INHIBITORS OF THE BIOSYNTHESIS AND PROCESSING OF N-LINKED OLIGOSACCHARIDES

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I. INTRODUCTION

Among the common types of glycoproteins that are found in eukaryotic cells, both as cell surface proteins and as secreted proteins, are those having N-linked or asparagine-linked oligosaccharides.[1,2] The oligosaccharide portion of these glycoproteins may be either of the high-mannose, the complex, or the hybrid type, as shown in Figure 1. Each of these structures contains the same basic core region, which is a pentasaccharide composed of a branched trimannose region (shown in the box) linked to an *N,N'*-diacetylchitobiose that is, in turn, linked to the amide nitrogen of asparagine. In the high-mannose structure shown in Figure 1, this pentasaccharide is further substituted by six α-linked mannose residues, but some high-mannose glycoproteins may have fewer mannose residues. On the other hand, in the complex structures, the pentasaccharide is elongated by the trisaccharide, sialic acid→galactose→GlcNAc. These complex oligosaccharides may have two (biantennary), three (triantennary), or four (tetraantennary) of these trisaccharide units. Finally, the hybrid structures, which have only recently been observed in cells, are combinations of the high-mannose and complex types and may have several mannose residues on one branch as well as one or more trisaccharide units on another branch. These oligosaccharides may also contain L-fucose as well as other GlcNAc residues.[3,4]

The biosynthesis of the core region of each of the oligosaccharides shown in Figure 1 appears to involve a common series of reactions which are outlined in Figure 2.[5-7] This pathway is commonly referred to as the dolichol pathway since it involves the participation of lipid-linked saccharides and dolichyl phosphate serves as the carrier lipid. The pathway is initiated by the transfer of GlcNAc-1-P from UDP-GlcNAc, to dolichyl-P to form the first lipid intermediate, GlcNAc-pyrophosphoryl-dolichol. This GlcNAc-1-P transferase is the site of inhibition by the antibiotic tunicamycin (see Section II.A). A second GlcNAc is then added, from UDP-GlcNAc, to form *N,N'*-diacetylchitobiosyl-PP-dolichol, and this lipid serves as the acceptor of mannose and glucose residues to give the lipid-linked oligosaccharides. Studies with the antibiotics, amphomycin and tsushimycin, as well as studies with a mammalian cell mutant and with solubilized enzymes (see Section II.D) suggest that the first five mannose residues come directly from GDP-mannose to give the heptasaccharide-lipid, $Man_5GlcNAc_2$-PP-dolichol. The next four mannose residues are apparently donated by the saccharide-lipid, mannosyl-P-dolichol to form the $Man_9GlcNAc_2$-PP-dolichol, and then three glucose residues are added from glucosyl-P-dolichol to form the lipid-linked oligosaccharide, $Glc_3Man_9GlcNAc_2$-PP-dolichol.[8,9] This oligosaccharide is finally transferred from its lipid-carrier to protein, while the polypeptide is being synthesized on membrane-bound polysomes.[10-14] The enzymes involved in the "dolichol pathway" are all membrane-bound glycosyltransferases and these reactions occur in the endoplasmic reticulum.[15-17]

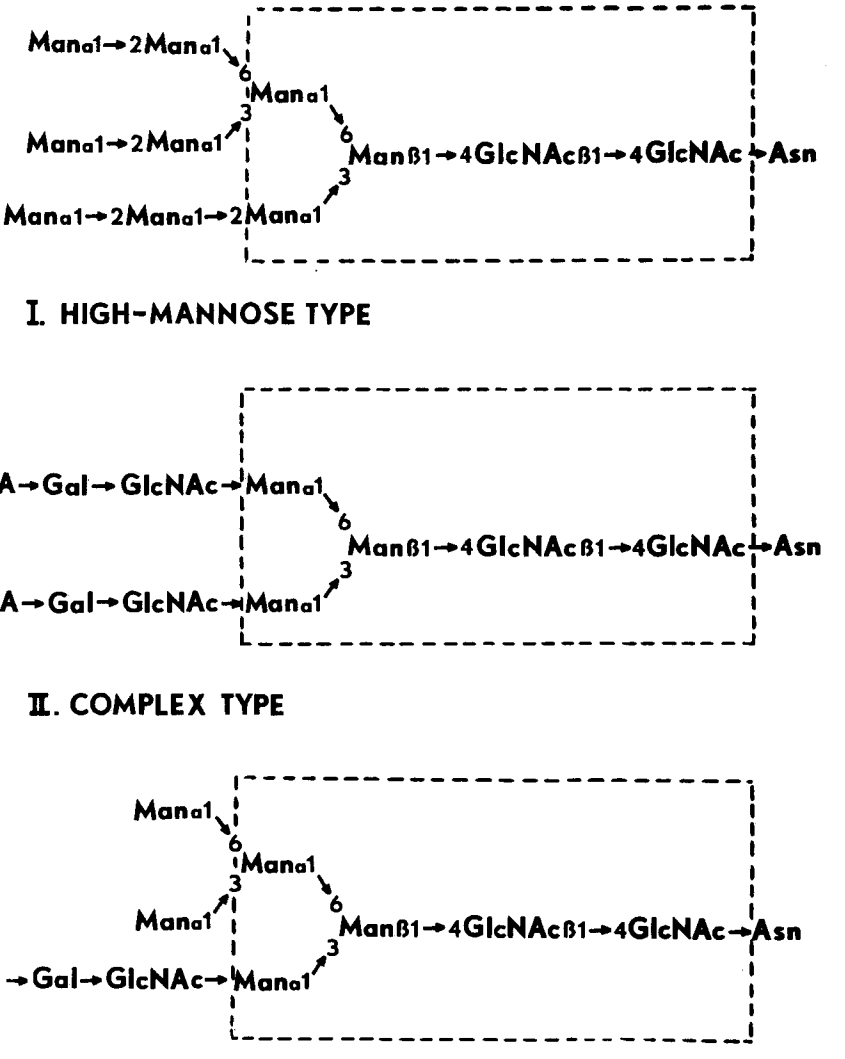


FIGURE 1. Structures of the oligosaccharide chains of the asparagine-linked glycoproteins.

Following the transfer of oligosaccharide to protein, the oligosaccharide undergoes a number of processing or trimming reactions. The initial processing reactions begin in the endoplasmic reticulum and continue as the protein is transported through the Golgi apparatus to its ultimate goal.[18,19] The processing reactions involved in glycoprotein biosynthesis are outlined in Figure 3. Very soon after attachment of oligosaccharide to protein, the glucose residues are removed from the glycoprotein by two membrane-bound glucosidases.[20-23] Glucosidase I removes the terminal α1,2-glucose[24,25] while glucosidase II removes the next two α1,3-glucose residues.[26,27] Several inhibitors of glucosidase activity have recently been described (see Section III. B, C, and D). Removal of all three glucose residues gives rise to a glycoprotein having a $Man_9GlcNAc_2$ structure. This glycoprotein may proceed directly to the high-mannose types, or it may be further processed to form the precursor to the complex types of oligosaccharides.[28] These additional processing reactions occur in the Golgi and involve the removal of four mannose residues by membrane-bound α1,2-mannosidases

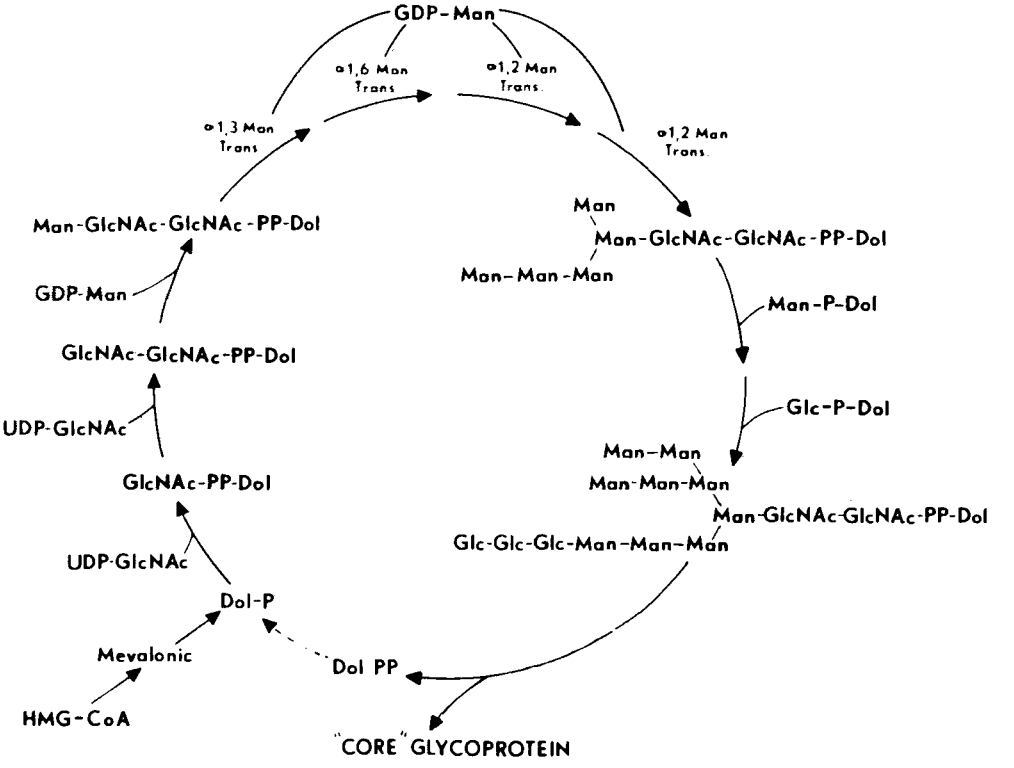


FIGURE 2. Pathway of biosynthesis of the oligosaccharide portion of the asparagine-linked glycoproteins. Dolichyl-P (Dol-P) serves as the lipid carrier and the sugars *N*-acetyl-glucosamine (GlcNAc), mannose (Man), and glucose (Glc) are added from their sugar nucleotide derivatives to this lipid to form the $Glc_3Man_9GlcNAc_2$-PP-Dol.

to give a $Man_5GlcNAc_2$-protein.[29-32] A GlcNAc residue is then added from UDP-GlcNAc to this $Man_5GlcNAc_2$-protein and this GlcNAc apparently is the signal for another α-mannosidase (mannosidase II) to remove the two branched mannoses.[33-35] An inhibitor of the mannosidase II and glycoprotein processing is also known (see Section III.A). After the removal of the final two mannose residues to give a GlcNAc-$Man_3GlcNAc_2$-protein, the remaining sugars of the complex chains, GlcNAc, galactose, and sialic acid, are added by the sequential transfer from their sugar nucleotides to form the completed glycoprotein,[3,4] However, all the details of these additions are not known. Nor is it known why some oligosaccharides remain as high-mannose whereas others become complex, or why some complex chains are biantennary while others are triantennary or tetraantennary.

One very fruitful approach to examining the biosynthetic steps in N-linked oligosaccharide formation is the use of inhibitors that block these pathways at specific steps. This approach can result in the accumulation of intermediates prior to the block, and such inhibitors may also cause the formation of altered and unusual glycoproteins. These abnormal glycoproteins may be quite useful for functional studies on the role of the complex or high-mannose structures. A number of inhibitors have been found that block individual steps in the formation of lipid-linked oligosaccharides and other inhibitors are now known that prevent various processing reactions from occurring. Some inhibitors of cholesterol biosynthesis also block the formation of dolichol and dolichyl-P, thereby limiting the levels of carrier lipid. Finally, some compounds appear to inhibit the movement of glycoproteins through the various intracellular compartments to their final destination. Each of these inhibitors is discussed in this review under the appropriate section with regard to the specific site of action.

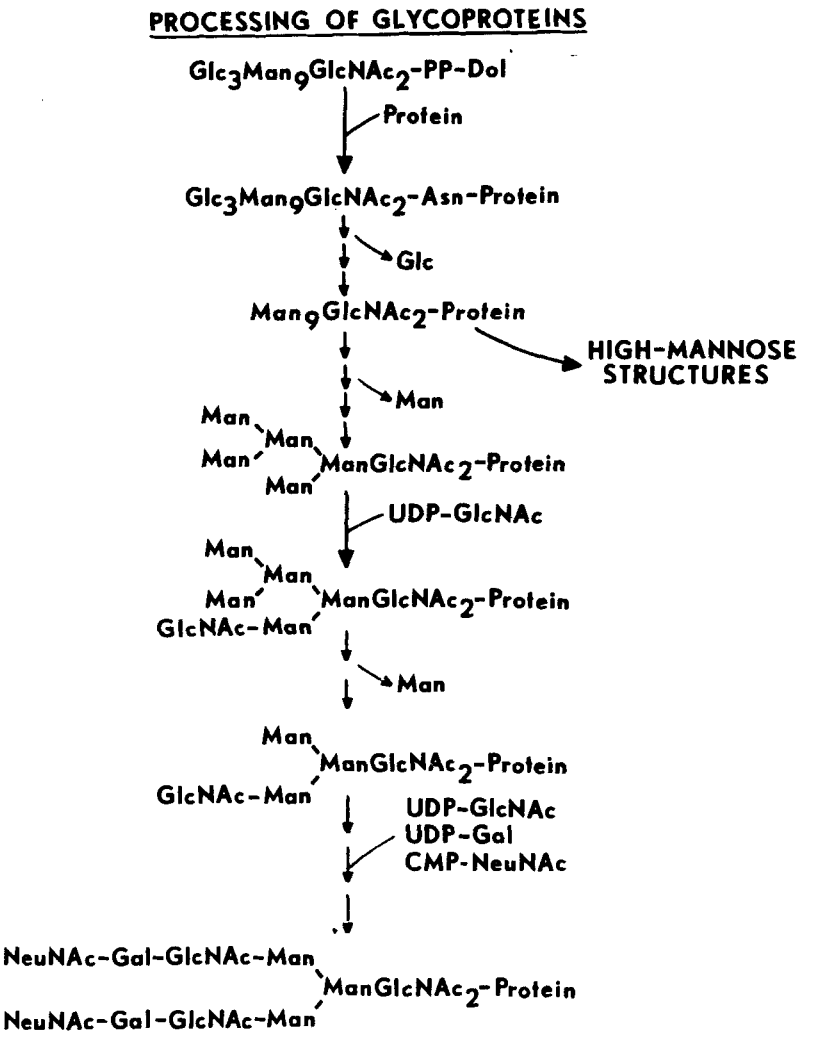
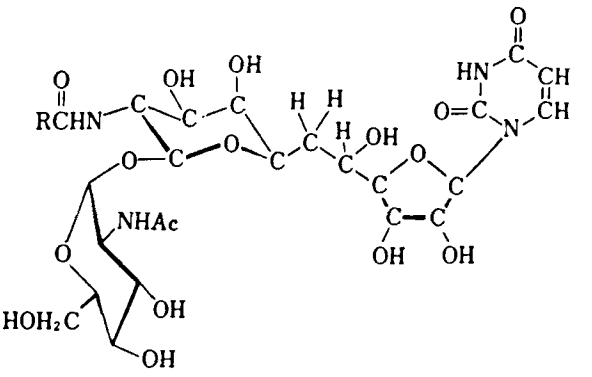


FIGURE 3. Reactions involved in the processing of the oligosaccharide chain of asparagine-linked glycoproteins. After transfer of oligosaccharide to protein, the oligosaccharide is subjected to a number of processing reactions that lead to removal of all of the glucose and some of the mannose units to give rise to complex chains.

II. INHIBITORS OF THE LIPID-LINKED SACCHARIDE (DOLICHOL) PATHWAY

A. Tunicamycin and Related Antibiotics

Tunicamycin is a nucleoside antibiotic that is produced by *Streptomyces lysosuperificus*. The antibiotic was originally isolated by Tamura and associates and was shown to be inhibitory towards Gram-positive bacteria, yeast, fungi, protozoa, enveloped viruses, and mammalian cells in culture.[36-38] The structure of the antibiotic was elucidated by Tamura and co-workers[39-41] largely through characterization of the the products resulting from acid hydrolysis (3 *N* HCl, 100°, 3 hr). As shown in Figure 4, the antibiotic is composed of uracil, fatty acid, and two glycosidically (N and O) linked sugars. The sugars are *N*-acetylglucosamine and a novel C_{11}-aminodeoxy dialdose which has been named tunicamine. The tunicamine is substituted at two positions; at the anomeric carbon an *N*-acetylglucosamine (GlcNAc) is attached in glycosidic linkage, while at the amino group a long-chain fatty acid



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 : $(CH_3)_2CH(CH_2)_{11}CH=CH-$

FIGURE 4. Structures of the various tunicamycin components. R refers to the different fatty acids indicated as I through X that may be attached to the tunicamine.

is linked in an amide bond. Tunicamycin is produced as a mixture of at least ten homologous antibiotics that can be separated from each other by high performance liquid chromatography.[41-43] These homologs differ in the structure of their fatty acid components, which may vary in chain length from C_{13} to C_{17} and may be either normal or branched, and saturated or unsaturated. The rest of the structure of these homologs appears to be identical to that seen in Figure 4. The molecular weights of the tunicamycins vary from about 802 to 872.[41,43]

The site of inhibition of tunicamycin in animal cell glycoprotein biosynthesis was demonstrated using particulate enzyme preparations from mammalian cells and tissues. This antibiotic was shown to inhibit the first reaction in the lipid-linked saccharide pathway (Figure 2), i.e., the transfer of GlcNAc-1-P from UDP-GlcNAc to dolichyl-P to form GlcNAc-PP-dolichol.[44,45] This GlcNAc-PP-dolichol serves as the acceptor for the remaining sugars in the lipid-linked saccharide pathway with the ultimate formation of $Glc_3Man_9GlcNAc_2$-PP-dolichol. Since this oligosaccharide-lipid is the ultimate donor of the carbohydrate chain to protein and its formation is blocked in the presence of sufficient amounts of tunicamycin, the protein is not glycosylated. Thus, in the presence of tunicamycin, at levels of 0.1 to 5 $\mu g/m\ell$, the incorporation of [2-^{3}H]mannose into lipid-linked oligosaccharides and into glycoproteins is inhibited by 90 to 95%. Tunicamycin was also shown to block protein glycosylation in vivo by the same mechanism as demonstrated in vitro.[46] However, tunicamycin does not interfere with the addition of the second GlcNAc to form GlcNAc-GlcNAc-PP-dolichol[47] nor does it interfere with the addition of GlcNAc residue to the nonreducing ends of the oligosaccharide.[46]

The UDP-GlcNAc-Dolichyl-P:GlcNAc-1-P transferase that catalyzes the first reaction in the lipid-linked saccharide pathway was solubilized from aorta tissue[48] and from hen oviduct membranes.[49] Tunicamycin bears a close structural relationship to UDP-GlcNAc, one of the substrates in the reaction, and therefore might be expected to be a competitive inhibitor of this enzyme. However, with the solubilized aorta enzyme, Lineweaver-Burk plots of UDP-GlcNAc concentration vs. velocity at various tunicamycin concentrations suggested that the inhibition was of a noncompetitive nature. The suggestion was made that tunicamycin might act as a substrate-product transition state analog binding irreversibly to the enzyme.[48] On the

other hand, with the hen oviduct GlcNAc-1-P transferase, kinetic analysis were consistent with tunicamycin acting as a tight-binding reversible inhibitor. UDP-GlcNAc, but not dolichyl-P, was able to protect the enzyme from tunicamycin inhibition and it was suggested that the inhibition was probably competitive with respect to UDP-GlcNAc.[49] Furthermore, when the inhibitory effect of tunicamycin was examined using chick embryo microsomes, it was found that less inhibition occurred at higher UDP-GlcNAc concentrations. The kinetics in this case suggested competitive inhibition.[50] Streptovirudin, an analog of tunicamycin having a 100-fold lower affinity for the GlcNAc-1-P transferase (see below), also shows kinetics with respect to UDP-GlcNAc concentration that are suggestive of competitive inhibition.[51] Taken together, these data suggest that tunicamycin binds very tightly to the GlcNAc-1-P transferase and is only displaced with some difficulty by high substrate concentrations.[52] The Ki for tunicamycin has been estimated at about 5×10^{-8} *M*.[50] Tunicamycin also inhibits peptidoglycan biosynthesis and teichoic and teichuronic acid formation in bacteria. In these cases, as well as in glycoprotein biosynthesis, the enzymes involved are phosphotransferases that catalyze the translocation of *N*-acetylhexosamine-1-P. Presumably, the interaction of antibiotic with enzyme involves recognition of an α-linked GlcNAc residue, but other facets of the antibiotic structure are apparently also involved.[52]

Several other antibiotics have been isolated that are closely related in structure to tunicamycin and appear to have the same site of action.[53] These antibiotics include streptovirudin,[54] mycospocidin,[55] antibiotic 24010,[56] and antibiotic MM 19290.[57] Although, in most cases, these compounds have not been characterized to the same extent as tunicamycin, the methods used in their purification and their chromatographic properties indicate that they are closely related to tunicamycin.[52] Also, the IR and UV spectra of several of these antibiotics are closely related to that of tunicamycin. Streptovirudin was, however, separated into two distinct series, called series I and II, by gel filtration and reverse-phase high-performance liquid chromatography. Field desorption mass spectrometry showed that series I streptovirudins were homologously related (molecular weights 790 to 818) with molecular masses 2 daltons greater than those of corresponding members of series II. This difference in mass was shown to be due to the replacement of the uracil of series II components by dihydrouracil in the series I homologs. The molecular weights of the isolated streptovirudins were as follows: A_1, 790; A_2, 788; B_1, 804; B_2, 802; C_1, 818; and C_2, 816.[51] Thus this series of antibiotics appear to be closely related to the tunicamycins, but have shorter-chain fatty acids. Mycospocidin was also examined by reverse-phase HPLC and found to be composed of a number of components that were indistinguishable from those of tunicamycin. Chemical studies also revealed that they were similar antibiotic complexes.[58]

Streptovirudin, antibiotic 24010, and mycospocidin were all shown to inhibit the in vitro synthesis of GlcNAc-pyrophosphoryl-dolichol in various eukaryotic tissues.[55,58,59] Of interest is the fact that this group of antibiotics, like tunicamycin, appear to be relatively specific for the GlcNAc-1-P transferase, at least when tested at relatively low concentrations. However, high concentrations of tunicamycin and streptovirudin also inhibited in vitro formation of glucosyl-phosphoryl-dolichol.[59] In this case, streptovirudins of series II inhibited this reaction (50% inhibition at 0.5 to 1.5 μg/mℓ) but the series I compounds were much less active (i.e., 50% inhibition at 50 to 100 μg/mℓ).

Tunicamycin has been used with a number of different cells and tissues (usually at concentrations of 0.5 to 5 μg/mℓ) to determine the role of carbohydrate in secretion of glycoprotein, or in membrane and viral capsid assembly, or in various other biological phenomena. Since these antibiotics also prevent protein glycosylation in vivo by the same mechanism as seen in vitro,[46] one generally finds that cells incubated in tunicamycin produce the unglycosylated form of the N-linked glycoprotein. Thus, in most cases, the protein portion of the molecule is still synthesized in the presence of antibiotic, but it may be subjected to more rapid degradation (see below). Although these results would suggest that

synthesis of the protein is not linked to glycosylation, such regulatory link has been postulated in some cases. For example, in studies on yeast carboxypeptidase Y,[60] and barley α-amylase,[61] the inhibition of glycosylation by tunicamycin also led to decreased amounts of the protein. In the case of yeast carboxypeptidase, the unglycosylated form of the enzyme was as stable in cell extracts as the normal glycoprotein, indicating that the decreased levels of protein were not due to more rapid proteolysis. The secretion and accumulation of the protein portion of IgA and IgE were also inhibited by tunicamycin.[62] A significant difference between in vivo and in vitro inhibition of protein synthesis was observed with regard to thyroglobulin synthesis.[63] In this case, tunicamycin did not significantly inhibit protein synthesis in vitro (>4%), whereas in vivo it was inhibited 33%. While in some cases, these kinds of results could be due to decreased stability and more rapid degradation of the protein, there may also be a regulatory link between synthesis of carbohydrate and protein.

The consequences of the protein not being glycosylated may vary widely, depending on the glycoprotein in question. Thus in many cases, the absence of glycosylation causes marked alterations in the properties and fate of the molecule. For example, the immunoglobulins IgA, IgE, and IgM are inefficiently secreted when these proteins are synthesized in the presence of tunicamycin.[62] In this case, electron microscopic studies and direct immunofluorescence showed the presence of distended RER that contained a dense granular precipitate identified as immunoglobulin. The authors postulated that the absence of carbohydrate caused an alteration in the physical properties of the immunoglobulins and led to decreased solubility and aggregation. The migration of the envelope proteins of vesicular stomatitis virus (G protein) and of Sindbis virus from the endoplasmic reticulum to the plasma membrane is also impaired when these proteins are synthesized in the presence of tunicamycin.[64] However, tunicamycin did not change the amount of the major cell surface glycoprotein appearing at the surface of chick embryo fibroblasts, but it did decrease the amount present in fibroblast cultures.[65] This decrease in total cell surface glycoproteins was apparently due to an increased degradation in cells treated with tunicamycin.

The idea that the carbohydrate chain may play a role in protecting the protein from proteolytic attack is also indicated from other studies. Thus, the carbohydrate-free form of chick fibronectin,[65] and the hemagglutinin of fowl plaque virus,[67] are unusually susceptible to proteolytic digestion in vivo. In the case of the fowl plaque virus, the unglycosylated hemagglutinin could be found when a protease inhibitor such as *N*-α-*p*-tosyl-L-lysine-chloromethylketone (TLCK was included in the incubation medium. On the other hand, the envelope protein of Rauscher murine leukemia virus is not proteolytically processed unless it has been glycosylated[68] Also, in mouse pituitary cells inhibited with tunicamycin, the α- and β-subunits of thyroid-stimulating hormone are synthesized, but these subunits apparently require the carbohydrate to form the active hormone.[69] Thus, in these cases, the carbohydrate chain appears necessary for proper functioning of the glycoprotein.

However, in a number of cases, lack of carbohydrate had little or no effect on the protein in question. For example, the secretion of the serum glycoproteins, transferrin and VLDL, by cultured hepatocytes was not inhibited by tunicamycin though the proteins were shown to be devoid of carbohydrate.[70,71] Likewise, the secretion of interferon,[72,73] IgG,[74] ovalbumin,[75] and the α-subunit of human chorionic gonadotropin[76] are not impaired in tunicamycin-treated cells. In addition, the signal peptides of placental prelactogen and the α-subunit of human chorionic gonadotropin are proteolytically processed in the presence of tunicamycin even though they lack carbohydrate.[77] Various glycoprotein enzymes, such as alkaline phosphatase of yeast,[78] are still active even in the unglycosylated form.

At first glance, the above results appear to be contradictory and confusing. However, a plausible explanation for these varied results has been proposed.[79,80] Since biosynthetic studies have shown that the carbohydrate chain is added to protein while the peptide is still attached to nascent chains and is therefore a cotranslational event, the suggestion has been

made that one major role of glycosylation is to influence the folding of the polypeptide chain.[80] Thus, in the absence of glycosylation, protein conformation may be altered leading to decreased solubility, increased susceptibility to proteolytic attack, increased aggregation, inability to be secreted, and so on. However, not all proteins are influenced to the same extent by the presence of carbohydrate and some glycoproteins may be able to assume the proper conformation even in the absence of carbohydrate. This ability must reside in the amino acid composition and sequence of the protein allowing proper function in the absence of glycosylation.

Some evidence for the above postulation has been obtained using various temperature-sensitive variants of vesicular stomatitis virus (VSV).[79] With one of these VSV strains (San Juan), tunicamycin prevented viral replication more than 90% when the host cells were grown at 38° and almost the same when the cells were grown at 30°. However, in another VSV strain (Orsay), viral replication was again inhibited 85 to 90% at 38° in the presence of tunicamycin, but only 30 to 55% at 30°. With this VSV strain (Orsay) grown at 30°, the unglycosylated G protein could be detected at the host cell surface indicating that it had been synthesized and transported in the normal way. However, when Orsay was grown at 37°, no G protein was detected at the cell surface. Furthermore, differences were observed in the physical properties of the viral proteins formed at various temperatures in the presence of tunicamycin. Thus, the Orsay G protein formed at 30° in the presence of tunicamycin could be solubilized by Triton® X-100 whereas the protein formed at 37°, or the G protein from San Juan, remained insoluble. These data suggest that different proteins have different requirements for carbohydrate in order to assume the proper conformation.

B. Sugar Analogs

A number of analogs of glucose and mannose, such as 2-deoxyglucose and 2-fluoro-2-deoxy-D-glucose, have been found to interfere with protein glycosylation. With these compounds there is a problem in terms of lack of specificity since they also appear to affect other metabolic reactions. Nevertheless, they have been widely used and have provided some valuable insight into the mechanisms of biosynthesis and function of the oligosaccharide chains of the N-linked glycoproteins.[81] It is interesting that 2-deoxyglucose was initially discovered as an inhibitor of protein glycosylation because of its antiviral activity against enveloped viruses, and the reversal of this inhibition by mannose.[82] Subsequently, it was found that this antiviral activity was due to the fact that this compound blocked the glycosylation of viral envelope glycoproteins and thereby prevented the formation of the viral envelope.[83] These compounds are usually used at concentrations of 0.1 to 5 m*M*.

When 2-deoxyglucose, which is an analog of glucose and mannose, is given to cells in culture, this sugar is converted to both UDP-2-deoxyglucose and GDP-2-deoxyglucose as well as the dolichyl derivative, dolichyl-phosphoryl-2-deoxyglucose. Apparently, the inhibition of protein glycosylation is not the result of depletion of the sugar nucleotide pool because the levels of GDP-mannose and UDP-GlcNAc were actually found to increase in the presence of 2-deoxyglucose. In fact, GDP-2-deoxyglucose appears to be the major nucleotide involved in this inhibition since the inhibition could be reversed by the addition of mannose with a resultant reduction in the levels of GDP-2-deoxyglucose in the cells.[83,84]

The actual site of inhibition is probably at the level of the lipid-linked saccharides.[84] Thus, GDP-2-deoxyglucose was found to inhibit the formation of lipid-linked oligosaccharides in crude membrane preparations of chick embryo cells. Under these conditions, dolichyl-pyrophosphoryl-*N,N'*-diacetylchitobiosyl-2-deoxyglucose accumulated. It was not possible to add additional mannose residues to this trisaccharide-lipid, and protein glycosylation was inhibited. On the other hand, the effects of GDP-2-deoxyglucose were also examined under partially inhibitory conditions which were obtained using a mixture of GDP-2-deoxyglucose and GDP-mannose. In this case, the major glycolipid formed by these membranes was

dolichyl-pyrophosphoryl-$(GlcNAc)_2$-mannose-2-deoxyglucose. This oligosaccharide apparently could not be transferred to protein, nor could it be further elongated. Since the levels of dolichyl-P in the cells appear to be limiting, high levels of 2-deoxyglucose may tie up all of the available dolichyl-P and thus lead to inhibition of protein glycosylation. This is one plausible explanation for the 2-deoxyglucose inhibition, since it could be partially reversed by the addition of dolichyl-P.[85] However, since reversal was not complete, there may be other facets to the inhibition besides levels of dolichyl-P. In vivo, it was found that in the presence of 2-deoxyglucose the normally glycosylated K-46 chain produced by myeloma cells was not glycosylated.[86]

Fluoroglucose and fluoromannose (2-deoxy-2-fluoro-D-glucose or D-mannose) are other analogs of glucose and mannose that have been shown to inhibit protein glycosylation. In chick embryo cells treated with fluoroglucose, the formation of lipid-linked oligosaccharides did not go to completion and oligosaccharides with decreased amounts of glucose and mannose were formed.[87] This inhibition was reversed by the addition of glucose and mannose to the culture medium. In contrast to deoxyglucose, fluoroglucose was not incorporated into the lipid-linked oligosaccharides. However, this sugar analog did inhibit the formation in vivo of dolichyl-phosphoryl-glucose and dolichyl-phosphoryl-mannose, but it did not prevent the transfer of these sugars from their dolichyl-derivatives to the lipid-linked oligosaccharides. In the presence of fluoroglucose the pool size of UDP-glucose, but not that of GDP-mannose or UDP-GlcNAc, was decreased. Probably, the smaller-sized lipid-linked oligosaccharides formed in the presence of fluoroglucose are not transferred efficiently to protein.[88]

C. Aminosugars

Glucosamine (2-deoxy-2-amino-D-glucose) inhibits viral multiplication of a variety of enveloped viruses.[89] This inhibition required millimolar concentrations of this aminosugar. In the presence of glucosamine, the entire oligosaccharide chain of the glycoprotein was missing indicating that this inhibitor might be affecting the formation of the lipid-linked saccharides.[89] However, in one study, no aberrant forms of the lipid-linked oligosaccharides were detected.[90] Furthermore, at inhibitory concentrations of glucosamine, no unusual metabolites were formed and no evidence for glucosamine intermediates, such as UDP-glucosamine, were reported.[91] These studies suggested that glucosamine itself was necessary for inhibition and that this inhibition only occurred in vivo.

However, in another study, glucosamine (at millimolar concentrations) did cause dramatic alterations in the nature of the lipid-linked oligosaccharides.[92] Normally, the major lipid-linked oligosaccharide found in influenza virus-infected MDCK cells is the $Glc_3Man_9GlcNAc_2$-pyrophosphoryl-dolichol. However, at low glucosamine concentrations (0.5 to 1 m*M*), the $Glc_3Man_9GlcNAc_2$-lipid was replaced by a lipid-linked oligosaccharide having a smaller-sized oligosaccharide characterized as a $Man_7GlcNAc_2$. As the glucosamine concentration was increased to higher levels (2 to 10 m*M*), the $Man_7GlcNAc_2$-lipid disappeared and was replaced by a $Man_3GlcNAc_2$-lipid. These data indicate that these two oligosaccharides, $Man_7GlcNAc_2$ and $Man_3GlcNAc_2$, may represent control points in the lipid-linked saccharide pathway, or they may be places where there is a change in the nature of the glycosyl donor. In these studies, as in others described above, the inhibition by glucosamine was reversible. Thus, removal of glucosamine from the culture medium and washing the cell monolayers with glucosamine-free medium led to a restoration of normal lipid-linked oligosaccharide synthesis. The inhibitory effect of glucosamine could not be mimicked by other aminosugars such as galactosamine, mannosamine, or *N*-acetylglucosamine. However, mannosamine, also caused alterations in the formation of the lipid-linked oligosaccharides but these inhibitions were at different sites.[93] Thus, in the presence of low concentrations of mannosamine, the major oligosaccharide associated with the lipid-linked oligosaccharides of MDCK cells was a $Man_6GlcNAc_2$, while at higher mannosamine concentrations this was shifted to a

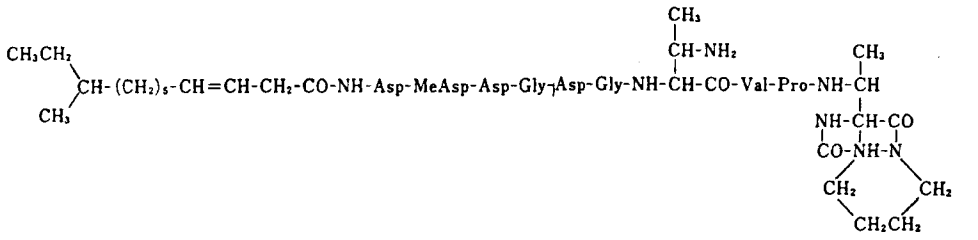


FIGURE 5. Structure of the peptide antibiotic, amphomycin.

$Man_2GlcNAc_2$. It is not clear what these inhibitions mean in terms of regulation, but the site of mannosamine inhibition appears to be distinct from that of glucosamine inhibition.

D. Amphomycin and Tsushimycin

Amphomycin is a lipopeptide antibiotic that is produced by *Streptomyces canus*.[94] Its structure is shown in Figure 5. This antibiotic was shown to be an undecapeptide containing either 3-isododecanoic or 3-anteisotridecanoic acid attached to the N-terminal aspartic acid in an amide linkage.[95] In Gram-positive bacteria, amphomycin inhibited the synthesis of the cell wall peptidoglycan by blocking the transfer of phospho-*N*-acetylmuramoyl-pentapeptide from its UMP-derivative to the lipid carrier, undecaprenyl phosphate.[96] Tsushimycin is another peptide antibiotic that also belongs to the amphomycin-glumamycin group of antibiotics.[97] Although the complete structure of this antibiotic has not been elucidated, it differs from amphomycin in amino acid and fatty acid composition. It also appears to act as an inhibitor of peptidoglycan synthesis.

Since amphomycin inhibits the formation of lipid-linked saccharides in bacteria, it was tested as an inhibitor of the lipid-linked saccharide pathway in animal tissue.[98] In these experiments, cell-free extracts of pig aorta were used as the tissue source since these extracts have the capacity to transfer mannose from GDP-mannose, GlcNAc from UDP-GlcNAc, and glucose from UDP-glucose into the various lipid-linked monosaccharides (i.e., dolichyl-phosphoryl-mannose, dolichyl-pyrophosphoryl-GlcNAc, etc.) and lipid-linked oligosaccharides (see Figure 2). With this enzyme preparation, amphomycin was a much better inhibitor of mannose incorporation into dolichyl-phosphoryl-mannose than of mannose incorporation into the lipid-linked oligosaccharides. Thus, at amphomycin concentrations of 50 to 200 μg per incubation mixture, the transfer of mannose from GDP-mannose to dolichyl-P was inhibited more than 95%, but mannose incorporation into the lipid-linked oligosaccharides was only inhibited by 60 to 70%. Under these conditions, essentially all of the labeled mannose was found in a single oligosaccharide that was characterized as a $Man_5Glc\text{-}NAc_2$.[98] These data suggested that some, if not all, of the first four α-linked mannoses in the $Man_5GlcNAc_2$ are derived from GDP-mannose rather than from dolichyl-phosphoryl-mannose. Data from several other experimental sources have also supported the idea that these mannoses come directly from GDP-mannose. For example, several studies have shown the direct transfer of mannose from GDP-mannose, but not from dolichyl-P-mannose, to lipid-linked oligosaccharide by solubilized and partially purified enzyme preparations.[99,100] With the solubilized enzyme system, the major product was a $Man_5GlcNAc_2$-lipid. In addition, a mutant mammalian cell line was isolated that had lost its capacity to synthesize dolichyl-phosphoryl-mannose.[101] This mutant cell synthesized the $Man_5GlcNAc_2$-lipid but was not able to elongate it, presumably because it lacked the mannosyl donor (i.e., dolichyl-phosphoryl-mannose) for the last four mannose residues. However, when extracts of the mutant cells were supplemented with dolichyl-phosphoryl-mannose, they could elongate the $Man_5GlcNAc_2$-lipid.

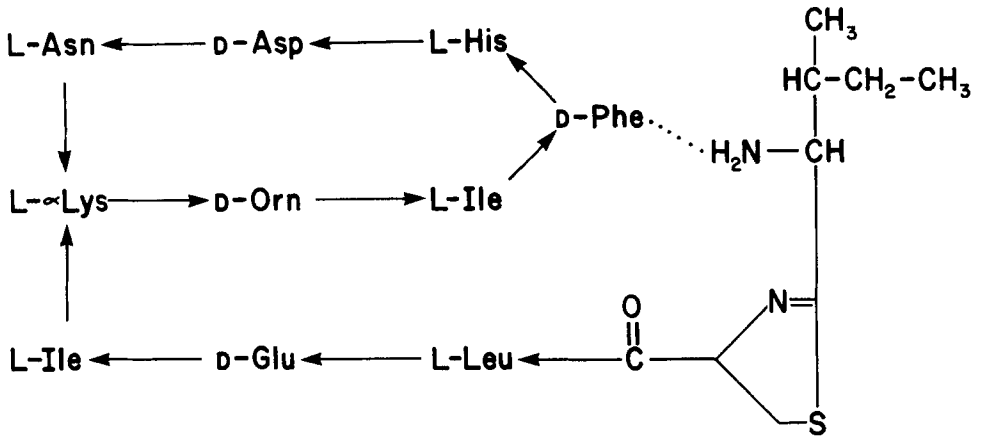


FIGURE 6. Structure of the peptide antibiotic, bacitracin.

Amphomycin has also been utilized as an inhibitor of lipid-linked saccharides in membrane preparations from brain tissue.[102] These studies gave similar results to those described above and also demonstrated that amphomycin blocks the transfer of glucose from UDP-glucose to dolichyl-P. However, the antibiotic did not affect glucose transfer from UDP-glucose to particle-bound glucan or to ceramide. Also, in these studies, the transfer of GlcNAc-1-P from UDP-GlcNAc to dolichyl-P was inhibited by this antibiotic. These workers suggested that amphomycin may form a complex with dolichyl-P and in this way obstruct glycosylation reactions. Amphomycin was also shown to inhibit similar types of glycosylation reactions in plants.[103]

Tsushimycin is closely related to amphomycin chemically, and it appears to have the same site of action on the lipid-linked saccharide pathway.[104] Thus, at 50 to 200 μg of tsushimycin per incubation mixture, the formation of dolichyl-phosphoryl-mannose was almost completely inhibited, but mannose, from GDP-mannose, was still incorporated into the $Man_5GlcNAc_2$-lipid. Unfortunately, amphomycin and tsushimycin do not inhibit these reactions in intact mammalian cells, probably because they are not able to enter the cells.

E. Bacitracin

Bacitracin is a polypeptide antibiotic that is produced by certain strains of *Bacillus licheniformis*. Its structure is shown in Figure 6. This antibiotic was shown to inhibit cell wall peptidoglycan biosynthesis in bacteria by preventing the dephosphorylation of undecaprenyl pyrophosphate.[105] This undecaprenyl-PP must be converted to undecaprenyl-P in order to recycle as a carrier of the carbohydrate in peptidoglycan synthesis. It has also been reported that bacitracin inhibits the biosynthesis of squalene and sterols from mevalonate,[106] the formation of ubiquinones,[107] and the degradation of thyrotropin-releasing factor and leutinizing hormone-releasing factor.[108] Bacitracin has also been tested in a number of mammalian systems as an inhibitor of N-linked glycoproteins. The results in these cases have been quite variable and may depend on the system in question.

When hen oviduct membranes were incubated with UDP-[^{14}C]GlcNAc in the presence of 1 m*M* bacitracin, a trisaccharide-lipid accumulated. The trisaccharide was characterized as a Man-β-$GlcNAc_2$ indicating that the antibiotic blocked the addition of the first α-linked mannose residue.[109] On the other hand, 0.2 to 1 m*M* bacitracin inhibited the formation of dolichyl-pyrophosphoryl-GlcNAc by calf pancreas microsomes, but had no effect on the synthesis of dolichyl-PP-$(GlcNAc)_2$, dolichyl-P-mannose or dolichyl-P-glucose.[110] In yeast membrane preparations, 0.33 m*M* bacitracin caused an inhibition of the formation of dolichyl-

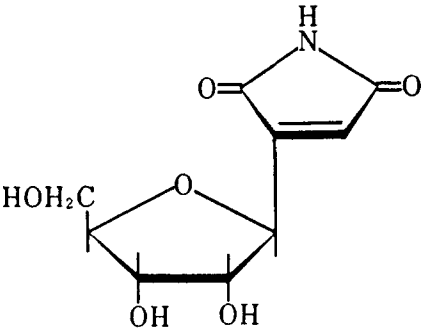


FIGURE 7. Structure of the nucleoside antibiotic, showdomycin.

PP-$(GlcNAc)_2$, but in this case no inhibition of dolichyl-PP-GlcNAc was reported.[111] Thus, these studies suggest at least three different sites of action of this antibiotic.

It seems likely that the action of bacitracin in these eukaryotic sytems is due to the formation of a complex with the lipid carrier, dolichyl-P, as has been shown for the microbial systems (i.e., with undecaprenyl-P). Thus, using a particulate or solubilized enzyme preparation of pig aorta, 0.1 to 0.2 m*M* bacitracin blocked the incorporation of both mannose and GlcNAc from their sugar nucleotide derivatives into lipid-linked monosaccharides (dolichyl-P-mannose and dolichyl-PP-GlcNAc), and into lipid-linked oligosaccharides. The inhibition of dolichyl-P-mannose formation could be overcome by the addition of high concentrations of dolichyl-P but this lipid could not reverse the inhibition of dolichyl-PP-GlcNAc synthesis. Bacitracin also blocked the transfer of mannose from GDP-mannose to lipid-linked oligosaccharides by the particulate enzyme, as well as the transfer of mannose from dolichyl-P-mannose to lipid-linked oligosaccharides.[112] Similar types of inhibitory reactions were observed when bacitracin was tested with a particulate enzyme preparation from mung bean seedlings.[113] These data indicate that bacitracin may block many of the steps in the lipid-linked saccharide pathway.

F. Showdomycin

Showdomycin is a broad spectrum, nucleoside antibiotic that is elaborated by *Streptomyces showdoensis*.[114] Its structure is shown in Figure 7. The antibiotic is moderately active against Gram-positive and Gram-negative bacteria and also shows remarkable activity against Ehrlich ascites tumor in mice and against Hela cells.[114] This antibiotic strongly inhibited bovine liver UDP-glucose dehydrogenase and this inhibition was attributed to its alkylating action on the enzyme. Preincubation of showdomycin with cysteine completely prevented the inhibition.[115]

With the aorta particulate enzyme preparation, showdomycin effectively inhibited the formation of dolichyl-phosphoryl-glucose, but this inhibition was much more pronounced in the presence of the nonionic detergent, NP-40. At 0.25% NP-40, 50% inhibition of this activity required about 10 μg/mℓ of antibiotic. The antibiotic also inhibited transfer of mannose from GDP-mannose to both dolichyl-phosphoryl-mannose and to lipid-linked oligosaccharides, but in these cases inhibition was only evident in the presence of detergent and required much larger amounts of antibiotic than necessary for glucose inhibition. Relatively little inhibition of GlcNAc transfer was observed either in the presence or absence of detergent.[116] Although showdomycin inhibited glucolipid formation in aorta, it greatly stimulated glucose incorporation into lipid in yeast membrane preparations. This glucolipid had chemical and chromatographic properties like those of glucosylceramide. The stimulation of this glucosylceramide appeared to be due to a protection of the substrate, UDP-glucose,

from degradation and suggested that showdomycin may inhibit one or more of the enzymes involved in catabolism of UDP-glucose.

With membrane preparations from *Volvox cartere* f. *nagaraensis,* both the formation of dolichyl-phosphoryl-glucose and dolichyl-pyrophosphoryl-GlcNAc were sensitive to showdomycin to about the same extent in the presence of Triton® X-100.[117] This inhibitory effect of showdomycin was lost when an excess amount of dithiotheitol was added. These enzymes were also inactivated by *N*-ethylmaleimide, and inhibition by this reagent was comparable to that of showdomycin. The addition of UMP to incubation mixtures protected the enzymes from inhibition by either *N*-ethylmaleimide or showdomycin. The conclusion of these studies was that inhibition by showdomycin is probably caused by the irreversible reaction of the maleimide structure of the antibiotic with thiol groups on the protein.[117]

Showdomycin was used as a tool to examine the regulation of GlcNAc-lipid synthesis by dolichyl-P-mannose.[118] Previous studies had shown a great stimulation in synthesis of GlcNAc-lipid when exogenous dolichyl-P-mannose was added to the incubation mixtures. Thus, showdomycin (and diumycin) were added to incubation mixtures at concentrations that partially inhibited the formation of dolichyl-P-mannose. Under these conditions, the stimulatory effect was also blocked. However, when dolichyl-P-mannose was added alone with the showdomycin, stimulation of GlcNAc-lipid still occurred. In the presence of showdomycin or diumycin, the formation of the chitobiosyl- and mannose-containing trisaccharide lipids were completely suppressed, but not that of GlcNAc-PP-dolichol. The author suggests that the target of activation by dolichyl-P-mannose is the glucosaminyl transferase that forms GlcNAc-PP-dolichol.

G. Diumycin

The structure of diumycin is not currently known, but acid hydrolysis of the antibiotic releases glucosamine, glucose, ammonia, acetic acid, phosphate, and a C_{25} fatty acid. Diumycin is in the same group of antibiotics as are moenomycin, prasinomycin, macarbomycin, 8036RP, 11837RP, and 19402RP, all of which affect the lipid-linked saccharide pathway of bacterial cell wall peptidoglycan biosynthesis.[119,120] This antibiotic was originally isolated from fermentation broths of *Streptomyces umbrinus*[121] and was shown to inhibit cell wall biosynthesis in *Staphylococcus aureus.*[122]

Diumycin inhibited the formation of dolichyl-P-mannose with both a particulate and a solubilized enzyme preparation of *Saccharomyces cerevesiae.*[123] Inhibition was somewhat more pronounced with the solubilized enzyme, and 90% inhibition of the above reaction was observed at 150 μg/mℓ of antibiotic. Transfer of mannose to preformed dolichyl-PP-$(GlcNAc)_2$ still occurred in the presence of diumycin, but transfer of mannose from dolichyl-phosphoryl-mannose to serine (threonine) residues on the protein was also inhibited. However, the transfer of the mannose portion of GDP-mannose directly to serine or threonine residues was not prevented by diumycin. With a soluble enzyme preparation from *Acanthamoeba,* diumycin inhibited the transfer of mannose from GDP-mannose and GlcNAc from UDP-GlcNAc into the lipid-linked monosaccharides. In this case, 250 μg/mℓ of antibiotic inhibited dolichyl-P-mannose formation by 90% and dolichyl-PP-GlcNAc formation by 70%. The synthesis of dolichyl-P-glucose was only slightly affected by this antibiotic. In addition, diumycin also blocked the transfer of the second GlcNAc from UDP-GlcNAc to dolichyl-PP-GlcNAc, and this reaction was even more sensitive than was the formation of dolichyl-PP-GlcNAc. The kinetics of inhibition of dolichyl-P-mannose formation were of the mixed type, and suggested that the antibiotic may bind at a site other than the active site and alter the affinity of enzyme for the substrates.[124]

H. Other Inhibitors

It is known that glucose-starved mammalian cells (especially CHO cells) do not form the

$Glc_3Man_9GlcNAc_2$-PP-dolichol to any extent, but instead accumulate a $Man_5GlcNAc_2$-PP-dolichol.[125,126] This lipid may be glucosylated to give rise to a $Glc_3Man_5GlcNAc_2$-lipid which probably serves as the oligosaccharide donor for the protein. The mechanism of this inhibition is not known, but it may be related to the utilization of glucose as an energy source. Interestingly, CCCP (-chlorocarbonyl-cyanide phenylhydrazone) is an uncoupler of oxidative phosphorylation, and it causes energy depletion of cells when it is added to the culture media. In the presence of 10 m*M* CCCP, cultured mammalian cells do not produce dolichyl-P-mannose but can still synthesize dolichyl-P-glucose, dolichyl-PP-GlcNAc, and dolichyl-PP-$(GlcNAc)_2$. These cells incubated in the presence of CCCP produce a Man_5-$GlcNAc_2$-lipid and transfer this oligosaccharide to protein. Some of this $Man_5GlcNAc$-lipid may become glucosylated at the lipid stage and this species may be the one that is transferred to protein.[127] These studies agree with the in vitro experiments done with amphomycin indicating that dolichyl-P-mannose is necessary as mannosyl donor to elongate the Man_5-$GlcNAc_2$-lipid.

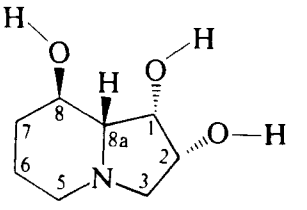
III. INHIBITORS OF GLYCOPROTEIN PROCESSING

Once the oligosaccharide portion of the lipid-linked oligosaccharide has been attached to protein, the oligosaccharide undergoes a number of processing reactions as outlined in Figure 3. These reactions involve the removal of all three glucose residues by membrane-bound glucosidases (glucosidase I and II), followed by the removal of four mannose residues by one or more α1,2-mannosidases. Following the addition of a GlcNAc residue to the α1,3-linked mannose, two more mannose residues are removed by mannosidase II and the remaining sugars of the complex chains are added. Since both the high-mannose glycoproteins and the complex glycoproteins share the same initial precursor, i.e., $Glc_3Man_9GlcNAc_2$-protein, inhibitors of processing might prevent the formation of complex types of oligosaccharides and give rise to glycoproteins that have altered types of oligosaccharides. Such inhibitors could, therefore, be valuable tools to examine the role of complex or high-mannose oligosaccharides in glycoprotein structure and function.

A. Swainsonine

Swainsonine is an indolizidine alkaloid that was initially isolated from the Australian plant, *Swainsona canescens*.[128] The structure of this alkaloid is shown in Figure 8. Livestock that eat this plant develop symptoms analogous to those of human α-mannosidosis,[129] and swainsonine was shown to be a potent inhibitor of lysosomal and jack bean α-mannosidase.[130] This alkaloid has recently been isolated from locoweed *(Astragalus lentiginosis)* that grows in the southwestern U.S.[131] and also from the fungus, *Rhizoctonia leguminicola*.[132]

Swainsonine was tested as an inhibitor of glycoprotein processing in cultured mammalian cells to determine whether it would inhibit the formation of complex oligosaccharides.[133] In these studies, MDCK cells were grown to confluency, incubated for several hours in the presence of swainsonine (100 to 500 ng/mℓ), and then incubated for several hours in the presence of either [2-^{3}H]mannose or [6-^{3}H]-glucosamine (also in the presence of swainsonine) to label the glycoproteins. In order to examine the nature of the oligosaccharide chains of these glycoproteins, the cell residues were digested with pronase and the labeled glycopeptides were purifid by gel filtration on Biogel® P-4 columns. Since it is difficult to resolve the complex types of glycopeptides from the high-mannose types on these columns, the entire glycopeptide fraction from swainsonine-treated and control cells were digested with endoglucosaminidase H and rechromatographed on the Biogel® P-4 column. This enzyme cleaves between the two internal GlcNAc residues (i.e., the *N,N'*-diacetylchitobiose) of high-mannose and hybrid glycopeptides, but does not act on the complex types of glycoproteins.[134] Thus, after this digestion, the high-mannose (and hybrid) structures are reduced in molecular



Swainsonine
(8aβ-indolizidine-1α,2α,8β-triol; mol.wt. 173.1)

FIGURE 8. Structure of the plant indolizidine alkaloid, swainsonine.

size, due to loss of GlcNAc-peptide, and can be easily resolved from complex chains. Cells incubated in swainsonine showed a considerable decrease in the amount of radioactive mannose or glucosamine in the complex types of glycopeptides, and a considerable increase in the amount of radioactivity in the glycopeptides that were sensitive to endoglucosaminidase H. These endoglucosaminidase H-sensitive structures appeared to be a mixture of the high-mannose and hybrid types of oligosaccharides.[133]

In cultured mammalian cells, it is not feasible to study glycoprotein synthesis from the start, nor it is possible to examine the synthesis of a single glycoprotein. However, various viruses produce envelope glycoproteins that have asparagine-linked oligosaccharides, and these systems offer excellent models to examine the biosynthesis, processing, and transport of specific glycoproteins. Thus, the effect of swainsonine on the formation of the influenza viral hemagglutinin was examined.[135] The influenza viral hemagglutinin is a glycoprotein having both complex and high-mannose chains.[136] MDCK cells were infected with influenza virus and swainsonine was added to some flasks. After an incubation of several hours to allow the alkaloid to take effect, [^{3}H]mannose or [^{3}H]glucosamine was added to label the glycoproteins. In the control virus grown without swainsonine, about 70% of the mannose incorporated into glycoprotein was in endoglucosaminidase H-resistant glycopeptides of the complex type, and 30% was in endoglucosaminidase H-sensitive structures of the high-mannose type. However, when virus was grown in swainsonine, almost 90% of the glycopeptides were digested by endoglucosaminidase H and these oligosaccharides were found to be of the hybrid and high-mannose types.[135]

The above in vivo studies are in accord with recent in vitro studies on the site of action of swainsonine. Thus, this alkaloid was shown to be a potent inhibitor of mannosidase II that removes the α-1,3- and α-1,6-linked mannose residues from the GlcNAc-Man_5-$GlcNAc_2$-protein, but it had no effect on the α-1,2-mannosidase(s) that removes the first four α-1,2-linked mannoses.[137] When various mammalian cell lines were grown for several days in the presence of swainsonine, these cells showed a considerable increase in their ability to bind [^{3}H]Concanavalin A and other ligands that recognize high-mannose structures, and a considerable decrease in the binding of lectins that recognize sugars of the complex chains (i.e., wheat germ agglutinin and ricin). However, swainsonine had no effect on cell growth and was not cytotoxic at the levels used in these studies.[138]

B. Castanospermine

Castanospermine is another plant alkaloid (1,6,7,8-tetrahydroxyoctahydroindolizine) that was isolated from the seeds of the Australian plant, *Castanospermum australe*.[139] The structure of this alkaloid is shown in Figure 9. Castanospermine was found to be a potent inhibitor of almond emulsin β-glucosidase but was without effect on yeast α-glucosidase, α or β-galactosidase, α-mannosidase, β-*N*-acetylhexosaminidase, β-glucuronidase or α-L-fucosi-

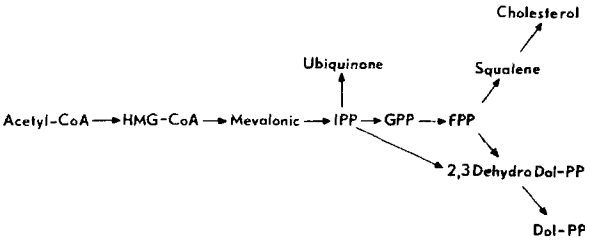


FIGURE 10. Pathway of biosynthesis of cholesterol and dolichol from acetyl-coenzyme A.

dolichyl-linked saccharides and glycoproteins. 25-Hydroxycholesterol, at 1 to 5 μM, also inhibited the incorporation of acetate into both cholesterol and dolichol in L-cell cultures, but in these studies the relationship between the concentration of 25-hydroxycholesterol and the extent of inhibition was different for the two lipids. Thus, large fluctuations in cholesterol synthesis were observed under conditions that only slightly affected the synthesis of dolichol. However, under conditions where sterol synthesis was repressed to levels below 25% of control cultures, further inhibition of cholesterol biosynthesis was accompanied by a proportionate decline in dolichol formation.[154] The authors postulated that a rate-limiting enzyme unique to the dolichol pathway may be saturated at a lower level of intermediate than is required to saturate the cholesterol pathway at the next rate-limiting step beyond the branch point. Therefore, HMG-CoA reductase activity and the rate of cholesterol synthesis may fluctuate with little change in dolichol synthesis, as long as the levels of these intermediates are sufficient to saturate the dolichol branch of the biosynthetic pathway.

Nevertheless, these studies do indicate that the rate of synthesis of dolichyl-P, and thus the levels of dolichyl-P in the cell, may play a role in the regulation of the biosynthesis of the oligosaccharide portion of glycoproteins. It seems likely that in some tissues, the synthesis of dolichyl-P is regulated at steps in its biosynthetic pathway that are unique to the dolichol branch, in addition to the regulation at the HMG-CoA reductase step. Thus, feeding animals a diet high in cholestyramine, a compound that causes increased activity of the HMG-CoA reductase, resulted in a great stimulation in acetate incorporation into cholesterol but not into dolichyl-P. This finding led these workers to conclude that in rat liver, the rate of dolichyl-P synthesis is not regulated by the activity of HMG-CoA reductase,[155] They suggested that regulation of this lipid may be at the level of the dolichyl-P synthetase; but animals fed a high cholesterol diet, which suppresses the biosynthesis of cholesterol, show increased incorporation of mevalonate into dolichol and dolichyl-pyrophosphoryl-oligosaccharides as well as increased activity of some of the glycosyl transferases associated with glycoprotein synthesis.[156,157] In this case, inhibiting the cholesterol branch of the pathway may lead to increased concentrations of various intermediates (farnesyl-PP, isopentenyl-PP, etc.) and therefore to stimulation of dolichyl-P biosynthesis.

B. Compactin

A specific, nonsteroidal inhibitor of cholesterol called compactin, or ML-236B, shows great promise for some of these studies on the role of dolichyl phosphate levels on glycoprotein synthesis.[158] Compactin is produced by several fungal strains such as *Pencillium brevicompactin*,[159] and its structure (see Figure 11) includes a lactonized ring that resembles the lactone form of mevalonic acid. In CHO cells in culture, compactin at concentrations of 1 to 10 μM, blocks the synthesis of cholesterol by inhibiting the HMG-CoA reductase. These cells are thus dependent on external cholesterol.[160] When 1 to 5 μM compactin is

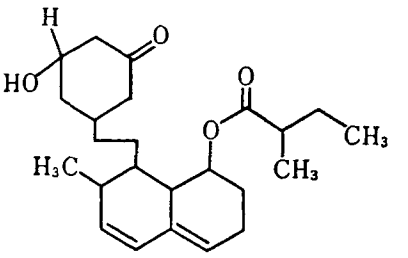


FIGURE 11. Structure of compactin.

given to developing sea urchin embryos, it induces abnormal gastrulation. This effect is apparently due to an inhibition of dolichyl phosphate synthesis since the embryos cultured in compactin show a decreased capacity to synthesize mannose-labeled glycolipids and asparagine-linked glycoproteins. However, the inhibitory effect of compactin on development and glycoprotein biosynthesis could be overcome by supplementing the embryos with exogenous dolichol or dolichyl phosphate, but not with cholesterol or Coenzyme Q.[161] Thus, compactin and 25-hydroxycholesterol are examples of inhibitors that can be used to study some of the steps in dolichyl phosphate synthesis. However, it would be valuable to have other inhibitors that block the biosynthetic pathway after the branch point, since such inhibitors would be much more specific for dolichol.

V. INHIBITORS OF GLYCOPROTEIN TRANSPORT (MOVEMENT)

The synthesis of the oligosaccharide chain ($Glc_3Man_9GlcNAc_2$) via the lipid-linked saccharide pathway occurs in the endoplasmic reticulum and this is also the location where transfer of oligosaccharide to protein occurs. Following this attachment of carbohydrate, the newly formed glycoprotein is transported, probably in vesicles, to the Golgi appratus. During this transport, and in the Golgi, the glycoprotein undergoes a number of processing or trimming reactions (see Figure 3), and the sugars of the complex chains are added. The protein is then transported, again probably in vesicles, to the cell surface where it is inserted into the membrane or secreted from the cell. Although the signals involved in these various transport reactions are not known, there are several very interesting and important compounds, called ionophores, that perturb the movement of glycoproteins to the plasma membrane.

Ionophores were first recognized through their effect of stimulating energy-linked transport in mitochondria. These studies thus provided valuable tools for studies on the linkage between metabolism and transport, and also stimulated extensive in vitro studies to examine the molecular basis of ionophore action. Ionophores are compounds of low-to-moderate molecular weight (200 to 2000) that form lipid-soluble complexes with polar cations of which K^+, Na^+, Ca^{++}, Mg^{++}, and the biogenic amines are the most significant from a biological standpoint. Although these compounds were first isolated from fermentation broths of microorganisms, several synthetic ionophores were later discovered to have equivalent molecular properties.[163] Some of these ionophores have been utilized to study the transport of glycoproteins within the cell.

The effect of the ionophores A23187, monensin, and nigeracin on Ig secretion by plasma cells was examined.[164] A23187 has been shown to lower the intracellular Ca^{++} levels, whereas monensin and nigeracin induce partial Na^+/K^+ equilibration. In this study, the Ig molecules were labeled by pulsing plasma cells with [4,5-^{3}H]leucine for 1 hr and then following the secretion of ^{3}H-Ig during a chase of 20 hr. Ig secretion was markedly inhibited by these ionophores, and striking alterations in the ultrastructural appearance of the Golgi

complex were observed.[164] The authors postulated that under conditions of Ca^{++} depletion (i.e., in the presence of A23187), ^{3}H-Ig passes to the Golgi vesicles, but these vesicles are incapable of fusion or migration and therefore accumulate in exaggerated numbers in the Golgi. On the other hand, when Na^+/K^+ equilibration occurs (i.e., in the presence of monensin or nigeracin), the ^{3}H-Ig also passes to Golgi elements to give rise to large vacuoles which contain increasing amounts of Ig. These data are consistent with the hypothesis that Ig secretion is medicated by constant movement of smooth vesicles to the cell surface where Ig is discharged by exocytosis. The inhibition by ionophores is accompanied by dramatic alterations of the Golgi complex which can be ascribed to disturbances of the ongoing vesicular traffic of Ig between the RER, the Golgi, and the plasma membrane.[164]

Procollagen and fibronectin are major products of cultured fibroblasts and both are secreted from cells. The secretion of these two proteins could be reduced to 20% of normal in the presence of low concentrations (0.1 to 1 μM) of monensin, an ionophore having an affinity for monovalent cations. However, ionophore A23187, which binds divalent cations, had little effect on the secretion.[165] Electron microscopy showed that the inhibition of secretion was accompanied by the accumulation of membrane vacuoles. The authors suggested that monensin blocked secretion by affecting the secretory structures rather than the proteins themselves. In support of this idea, the ionophore had no effect on hydroxylation or glycosylation of procollagen or on glycosylation of fibronectin, nor were significant changes in cellular amino acid incorporation observed. Pulse-chase studies indicated that the rates of secretion were impaired by the ionophore without enhancing intracellular degradation. Secretion could be restored by removing the monensin from the culture medium. The distribution of procollagen and fibronectin was compared in control and monensin-inhibited cells by immunofluorescence microscopy.[166] In control cells, both proteins were present throughout the cytoplasm and in the Golgi zone. Treatment of cells with monensin caused intracellular accumulation of procollagen and fibronectin in the Golgi vacuoles, but normal Golgi complexes were not found. These proteins were also found in more peripheral deposits corresponding to RER in monensin-treated cells.

Monensin (and other ionophores) have also been found to inhibit the appearance of viral glycoproteins at the surface of the host cell or to effect their normal maturation.[167-170] In studies with Friend murine leukemia virus,[167] or with Mason-Pfizer monkey virus,[168] Mammalian cells treated with monensin at concentrations of 10^{-6} to 10^{-7} M continued to synthesize viral particles, and these particles appeared to bud normally from the plasma membrane of the cell as viewed by electron microscopy. However, the particles released from cells had an altered buoyant density in sucrose gradients and were noninfectious. Furthermore, the noninfectious particles had a normal compliment of proteins but showed a significant reduction in the amount of glycosylated proteins. It appeared that the precursor protein for the viral glycoproteins was synthesized normally in the presence of monensin, but was not cleaved to the mature glycoproteins. The uncleaved molecule, however, was found at the cell surface.

However, the glycoproteins of Sindbis and vesicular stomatitis virus grown in BHK and chicken embryo fibroblasts were synthesized in normal amounts in the presence of monensin, but these glycoproteins did not appear at the surface of the infected cells. Proteolytic cleavage of Sindbis virus glycoprotein PE2 to E2 was inhibited in the ionophore-treated cells, but the attachment of fatty acid to PE2 proceeded normally. Also, fatty acid attachment to the VSV G protein and processing of the oligosaccharide portion occurred in the drug-treated cells. Electron microscopy indicated that the ionophores blocked the movement of viral glycoproteins from the Golgi apparatus to the cell surface membrane where budding and release of viruses occur.[170]

VI. INHIBITORS OF PROTEIN SYNTHESIS

One major question that is still open with regard to the assembly of the asparagine-linked glycoproteins is the problem of how the formation of the oligosaccharide is regulated. That is, is there some control at the level of the lipid-linked oligosaccharides, and is the formation of the core oligosaccharide in any way linked to the synthesis of the protein portion of the molecule, or vice versa. As indicated earlier, studies with tunicamycin have suggested that the formation of the protein is linked to oligosaccharide synthesis, since inhibition of lipid-linked saccharide formation also resulted in cessation of the protein portion of the glycoprotein. The question of whether the synthesis of oligosaccharide is linked to formation of the protein has also been approached with the use of inhibitors of protein synthesis.

In one study, MDCK cells were grown in [2-^{3}H]mannose in the presence of cycloheximide or puromycin, and the effects of these inhibitors on the synthesis of lipid-linked saccharides and proteins were examined.[171] In this study, the inhibition of protein synthesis resulted in a substantial inhibition in the incorporation of [^{3}H]mannose into lipid-linked oligosaccharides. However, under these conditions, the formation of dolichyl-phosphoryl-mannose was only slightly affected. Cycloheximide had no affect on the in vitro incorporation of mannose from GDP-[^{14}C]mannose into lipid-linked saccharides by membrane preparations of pig aorta. The inhibition of lipid-linked oligosaccharide formation did not appear to be caused by a decrease in the amount of the various glycosyltransferases as a result of inhibition of protein synthesis, nor was it the result of more rapid degradation of the lipid-linked oligosaccharides. The two most likely explanations for these results were that either the amount of dolichyl phosphate was limiting, and therefore this lipid was not available as a carrier for the oligosaccharide, or that the synthesis of lipid-linked oligosaccharide is subject to feedback control. However, since the synthesis of dolichyl-phosphoryl-mannose was not inhibited in the presence of cycloheximide, the levels of dolichyl phosphate are apparently not limiting, unless there are two different pools of this lipid. Therefore, the formation of lipid-linked oligosaccharides may be regulated, at least in part, by end-product inhibition.

In another study, oligosaccharide-lipid synthesis was examined in cells incubated in the presence of 1 μg/mℓ of actinomycin D to depress levels of mRNA, or in the presence of 100 μg/mℓ of cycloheximide to abolish protein synthesis.[172] The results indicated that the synthesis of lipid-linked oligosaccharides was proportional to the rate of protein synthesis. The regulated step appeared to be prior to the formation of $Man_5GlcNAc_2$-pyrophosphoryl-dolichol, leading these workers to suggest that the most likely control point was the availability of dolichyl phosphate. These studies, as well as others described previously suggest that there may be several control points in the pathway of oligosaccharide assembly, one of which involves levels of the lipid carrier, and that the use of inhibitors may help to delineate these steps.

VII. CONCLUSIONS

A great deal of information has accumulated about the pathway of biosynthesis of the oligosaccharide portion of the N-linked glycoproteins. With regard to the lipid-linked saccharide pathway, some of the information has come through the use of inhibitors that block specific steps in the pathway. For example, tunicamycin blocks the first step by preventing the formation of dolichyl-pyrophosphoryl-GlcNAc and consequently inhibits the synthesis of the $Glc_3Man_9GlcNAc_2$-PP-dolichol which is the ultimate donor of oligosaccharide to protein. Thus, cells grown in tunicamycin lack the ability to glycosylate their proteins. The antibiotics, amphomycin and tsushimycin, and some of the sugar analogs, inhibit the formation of dolichyl-phosphoryl-mannose which is the mannosyl donor for the last four mannose residues of the $Glc_3Man_9GlcNAc_2$-PP-dolichol.Thus, in the presence of these inhibitors,

cell-free extracts, or in some cases cells in culture, accumulate a Man_5-$GlcNAc_2$-PP-dolichol. Other inhibitors are also known which block these reactions, but some of these have proven to be less specific.

One problem with regard to some of these compounds, such as amphomycin and tsushimycin, is that they are not able to enter cells and therefore are not useful for in vivo experiments. Thus, the ideal inhibitor would be one that is specific for a single step in the lipid-linked saccharide pathway, and is able to penetrate cells and cause its effect in vivo. Such a compound would be useful for causing alterations in the oligosaccharide composition of glycoproteins and for examining functional aspects of the indicated glycoprotein. Although tunicamycin fits this description and is able to exert its effects in cultured cells, it completely prevents glycosylation of the protein. Thus, in many cases, the protein portion of the glycoprotein is significantly altered to such a degree that it may be much more insoluble or much more rapidly degraded. While tunicamycin is still of considerable interest and has been widely used to show that various proteins are N-glycosylated, it would be valuable to have other inhibitors which cause alterations in the oligosaccharide chains after or during their transfer to protein.

Several compounds have recently been described that inhibit specific steps in the processing pathway and these inhibitors may provide a means to prevent the formation of complex chains and allow aberrent glycoproteins to be produced. Swainsonine, a plant indolizidine alkaloid, inhibits the processing mannosidase II that removes the α1,3- and α1,6-linked mannoses from the GlcNAc-$Man_5GlcNAc_2$-protein. Thus, cells grown in this alkaloid form glycoproteins having hybrid types of oligosaccharides with a partial high-mannose and a partial complex structure. Castanospermine is another plant indolizidine alkaloid that inhibits glucosidase I, the processing enzyme that removes the first α1,2-glucose from the $Glc_3Man_9GlcNAc_2$-protein. Apparently, in the presence of this inhibitor, several mannose residues can still be trimmed from the $Glc_3Man_9GlcNAc_2$-protein so the major oligosaccharide is apparently a $Glc_3Man_7GlcNAc_2$-protein. Another inhibitor of glucose processing is the antibiotic, deoxynojirimycin, which in vitro inhibits both glucosidase I and II. Cells incubated in the presence of this inhibitor fail to form complex oligosaccharides and render the high-mannose structures less susceptible to the action of α-mannosidase. Finally, a synthetic glucose analog, bromoconduritol, has also been shown to inhibit the processing glucosidases and to give rise to increased amounts of glucose-containing oligosaccharides attached to proteins. In this case, the major oligosaccharides associated with viral proteins were $GlcMan_9GlcNAc_2$, Glc $Man_8GlcNAc_2$, and Glc $Man_7GlcNAc_2$. These types of inhibitors should be useful to modify the oligosaccharides of viral and membrane glycoproteins and provide useful information on the role of complex oligosaccharides.

As indicated in the introduction, the formation of N-linked glycoproteins is a complex process and involves synthesis of dolichyl phosphate, attachment of sugars to this lipid, transfer of oligosaccharide to protein, processing of the oligosaccharide, and movement of the glycoprotein through the various cellular compartments. Several inhibitors of cholesterol biosynthesis also block the formation of dolichyl phosphate (hydroxycholesterol and compactin). Since cells grown in these compounds are unable to form dolichyl phosphate, the outcome of these treatments are similar to those of tunicamycin, i.e., nonglycosylated proteins. The difficulty with these inhibitors is that they also block cholesterol formation and this steroid is an essential membrane component. Thus, over a long period of time, these inhibitors may cause alterations in membrane structure. Another group of intriguing compounds are the ionophores, which have recently been shown to block the movement of glycoproteins from the Golgi apparatus to the cell surface. These compounds should be of considerable interest in tracing glycoprotein pathways within the cell as well as for studying alterations in cell behavior.

Finally, although not discussed in this review, it should be mentioned that an alternate

approach to the use of inhibitors to study glycoprotein biosynthesis is the use of mutant cell lines which are missing specific enzymes in the lipid-linked saccharide or processing pathways. For example, a mutant cell line was isolated that was missing the enzyme that is necessary to form dolichyl-phosphoryl-mannose. This mutant accumulated the $Man_5GlcNAc_2$-PP-dolichol, but was unable to further elongate it, apparently because of lack of the proper mannosyl donor.[101] Thus, this mutant organism gives results analogous to those found with the antibiotic, amphomycin. Another example is the recent isolation of a mutant organism that is missing the glucosidase II that removes the inner two glucose residues from the $Glc_3Man_9GlcNAc_2$-protein. The major glucosylated oligosaccharides found in this cell line were identified as $Glc_2Man_9GlcNAc_2$ and $Glc_2Man_8GlcNAc_2$.[173]

A number of mutant cell lines have now been isolated, largely on the basis of their resistance to specific plant lectins. These mutants will provide novel biological tools with which to study membrane structure/function relationships as well as the various roles of membrane-bound carbohydrate. This area has recently been reviewed.[174]

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