol. However, it did not increase the incorporation of acetate into dolichyl-P (241). On the other hand, feeding animals a diet high in cholesterol would be expected to suppress cholesterol synthesis at the level of the HMG CoA reductase. However, this treatment caused a significant increase in the incorporation of mevalonate into dolichol and dolichol-linked oligosaccharides as well as increased activity of some of the glycosyltransferases that are required for glycoprotein synthesis (242). The explanation for these data is not clear, but perhaps inhibiting the cholesterol branch of the polyprenol pathway results in the accumulation of certain intermediates that are key players in other branches of the biosynthetic pathway.

Compactin is a fungal product that is a competitive inhibitor of HMG CoA reductase (243). Compactin, and a number of chemically synthesized derivatives of this structure (i.e., mevinolin), have been studied extensively because of their enormous chemotherapeutic potential in lowering serum cholesterol levels. In one study, when rat hepatocytes were cultured in compactin for 24 hr, the synthesis of dolichyl-P was decreased by 75–90%, suggesting that dolichyl-P was mainly synthesized by the *de novo* pathway and that the CTP-mediated phosphorylation of dolichol was of limited use. However, another study (244) indicated that the dolichol kinase and the dolichyl-P phosphatase played key roles in regulating the cellular levels of dolichyl-P. When compactin, at concentrations of 1–5 μM, was fed to sea urchin embryos, it induced abnormal gastrulation. This effect appeared to be due to an inhibition in dolichyl-P formation because these embryos also showed a decreased capacity to synthesize lipid-linked oligosaccharides and glycoproteins. Furthermore the inhibitory effect of compactin on development could be overcome by supplementing the embryos with dolichyl-P, but the addition of cholesterol or coenzyme Q could not overcome the inhibition (245). Similar results were obtained with mouse embryos, in which compactin and an oxygenated sterol arrested development at the 32-cell stage and left the blastomeres decompacted. Here also, N-glycosylation was effected and this affect could be overcome by adding mevalonate (246).

VIII. Inhibitors of Golgi Trafficking Affect N-Linked Glycosylation

During their synthesis and processing, the N-linked glycoproteins are transported from the initial site of synthesis in the endoplasmic reticulum of the cell through the various Golgi stacks (i.e., from cis-Golgi, through medial Golgi, to trans-Golgi) to their final destination in the plasma membranes, in lysosomes or in other locations in the cell. While the signals that direct these proteins to specific sites are known in some cases (e.g., the mannose-6-phosphate receptor for lysosomal targeting has been elegantly described (247)), in other cases the signals are just beginning to become known. Nevertheless, a number of compounds have been identified that perturb the transport or migration of proteins through the various compartments of the cell. Most of these pertubants are ionophores that affect the concentrations of various ions in some cellular compartments and may also affect the internal pH of these compartments. For example, the secretion of immunoglobulins by plasma cells was markedly inhibited by monensin, and striking alterations in the ultrastructural appearance of the Golgi apparatus were seen (248). The suggestion was made that depletion of Ca^{2+} levels by this ionophore rendered the Golgi vesicles incapable of fusing, which resulted in a disruption in the flow of glycoproteins from the ER to the Golgi (249).

The transport of other proteins, such as fibronectin and procollagen, is also inhibited by ionophores (250), as is the transport of various viral envelope glycoproteins (251). In these experiments, membrane vacuoles could be visualized by electron microscopy, and large accumulations of procollagen and fibronectin could be seen in these vacuoles by immunofluoresence. In one interesting study, monensin was used as a tool to isolate those cisternae that contained accumulations of viral capsids because they sedimented at a higher density that normal cisternae (252). In the presence of monensin, terminal glycosylation is inhibited, i.e., addition of complex sugars such as galactose, sialic acid, fucose, etc., but the incorporation of amino acids into protein and the hydroxylation of proline were not affected. Thus, in monensin-treated cells, newly synthesized proteins accumulate in intracellular vesicles that appear to be derived from the Golgi apparatus, and some of the post-translational modifications are blocked. Apparantly there exists an acidic compartment in the Golgi that is necessary for the secretory process (253).

A recently discovered and very useful antibiotic, called brefeldin A, also inhibits the secretion of glycoproteins in hepatocytes but does not affect protein synthesis. Brefeldin inhibited the terminal glycosylation

of α_1-protease inhibitor and haptoglobulin, as well as the conversion of proalbumin to albumin. Both of these modifications appear to occur in the trans-Golgi (254). On the other hand, the proteolytic processing of haptoglobulin occurs in the endoplasmic reticulum and this modification was not affected by brefeldin (255).

The effect of brefeldin on the Golgi complex and endoplasmic reticulum of retrovirus-transformed murine erythroleukemia (MEL) cells was studied. Within 5 min of addition of this compound, most stacked cisternae were converted to vesicles that were scattered throughout the centrosphere region, and by 30 min, the Golgi complexes were completely disassembled. Over the next few hours, the number of vesicles in the Golgi area decreased concomitantly with an expansion of a predominantly smooth endoplasmic reticulum that consisted of a network of dilated tubules in continuity with regular RER cisternae, annulate lamellae, and the nuclear envelope. By electron microscopy, viral glycoproteins appeared to accumulate on these membranes and immature virions budded preferentially into the cisternal space. These findings suggest that brefeldin blocks the intracellular transport of newly synthesized cellular and viral proteins immediately distal to the distinct compartment of the ER. The effects of brefeldin were completely reversible and, within a short time after removal of the drug, Golgi cisternae bergan to reappear (256).

In a cell free system, brefeldin A prevented the assembly of non-clathrin-coated vesicles from Golgi cisternae. Furthermore, when coated vesicle assembly is blocked, extensive tubule networks form. These connect previously separate cisternae and stacks into a single topological unit that allows the mixing of the contents of Golgi cisternae (257). These studies are consistent with *in vivo* studies that showed that the earliest detectable effect of brefeldin was the displacement from the Golgi stacks of a 110-kDa protein (258, 259), and this protein was found to be identical to one of the major coat protein subunits (COPS) of Golgi non-clathrin-coated vesicles, β-COP (260). Thus, a simple explanation for the effect of this interesting and very useful compound is that it acts by blocking the budding of coated transport vesicles. Brefeldin A is now being widely used in cell biology and biochemistry and its use will undoubtedly provide important insight into mechanisms of protein transport and Golgi assembly and disassembly.

IX. Inhibitors of the Glycan Portion of Membrane Anchors

As indicated earlier, many membrane proteins are anchored to the cell surface by a glycosylphosphatidylinositol (GPI) anchor in which

the protein is linked from its carboxyl-terminal region to the glycan via a phosphoethanolamine bridge. Since the glycan portion of the molecule is essential to this anchoring mechanism, inhibitors of glycan formation should be of considerable interest for determining the role of this anchoring mechanism, as well as for understanding the function of the anchored proteins. As discussed in a previous section, the mannosyl residues of the anchor are donated via dolichyl-P-mannose, so inhibitors that block the formation of this glycolipid should affect anchor formation. Thus, amphomycin was effective in cell-free experiments to demonstrate that the mannosyl donor for the three mannose units of the glycan portion of the anchor was indeed dolichyl-P-mannose (261). The sugar analog, 2-fluoro-2-deoxyglucose, which was previously shown to inhibit dolichyl-P-mannose formation and lipid-linked oligosaccharides (154), also altered the synthesis of the GPI-anchored protein alkaline phosphatase in JEG-3 cells and caused the accumulation of a proform of the enzyme. This proform was gradually processed into a secretory form by proteolytic removal of the carboxy-terminal hydrophobic peptide (262). Thus, both of these studies indicated that inhibition of the synthesis of dolichyl-P-mannose can block protein–anchor assembly.

Another recently reported inhibitor of protein–anchor formation is mannosamine. Mannosamine was previously shown to be an inhibitor of lipid-linked saccharide formation and to cause the production of truncated lipid-linked oligosaccharides in MDCK cells incubated with micromolar concentrations of this compound (156). When mannosamine was included in the growth medium of MDCK cells, it blocked the incorporation of glycosylphosphatidylinositol into GPI-anchored proteins. Thus, in polarized MDCK cell cultures, this amino-sugar markedely reduced the appearance at the cell surface of a recombinant GPI-anchored protein and converted this apically oriented membrane protein to an unpolarized secretory protein. It also blocked the surface appearance of other endogenous GPI-anchored proteins. In this system, mannosamine also inhibited the incorporation of [^{3}H]ethanolamine into GPI-anchored proteins and GPI intermediates, suggesting that this amino-sugar inhibits the assembly of the glycan portion of the anchor (263).

The effect of mannosamine on the biosynthesis of intermediates involved in anchor formation was also examined in MDCK cell cultures by incubating these cells with [2-^{3}H]mannose in the presence of various amounts of mannosamine. This amino-sugar inhibited the incorporation of mannose into GPI intermediates in a dose-dependent and reversible manner. Mannosamine was shown to be incorporated into the glycan as mannosamine and probably mostly in the second mannose position. Thus, it appears to block further addition of mannose because

the next mannose is attached to the 2-hydroxyl of this second mannose. Various smaller sized intermediates were detected in cells incubated in the presence of this drug. As a result, ethanolamine-P could not be added, nor could the protein be transferred to this anchor. This inhibition was specific for mannosamine, and other amino-sugars such as galactosamine and trehalosamine were not inhibitory (264). Further studies with mannosamine or with other inhibitors as they become known should help us to understand the physiological significance of this anchoring mechanism.

X. Summary and Future Directions

Inhibitors are certainly not a panacea for all biochemical studies, and as indicated earlier there are potential problems in their utilization, such as lack of specificity or interference with other key reactions. Nevertheless, they can provide valuable insights into understanding specific steps in a biochemical pathway as well as in determining the physiological function of various biochemical molecules. For example, in terms of the biosynthesis of the N-linked oligosaccharides, inhibitors of the lipid-linked oligosaccharide pathway, such as amphomycin, have helped to establish that the mannosyl donor for the last four mannose residues of the $Glc_3Man_9(GlcNAc)_2$-PP-dolichol was dolichyl-P-mannose, rather than GDP–mannose. Other inhibitors of this pathway, such as tunicamycin, have provided evidence to suggest that the carbohydrate portion of the asparagine-linked glycoproteins may play a number of different roles, including enhancing the stability of the glycoprotein, affecting the conformation of the protein, providing a recognition signal for targeting the protein to specific sites in the cell or to uptake from the circulation, providing a receptor molecule for cell–cell adhesion or interactions and so on. The actual role of the carbohydrate depends on the amino acid sequence of the protein in question.

The second group of important inhibitors are those that affect glycosidases. These types of enzymes are widespread in nature and are involved in many natural processes such as cell division and cell separation, turnover of macromolecules, N-linked glycoprotein processing, and so on. Thus, inhibitors of these enzymes have great potential for use as biochemical tools or even as therapeutic agents, or they may also be lethal as shown for the glucosidase and mannosidase inhibitors castanospermine and swainsonine. Again these latter compounds have gained widespread use for studies on the role of carbohydrate in N-linked glycoprotein function.

However, all of these studies point out the need for additional inhibitors as well as for structurally different inhibitors. That is, the studies

with glycosidase inhibitors have shown that even the best of these compounds is still somewhat nonspecific and inhibits more than one enzyme. For example, castanospermine inhibits the processing enzymes glucosidase I and glucosidase II, as well as lysosomal α-glucosidase, and other glucosidases. Swainsonine, which is somewhat more specific, still inhibits mannosidase II and lysosomal α-mannosidase. Can we find, or synthesize, more specific inhibitors that act only on one enzyme? Of course, the answer to that question is not known at this time since the mechanism of catalysis has not been demonstrated for most of these enzymes. However, even if some of these enzymes share a common catalytic mechanism, it should still be possible to narrow the site of action of these inhibitors to groups of similarly acting enzymes. Since there are now several inhibitors that appear to distinguish between glucosidase I and glucosidase II, these two enzymes must differ in their catalytic mechanism or in their active site. Thus, such studies provide hope that more specificity can be obtained with the right inhibitors.

How can we find better inhibitors? It seems that there are a number of approaches that could be used. One of the most logical, of course, would be to work out and understand the catalytic mechanism of each of the enzymes to be inhibited and then design inhibitors based on this knowledge. While this approach is certainly possible, it is not likely to be feasible in the immediate future. Most of the enzymes involved in the biosynthesis of N-linked oligosaccharides or membrane anchors appear to be present in cells in relatively low abundance, and therefore even the purification of these enzymes to homogeneity is difficult. Actually, several of these proteins have now been purified to homogeneity, and perhaps with modern approaches of molecular biology, they can be cloned and produced in sufficient amounts for crystallization.

A second approach is to design new inhibitors based on the structures of already known prototypes. In this regard, various laboratories are currently conducting modeling studies with the known inhibitors to provide the basis for developing analogs of already-known compounds. While this is a viable and logical way to develop new inhibitors, it does have one major shortcoming, which is that this approach cannot provide completely new and novel structures that could lead to even better inhibitors than those that we already have available. In that respect, perhaps we need to take a lesson from nature since evolution has had millions of years to develop all sorts of unusual chemical structures. It is unlikely that penicillin, streptomycin, or bleomycin, to name a few, could have been chemically synthesized *de novo* without first having been isolated and characterized from natural sources. Thus, the third approach to better inhibitors that should not be ignored is to look

for new inhibitory compounds from natural sources, because this seems like the most likely way to find new and previously unknown structures.

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