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# Carbohydrate Analysis of Glycoproteins

## A Review

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## ABSTRACT

Many of the products prepared by biotechnological approaches, including recombinant genetic engineering, cell tissue culture, and monoclonal technologies, are glycoproteins. As little as five years ago, glycosylation was believed to play no significant role in the function of glycoproteins. Recent large scale testing of glycoprotein-based pharmaceuticals has indicated that both the extent and type of glycosylation can play a central role in glycoprotein activity. Although methods for compositional and sequence analysis of proteins and nucleic acids are generally available, similar methods have yet to be developed for carbohydrate oligomers and polymers. This review focuses on new, developing methods for the analysis and sequencing of the carbohydrate portion of glycoproteins. Included are: (1) the release of oligosaccharides and hydrolysis of carbohydrate chains using enzymatic and chemical methods; (2) fractionation by LPLC, electrophoresis, HPLC, and lectin affinity chromatography; (3) detection through the preparation of derivatives or by new electrochemical methods; (4) analysis by spectroscopic methods, including MS and high-field NMR; and (5) their sequencing through the use of multiple, well-integrated techniques. The ultimate goal of the analytical approaches discussed is to firmly establish structure and, thus, permit

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the study of structure–function relationships and eventually to allow the intelligent application of carbohydrate remodeling techniques in the preparation of new glycoproteins.

**Index Entries:** Glycoproteins; carbohydrate remodeling techniques; glycosylation.

## INTRODUCTION

Glycoproteins are proteins to which carbohydrate moieties are covalently linked through glycosidic bonds. Glycosylation is a very common modification of extracellular and integral membrane proteins of eukaryotes (*1*), but has been recently observed in prokaryotes as well (*2*). Glycoproteins may contain from 4 to more than 60% carbohydrate (*3*); the carbohydrate moieties vary in size, may be branched or linear, and can be located at various positions on the polypeptide chain. The linkages between the oligosaccharide and the protein are either *N*-glycosidic (carbohydrate linked to the amido nitrogen of asparagine) or *O*-glycosidic (carbohydrate linked to the hydroxyl oxygen of L-serine, L-threonine, and occasionally 5-hydroxy-L-lysine or 4-hydroxy-L-proline). Three major types of asparagine-linked oligosaccharides have been determined as high-mannose type or simple type, complex type, and hybrid type (Fig. 1).

The biosynthesis of glycoproteins occurs within the internal membrane systems of cells, the endoplasmic reticulum, and the Golgi apparatus (*4*). Glycoprotein oligosaccharide units are assembled and modified as the molecules move through successive subcellular compartments en route for destinations outside the cell, as membrane components of the cell surface, or as components of membranes or contents of cellular organelles (*5*).

The biological roles of carbohydrate units include: protection of peptide chains against proteolytic attack (*6,7*), facilitation of the secretion of certain proteins or their mobilization to the cell surface (*8*), induction and maintenance of the protein conformation in a biologically-active form (*9*), clearance of glycoproteins from plasma (*10*), direction of the immune response and acting as immune decoys (*11,12*), and their importance as antigenic determinants in differentiation and development (*13*).

Many of the protein products of modern biotechnology, particularly human proteins of pharmaceutical relevance, are glycosylated (*14*). Whether a glycoprotein is prepared by a microorganism using recombinant genetic engineering or in mammalian or human tissue culture, several questions regarding glycosylation are often raised. These include: (1) the level of glycosylation, the position of glycosylation, and the carbohydrate composition and sequence; (2) the heterogeneity of the glycoprotein product, i.e., how many isoforms or structural variants are present; (3) the function of glycosylation and how carbohydrate structure relates to

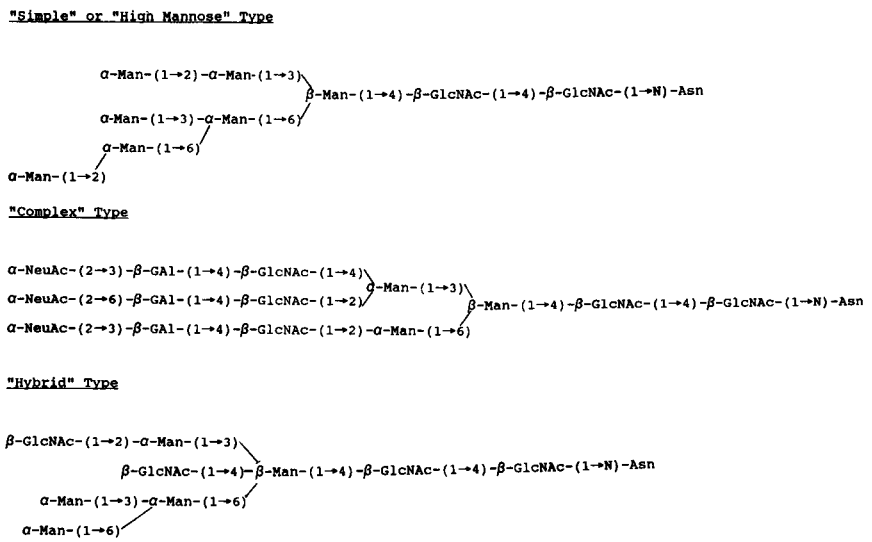



Fig. 1. Examples of simple, complex, and hybrid types of asparagine-linked oligosaccharides. Abbreviations are: Man = mannose, GlcNAc = *N*-acetyl-glucosamine, and Asn = asparagine.

function; and (4) control of glycosylation during glycoprotein biosynthesis and how undesirable (or absent) glycosylation might be modified by carbohydrate remodeling. Before structure–function relationships can be established or carbohydrate remodeling can be attempted, it is necessary to unambiguously establish carbohydrate structure (*15*). This review addresses, in detail, the analysis of the carbohydrate portion of glycoproteins.

## GENERAL METHODS FOR CARBOHYDRATE SEQUENCING OF GLYCOPROTEINS

### Release of Oligosaccharide and Hydrolysis of Carbohydrate Chains

There are two general strategies to enzymatically cleave a glycoprotein into glycopeptides. One is the specific cleavage of glycoproteins using a protease, such as trypsin, chymotrypsin, and endoproteinase Glu-C. (*16*). A second involves the use of nonspecific proteases, such as pronases (*17–22*). Both of these methods result in the preparation of glycopeptides that, following fractionation and purification, are suitable for carbohydrate compositional and sequence analysis.

Glycosidases are excellent tools for: the elucidation of the primary structure of sugars chain by sequential degradation (*19,23–28*); the determination of the anomeric linkage of each monosaccharide unit (*19,22*);

Table 1
Exoglycosidases

| Enzyme | Source | Specificity | References |
|---|---|---|---|
| α-L-fucosidase (EC 3.2.1.51) | *Charonia lampas* | very broad, all fucosyl linkages | (*145,146*) |
| α-D-galactosidase (EC 3.2.1.22) | coffee bean | very broad aglycone | (*147*) |
| α-mannosidase (EC 3.2.1.24) | Jack bean | Man-α-(1→2)-Man, Man-α-(1→6)-Man with 100%, and Man-α-(1→3)-Man with 7% reaction rate | (*148*) |
| α-glucosidase (EC 3.2.1.20) | Yeast | α-(1→2), α-(1→3), and α-(1→4) glycopyranosides, whereas α-(1→6) bonds are only attacked slowly | (*149*) |
| Neuraminidase (EC 3.2.1.18) | *Clostridium perfringens* | NeuAc-α-(2→3)-GA1 NeuAc-α-(2→6)-GA1, and NeuAc-α-(2→6)-GAlcNAc | (*150*) |
| β-glucuronidase (EC 3.2.1.31) | *E. coli* K 12 | β-D-glucuronides | (*151*) (*151*) |
| β-glucosidase (EC 3.2.1.21) | *Amyodalae dulces* | β-D-glucosides of phenols, salicyl alcohol, vanilin, 2-cresol and 4-cresol | (*152*) |
| β-galactosidase (EC 3.2.1.23) | Bovine testis | Gal-β-(1→3)-GlcNAc Gal-β-(1→4)-GlcNAc Gal-β-(1→4)-GAlNAc | (*153*) |
| | *Streptococcus pneumoniae* | Gal-β-(1→4)-GlcNAc No cleavage of Gal-β-(1→3)-GlcNAc or Gal-β-(1→6)-GlcNAc | (*154*) |
| β-*N*-acetyl-D-glucosaminidase (EC 3.2.1.30) | beef kidney | *N*-acetyl-β-D-glucosaminides, *N*-acetyl-β-D-galactosamindes | (*155*) |
| β-*N*-acetyl-galactosaminidase (EC 3.2.1.4) | *Aspergillus niger* | GalNAc-ser link | (*156*) |

the controlled modification of glycoprotein oligosaccharides to explore their biological role (*29*); and the preparation of specific acceptors for glycosyltransferase activity studies (*30*). Basically, two types of enzymes are used: exoglycosidases (Table 1), which hydrolyze glycosidic bonds of monosaccharides in terminal nonreducing positions and may achieve a stepwise degradation of the glycan (*31*) and endoglycosidases (Table 2),

Table 2
Endoglycosidases

| Enzyme | Source | Specificity | References |
|---|---|---|---|
| Endo-β-galactosidase (EC 3.2.1.103) | *Bacteriodes fragilis* | Internal β-galactosidic linkages with the structure Gal-β-(1→4) GlcNAc | (*157,158*) |
| Endoglycosidase F (EC 3.2.1.96) | *Flavobacterium meningosepticum* | High mannose, hybrid and complex glycans[a] from glycoproteins and glycopeptides | (*159*) |
| Endoglycosidase H (EC 3.2.1.96) | *Streptomyces griseus* | High mannose glycans from glycoproteins[a] and glycopeptides | (*160*) |
| Glycopeptidase F (EC 3.2.2.18) | *Flavobacterium meningosepticum* | High mannose, hybrid and complex glycans[a] from glycoproteins and glycopeptides between Asn and GlcNac | (*161*) |
| Endoglycosidase L (EC 3.2.1.96) | *Streptomyces plicatus* | *N*-Acetylchitobiose unit at low molecular weight | (*162*) |
| Endoglycosidase D (EC 3.2.1.96) | *Diplococcus pneumoniae* | *N*-linked complex type[a] | (*163*) |

[a]See Fig. 1.

which have been recently introduced and rapidly developed because of their very promising performance in the hydrolysis of internal glycosidic bonds, liberating intact oligosaccharides (*23,29,32–36*).

Chemical methods can be used to release oligosaccharides from glycoproteins and glycopeptides. Hydrazine cleaves the *N*-glycosidic linkages of *N*-glycosylpeptides and *N*-glycosylproteins and liberates the *N*-deacetylated glycans as their hydrazones (*37,38*). Thus, the procedure for obtaining the carbohydrate moieties is carried out in three steps: hydrazinolysis (Fig. 2); re-*N*-acetylation; and reduction with $NaB^3H_4$ to stabilize the molecule. At steps 2 and 3, a radiolabel can be easily introduced into the oligosaccharide chain (*39*). Hydrazinolysis may give rise to further degradation of *N*-linked oligosaccharide chains, thereby introducing more heterogeneity (*40*).

Treatment with (1:1) mixtures of trifluoroacetic anhydride and trifluroacetic acid at 100°C for 48 h also results in cleavage of the glycosylamine protein-carbohydrate linkage (*41,42*). Trifluoroacetolysis cleaves peptide bonds by transamidation and replaces the *N*-acetyl substituents of amino sugars by *N*-trifluoroacetyl groups. One drawback of this method is the loss of two glucosamine residues at the protein-carbohydrate linkage region. Milder trifluoroacetolysis (1:100 mixtures of trifluoroacetic

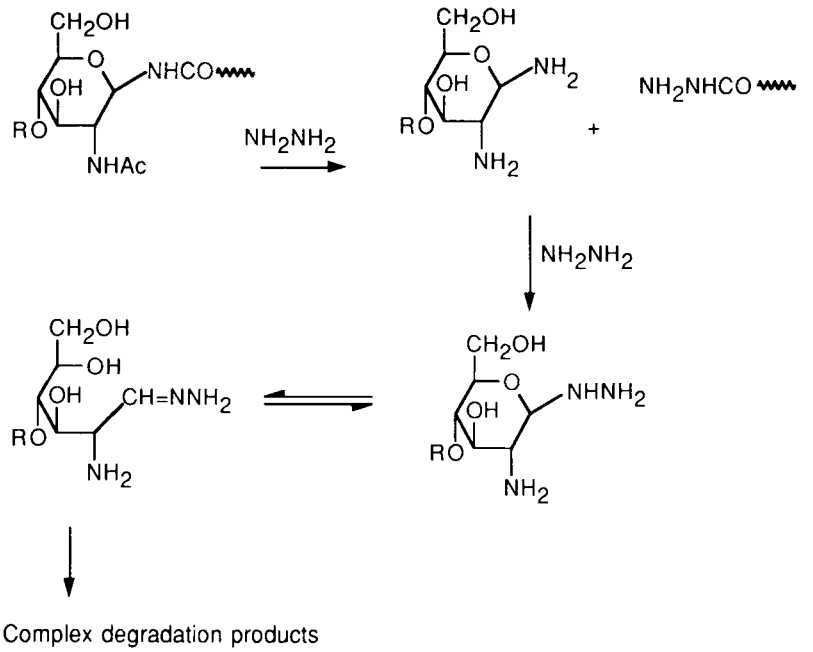



Fig. 2. Hydrazinolysis of an asparagine-linked oligosaccharide. "R" represents the remainder of the oligosaccharide chain.

acid anhydride and trifluoroacetic acid) can also release the oligosaccharide chain, but this method destroys fucose residues that may be attached to the linkage glucosamine and results in less than a 50% recovery of oligosaccharide (*43*).

*O*-glycosidic linkages between carbohydrate chain and the β-hydroxy-amino acids serine and threonine are easily cleaved using dilute alkali solution (0.05–0.1 *M* NaOH or KOH) under mild conditions (4–45°C for 0.5–6 d) by a β-elimination mechanism, as shown in Fig. 3 (*17,40,44–46*). This method offers a good recovery of oligosaccharide product.

Nonspecific hydrolysis of glycosidic linkages can be accomplished using acid. Aqueous hydrochloric or sulfuric acid hydrolysis (*47*) of specific glycosidic linkages has been largely replaced by more general methods, such as the use of 2*M* aqueous trifluoroacetic acid (*48*) or anhydrous hydrofluoric acid (*49*). If properly controlled, these methods can result in reproducible mixtures of the monosaccharide components of oligosaccharides, glycopeptides, or glycoproteins (*47*).

Acetolysis provides a complementary method to conventional acid hydrolysis as the types of glycosidic linkage cleaved preferentially by acetolysis and are often different from those split by acid hydrolysis. For example, (1→6) glycosidic linkage is highly susceptible to cleavage by acetolysis, whereas the (1→2) and (1→3) linkages are comparatively resistant (*50*). Compounds with glycosidic linkages in the α-configuration are more susceptible to acetolysis than compounds with β-glycosidic link-

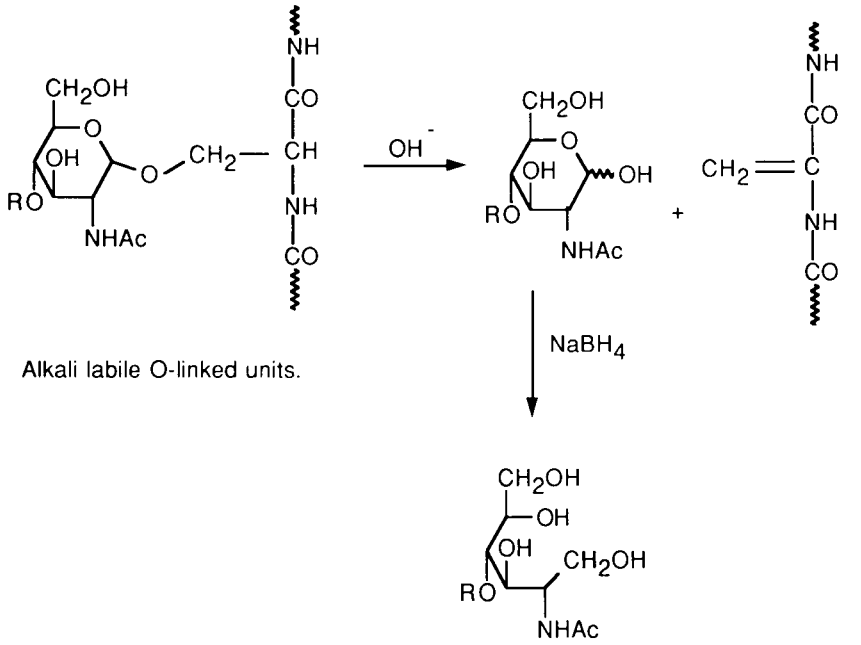



Fig. 3. Release of protein-bound *O*-linked oligosaccharides with alkali. "R" represents the remainder of the oligosaccharides chain.

ages. The acetolysis is performed using acetic anhydride/acetic acid/sulfuric acid generally in a ratio of 10/10/1.

## Fractionation of Oligosaccharides

Once oligosaccharides are released from a glycoprotein, they must be fractionated into their constituent components.

Low pressure gel permeation chromatography (LP-GPC) has been used to separate oligosaccharides with different degrees of polymerization or to separate small (i.e., salt) and large molecules (i.e., proteins) from oligosaccharides or glycopeptides. Sephadex G-25 or G-50, which can break down into soluble sugar contaminants, have been largely replaced with polyacrylamide-based supports, such as Biogel P4 or P6 (*24, 45,51–55*). The resolution of oligosaccharides by LP-GPC is enhanced because *N*-acetylhexosamines, hexoses, and methylpentoses have different effective sizes (*56*). Branching and difference in linkage position and stereochemistry also effect oligosaccharide size. Thus, oligosaccharides containing the same number of monosaccharide residues, but differing in the types of sugar present (or the linkages between sugars), can occasionally be separated in LP-GPC on Biogel P4. Although some improvements have been brought about of the introduction of HPLC-GPC, this method remains a low resolution technique (*55*).

Electrophoresis is a fractionation method of separation that depends on the property of charged molecules to migrate in an electric field. Gel electrophoresis was first applied to the sequencing of nucleic acids by Maxam and Gilbert in 1977 (*57*) and has now become a routine laboratory procedure. Over the past few years, several research groups (*58–60*) have successfully applied this method of fractionation to acidic oligosaccharides. The use of gel electrophoresis for the separation of glycoprotein-derived oligosaccharides, or their derivatives, has not yet been reported. The development of this technique rests on two important issues: the placement of a fixed charge into a neutral glycoprotein-derived oligosaccharide and the detection of the oligosaccharide at low concentrations in the gel. Because of the successful application of gel electrophoresis to the sequencing of other biopolymers, this is one approach that warrants a closer examination.

Higher resolution and reduced fractionated time is provided by the use of high performance liquid chromatography (HPLC). Detailed reviews have recently appeared on oligosaccharide fractionation using high performance liquid chromatography (HPLC) methods (*61,62*). Several types of HPLC techniques covered in these reviews, as well as several new methods, are currently applied to the separation of oligosaccharides.

Normal phase HPLC can be used for the fractionation of uncharged oligosaccharides released by hydrazinolysis or endoglycosidases from glycoprotein-containing, high-mannose or complex type oligosaccharide chains (*23,29,33*). The released oligosaccharides are usually radiolabeled by reduction with $NaB^3H_4$ and detected with high sensitivity by scintillation counting. Alternatively, a derivative can be prepared that permits detection by UV absorbance or fluorescence (*23*). Separation can be carried out on an anion-exchange column. Micro Pak AX-10 and AX-5 anion exchange columns (Varian Associates) are two such columns that give a separation of dianternary, trianternary, and tetraantennary oligosaccharides (*32,38,63,64*) (Fig. 4). The presence of a single fucose residue on any of these structures results in a peak with a distinctive retention time (*65*). High-manose type oligosaccharides, differing by a single mannose unit, can also be resolved using these columns. This method, however, is not suitable for direct application to charged oligosaccharides and requires the removal of sialic acid (usually using neuraminidase) from the nonreducing terminus prior to oligosaccharide fractionation.

The separation of pyridylamino derivatives of oligosaccharides has been carried out by reverse-phase (RP) HPLC (*23,66*). RP-HPLC also appears to be particularly well suited to the fractionation of permethylated oligosaccharides obtained from glycoproteins (*67*). Methylated oligosaccharides can be easily detected and analyzed using mass spectrometry (*68*).

Partition HPLC on primary amine-bonded silica can also be used to fractionate oligosaccharides. Amino-bonded silica columns, such as the Amino AS-5A (Brownlee Labs, Santa Clara, CA) column, has been applied to the separation of neutral and acidic oligosaccharides obtained by hydra-

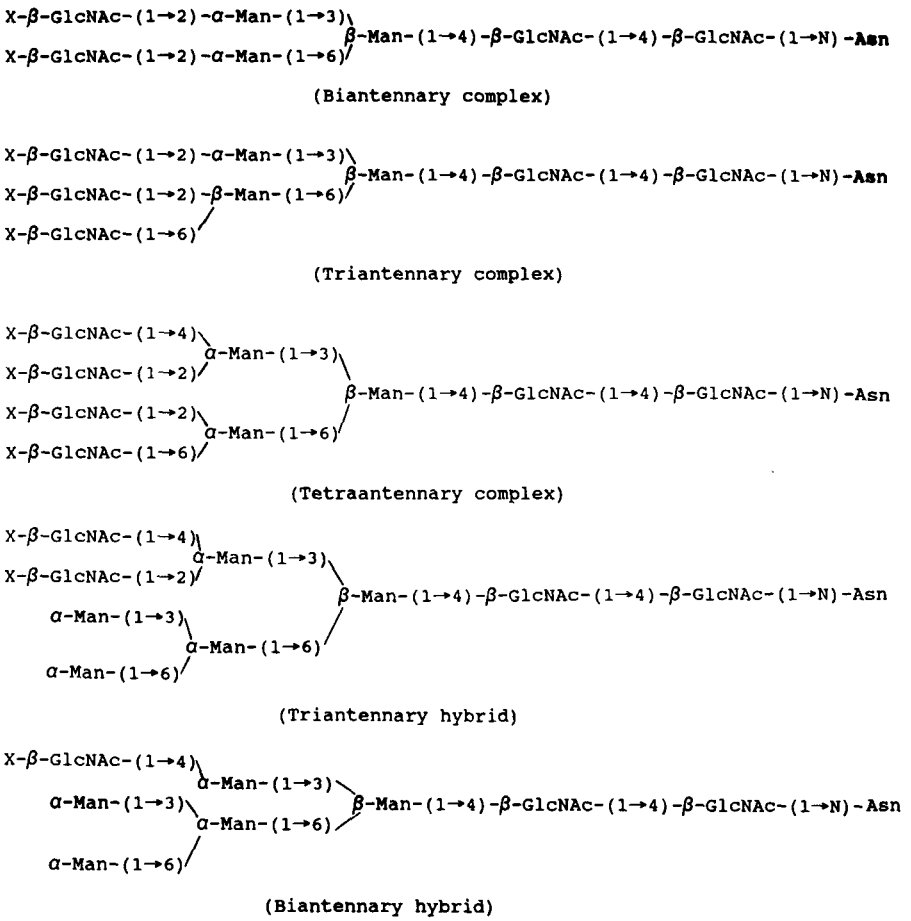


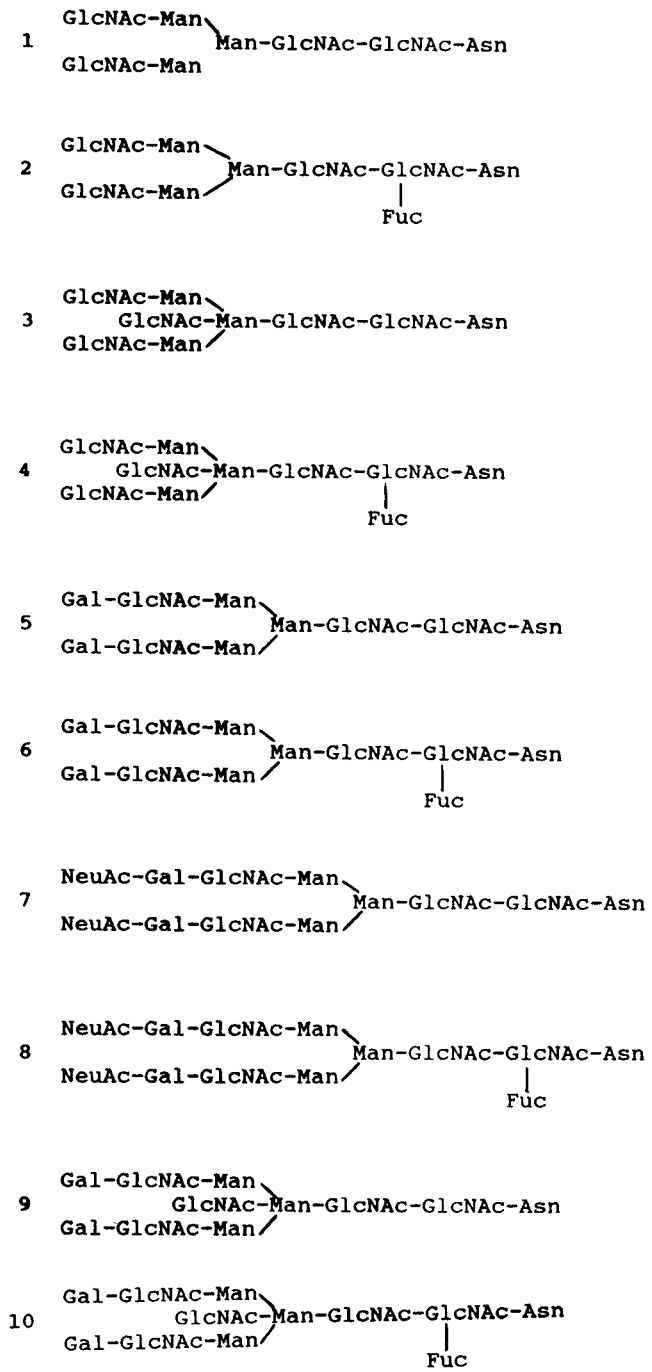

Fig. 4. Various types of oligosaccharide antennae. Abbreviations are: Man = mannose, GlcNAc = *N*-acetylglucosamine, Asn = asparagine, and "X" may represent a terminal sialic acid.

zinolysis of *N*-glycosylpeptides (*38*) and sialyloligosaccharides obtained by alkaline borohydride treatment of *O*-glycosylpeptides (*69*).

A two-dimensional sugar mapping method for structural analysis of *N*-linked oligosaccharides has been proposed by Tomiya et al. (*70*). The separation of pyridylamino oligosaccharides is performed on a reverse phase ODS-silica column and by size fractionation on a TSK-GEL Amide-80 column. The structure of an unknown oligosaccharide is then characterized from its position on the two-dimensional map.

The use of different types of support materials for LPLC and HPLC facilitates the separation of different types of oligosaccharides. However, in order to characterize oligosaccharide structure, standards are generally required. Low pressure liquid affinity chromatography (LPLAC) on immobilized lectins represents both a method to fractionate and obtain structural information on oligosaccharides (Table 3 and Fig. 5). Most of the *N*-glycosylpeptides can be fractionated into relatively homogeneous classes

Table 3
Structure of *N*-Glycosylpeptides
Used for Studying Immobilized Lectin Specificity



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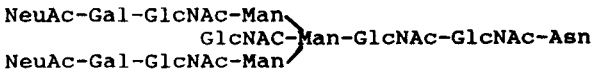


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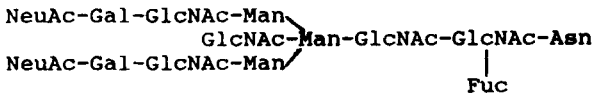


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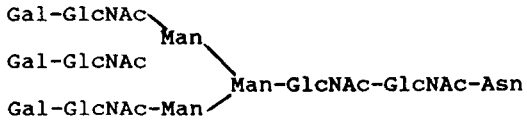


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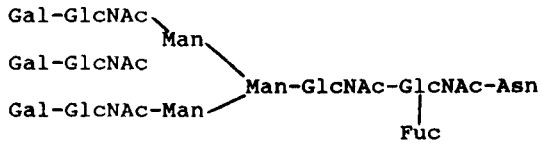


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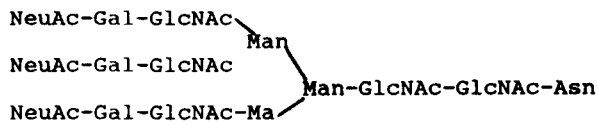


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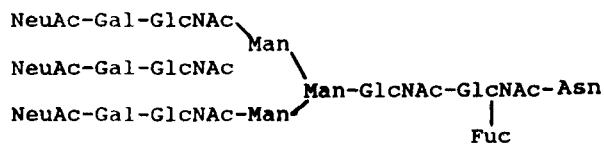


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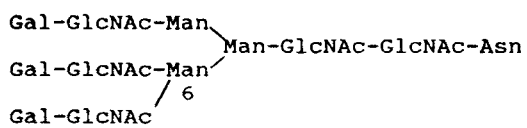


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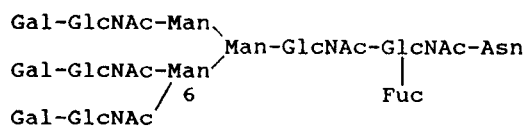

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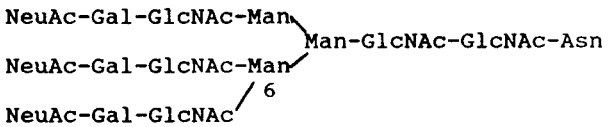


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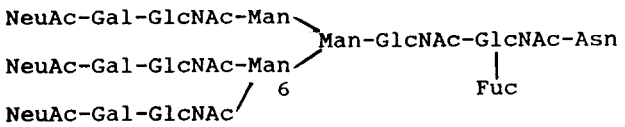


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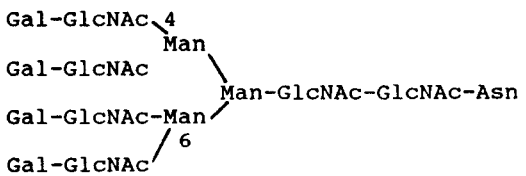


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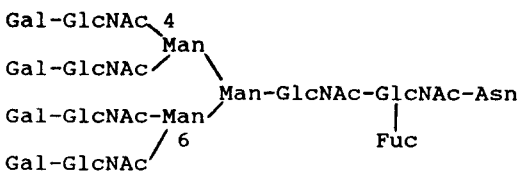


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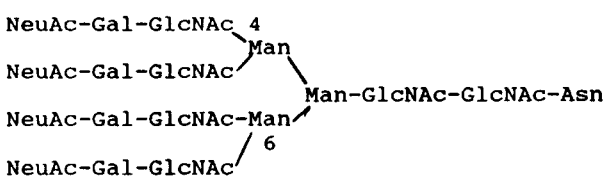


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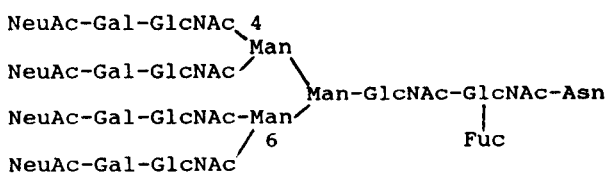

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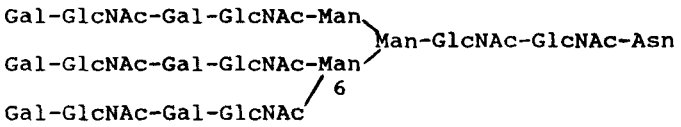


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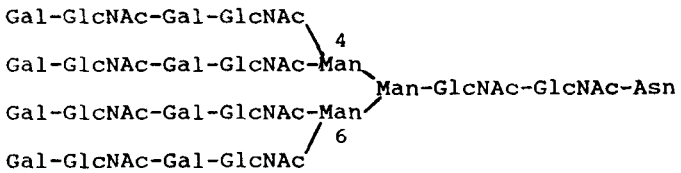


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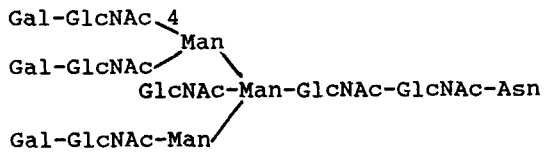


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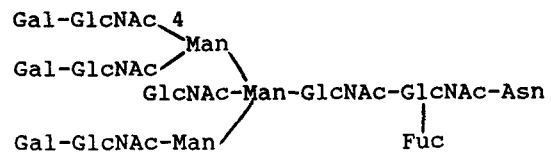


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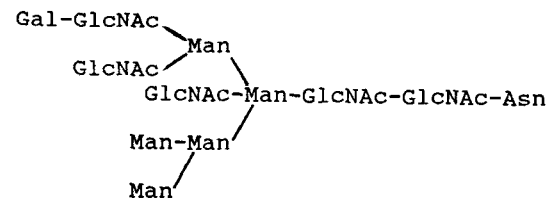


30 Oligosaccharidic Type
N-glycosylpeptide
$M_5$ to $M_9$

[a]Numbers given in the schemes refer to glycosidic linkages: 4: β-(1→4); 6: β-(1→6). In all schemes, the antennae linked to the α-(1→3) and α-(1→6) mannose residues are the upper and lower ones, respectively.

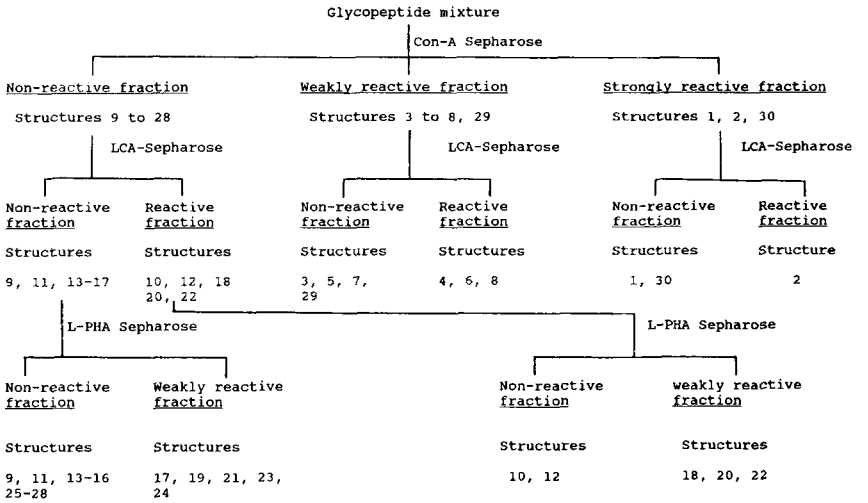


Fig. 5. Specificity of different lectins to glycopeptides. The structures associated with 1-30 are given in Table 3.

by sequential low pressure ligand affinity chromatography (*71,72*). Affinity chromatography quickly demonstrates whether a glycopeptide binds to a particular lectin, thus permitting deductions to be made about its structure. The association of Concannavalin A (ConA)-Sepharose LCA (*Lens culinaris* agglutinin)-Sepharose and $L_4$-PHA (*Phaseolus vulgaris* agglutinin)-Sepharose are most commonly utilized for fractionating the glycopeptides obtained from cell membrane glycoproteins (*73*). The methodology is rapid and sensitive, particularly when glycopeptides are radiolabeled by using $^{14}C$- and/or $^{3}H$-labeled precursors, by reduction using $NaB^3H_4$ (*44, 50,74–76*), or by *N*-acetylation carried out with [$^{14}C$ or $^{3}H$] acetic anhydride (*38*). The power of this technique lies in its usefulness to predict the primary structure of the bound glycopeptides or oligosaccharides. The recent availability of ConA-based HPLAC columns is bound to improve both the rapidity and resolution of this approach (*77*).

Two methods are available for introducing a tritium label specifically into the carbohydrate units of glycoproteins that contain nonreducing terminal residues of galactose, *N*-acetylgalactosamine, or sialic acid. Both techniques involve the initial oxidation of a monosaccharide residue to produce an aldehyde that is then labeled by reduction with $NaB^3H_4$. Sialic acid residues are selectively oxidized with low concentrations of periodate (*78–80*), whereas galactose and *N*-acetylgalactosamine can be oxidized enzymatically by galactose oxidase (*81,82*). Both methods were originally developed for the modification of soluble glycoproteins, but have been adapted for application to membrane-bound glycoconjugates (*83*). The galactose oxidase borotritide procedure has been extensively used for site specific labeling of cell surface glycoproteins and glycolipids (*80–82*). As previously discussed, it is also possible to radiolabel the reducing

end of oligosaccharides, released either enzymatically or chemically using $NaB^3H_4$.

Pellicular anion exchange resin-based columns (i.e., AS-6 columns from Dionex, Sunnyvale, CA), when used in combination with pulsed amperometric detection (PAD), permits the separation of both glycopeptides and oligosaccharides at alkaline conditions (pH = 13) in a single chromatographic step while affording detection without any pre- or postcolumn derivatization (*84*). Hardy et al. (*85*) report the impressive separation of positional isomers of oligosaccharides and glycopeptides using this method. PAD permits the detection of picomol amounts of both reducing and nonreducing sugars as intact carbohydrate chains. This technique promises rapid analysis of very small amounts of glycopeptides and oligosaccharides. One possible pitfall of this approach is the potential instability of glycopeptides and oligosaccharides at the high pH required for their sensitive detection using PAD.

## Characterization of Oligosaccharides/Monosaccharides

### *Methylation Analysis*

Methylation analysis is one of the most powerful tools available for elucidating the carbohydrate structure of glycoproteins (*16,19,24–26,46, 52,53,55,86,87*). It involves the permethylation of all free hydroxyl groups of the sugars, followed by the liberation of methylated monosaccharides by hydrolysis or methanolysis and the qualitative and quantitative analysis of the methylated derivatives. The positions of the free hydroxyl groups of the resulting partially-methylated monosaccharides give the positions in which the sugar residue was glycosylated. Hakomori's procedure (*88*) using sodium hydride and dimethylsulfoxide has been used for preparation of permethylated derivatives of glycopeptides or oligosaccharides released by hydrazinolysis or alkaline $\beta$-elimination (Fig. 3). This method leads to a rapid and complete *O*- and *N*-methylation (of the acetamido group of hexosamine residues) without any loss of *N*-acetyl groups.

### *Mass Spectrometry of Oligosaccharides*

Analysis of glycopeptides and oligosaccharides by mass spectrometry has the advantage that only small quantities of material are required. Mass spectrometry allows the detection of a few picomoles of partially methylated alditol acetates (*89*). Following derivatization of 100 $\mu$g or less of an oligosaccharide or glycopeptide, structure can be readily determined by chemical ionization or electron impact mass spectrometry (*90*). This method is potentially applicable to the very small quantities of sample (10 $\mu$g) that are often all that can be obtained from membrane preparations (*46*). However, the possibility of material loss by absorption to vessels and column fittings, as well as potential evaporation of methylated monomers, contamination with reaction products, solvents, or reagent impurities, imposes some limitations on this method (*87*). Unsubstituted oligosac-

charides are not volatile and decompose when heated; thus, it is essential to prepare permethylated or peracetylated derivatives when performing chemical ionization or electron impact mass spectrometry (*68,87,91*). Fast atom bombardent mass spectrometry (FAB-MS), which does not require heating to volatalize samples, has recently been introduced as an analytical method. FAB-MS can be applied to very small samples (5 μg) of underivatized oligosaccharides. Permethylated or acetylated oligosaccharides have also been analyzed using FAB-MS since they often result in an easily interpretable fragmentation pattern, permitting the assignment of linkage positions (*92*). Liquid secondary ion mass spectrometry has also been applied to the analysis of *p*-aminobenzoate oligosaccharide derivatives to determine the structure of very low abundance oligosaccharides isolated from heterogeneous biological samples (*93*).

### *NMR Spectroscopy of Oligosaccharides*

Nuclear magnetic resonance spectroscopy is a nondestructive analytical tool for structural analysis. The basic NMR parameters useful in the structural analysis are: spectral integration, chemical shift, coupling constant, nuclear Overhauser enhancement, and nuclear relaxation time. $^1$H NMR spectral integration of characteristic signals often provide a check on the purity of the sample. Effective use of the these parameters could lead not only to the primary structure of the carbohydrate, but even its secondary structure in liquid media based on solution NMR or in solid state using the CP-MAS NMR method (*94*). Thus, in view of the difficulties involved in crytallizing oligosaccharides or glycopeptides derived from glycoproteins, the role of molecular conformation in the physiological functions of carbohydrate chains of glycoproteins could be better studied by NMR than X-ray crystallography. In practice, however, analysis of the primary structure of carbohydrates of glycoproteins is more challenging than for other biopolymers. The variety of possible glycosidic linkages, the great number of assymetric centers present in every repeating sugar, each demanding correct stereochemical assingment and chain branching, pose a formidable task for unambiguous structure determination. No significant progress in the NMR-based structural analysis of carbohydrates from glycoproteins was made until 1970 owing to difficulties in obtaining reasonable amounts of these carbohydrates often limiting the sensitivity of NMR, and the poor resolution of signals arising from complex structures makes their interpretation extremely difficult at lower magnetic fields.

With the advent of high-field super-conducting NMR spectrometers offering improved sensitivity and spectral resolution, Wolf et al. reported 220 MHz $^1$H NMR spectra of oligosaccharides related to the *N*-acetyllactosamine type (*95*). Since 1984, a number of oligosaccharides have been studied using $^1$H NMR (*96–98*). The minimum amount of oligosaccharide needed for $^1$H NMR analysis at 360 MHz is of the order of 250 nmol and 25 nmol at 500 and 600 MHz.

A systematic approach to the application of high-field $^1H$ NMR for the determination of primary structure of complex oligosaccharides of glycoproteins was first introduced by Dorland et al. in 1977 (*99*). These authors observed that the essential information pertaining to the primary structure is born in the chemical shift, coupling constant, and line widths of proton signals resonating at clearly distinguishable positions in the high-field (360 or 500 MHz) spectrum. These 'structural reporter groups' include: (1) anomeric protons (H-1); (2) mannose H-2 and H-3 protons; (3) sialic acid H-3 protons; (4) fucose H-5 and $CH_3$ protons; and (5) galactose H-3 and H-4 protons and $CH_3$ protons of a N-Ac group in 2-acetamido-2-deoxyglucose and sialic acid. Even small structural variations markedly affect the chemical shifts, and the local mobility of protons affect the line width of the signals. When sample concentrations of the order of 50 μmol were used in conjunction with methylation analysis, structure elucidation of *N*-linked, as well as *O*-linked, glycans can be accomplished. A review by Vliegenthart et al. on the high-resolution $^1H$ NMR of 70 compounds derived from *N*-glycosidically-linked carbohydrate chains provides a detailed discussion of this approach (*100*). The successful application of the above method in recent years include structure elucidation of *O*-linked oligosaccharides from mucous glycoproteins (*101,102–104*), *N*-linked oligosaccharides (*44,105–107*), and *N*- and *O*-linked oligosaccharides (*41*). Even in a complex mixture of compounds that result from glycoprotein microheterogeneity, primary structure could be deduced for the components by the above $^1H$ NMR method when applied in combination with sugar and methylation analysis (*103,107*).

This approach has been adapted by others for the structural analysis of *O*-linked oligosaccharides of glycoproteins (*108*), polysialoglycoproteins (*109*), and pyridylamino derivatives of *N*-linked oligosaccharides (*110*). Independent approaches based on one-dimensional high-field $^1H$ NMR to the primary structure include the reports on glycopeptide oligosaccharides (10–200 nmol analyzed) (*111*), and glycoprotein oligosaccharides (*112,113*).

In yet another approach, Carver et al. found that a two-dimensional display formed by plotting a chemical shift of mannosyl H-1 vs H-2 demonstrated that these pairs of values in 63 glycopeptides and oligosaccharides of known structure are sensitive to long-range perturbations owing to remote substitution by hexoses, as well as to direct substitution effects (*114*). These authors report that, on this basis, the sequence and branching pattern for most structures could be derived. Recently, two-dimensional scalar shift correlated spectroscopy (COSY) has been applied in combination with one-dimensional methods for the elucidation of primary structure of highly microheterogenous oligosaccharide preparation (*115, 116*). Two-dimensional NMR methods at high fields have great potential in not only elucidating the oligosaccharide structure, but also in accomplishing almost complete $^1H$ and $^{13}C$ spectral assignment, as demonstrated

by Dabrowski et al., for *N*-linked glycopeptides derived from fetuin (*117*). A detailed discussion of the application of 2D NMR to the oligosaccharides is found in a recent review of Dabrowski et al. (*118*).

$^{13}C$ NMR spectroscopy is a powerful tool for studying the structure and dynamics of complex carbohydrates. Although it suffers from poor sensitivity compared to $^{1}H$ NMR, its large chemical shift range (~200 ppm) provides a great advantage. At high fields, good spectral resolution of carbon signals of carbohydrate residues, and those of protein, offers a unique method for analyzing intact glycoproteins. Information that otherwise would be difficult to obtain may be contained in the $^{13}C$ NMR spectrum of intact glycoproteins. For example, the presence of xylose or arabinose could be readily detected. These considerations motivated Allerhand et al. (*119*) to initiate a detailed $^{13}C$ NMR study in which structural information, as well as quantitative information, about the carbohydrate residues of intact glycoprotein, glucoamylase, was obtained. These authors used 0.6 mmol of denatured glucoamylose in their study. However, in order to make structural analysis, solely based on $^{13}C$ NMR, a complete understanding of the effect of glycosidic bond formation on the carbon chemical shifts of different carbohydrate residues is required. Toward this end, Allerhand et al. developed systematic approaches to the prediction of $^{13}C$ NMR spectra of α-D-mannopyranosyl residues in oligosaccharides (*120, 121*). Dill et al. have recently reviewed the natural abundance $^{13}C$ NMR studies of carbohydrates linked to amino acids and proteins (*122*). When a large enough directory of NMR spectra of oligosaccharides of glycoproteins has been built up, it has been suggested that other techniques for structural analysis might become obsolete (*123*).

Studies on the secondary structure of glycoprotein-derived oligosaccharides have only recently begun. The solution conformation of oligosaccharides can be determined through the combined use of internuclear distance data obtained from nuclear Overhauser enhancements (NOE) and tortional angles derived from coupling constants. The glycosidic angle is an important parameter that determines the relative orientation of the different antennae in the oligosaccharides.

The use of NOE for sequence determination in *N*-linked oligosaccharides has been reported (*124*). The prediction made by Montreuil (*125*) that biantennary *N*-linked oligosaccharides of glycoproteins would have well-defined, three-dimensional structures with the major site of flexibility being the mannose α1→6 linkage in the core was recently confirmed by Carver et al. based on quantitative proton NOE measurements and theoretical calculations (*126*). One-dimensional NOE data, in combination with circular dichroism results and conformational calculations, led Bush et al. to suggest that the nonreducing terminal sugar residues of blood group oligosaccharides adopt single, well-defined conformations that are determined mainly by nonbonded interactions and that the conformations are nearly identical in aqueous and nonaqueous solutions and are not dependent on temperature (*127,128*). Using 1-D NOE and 2-D NOESY,

Parker et al. determined solution conformation of the antifreeze glycopeptide Galactose-β-(1→3)-*N*-acetylgalactosamine-α-(1→O)Threonine-Alanine-Alanine. The disaccharide, which is a rigid unit, exists with a restricted orientation about the *N*-acetylgalactosamine-α-1 linkage whereas, the peptide backbone lies in an extended helical conformation (*129*).

The solution NMR structures of oligosaccharide components of glycopeptides may be different in the free (unbound) form than when complexed with a receptor. Molecular modeling approaches are a useful extension toward the determination of the structure of such complexes. Carver et al. (*130*) constructed a molecular model of a complex between a biantennary glycopeptide and ConA, based on X-ray crystallographic data and proton NOE measurements. They were able to show the glycopeptide-binding region and identify atoms responsible for both favorable and unfavorable interaction using this approach.

Future studies on the application of CP-MAS NMR for the determination of secondary structure in solid state can be anticipated (*131*). Although $^{1}H$ and $^{13}C$ NMR studies provide a wealth of structural information, NMR of other nuclei present in the oligosaccharides of glycoproteins remain largely unexamined. Application of $^{15}N$ NMR might throw more light on their secondary structure, whereas the phosphorylation sites in some glycoproteins might be studied by $^{31}P$ NMR.

## SEQUENCING APPROACHES

### Sequencing using Radiolabeling, Exoglycosidases, and LP-GPC

Sequential exoglycosidase digestion is becoming an indispensable procedure for the structural determination of sugar chains. In addition, the recent introduction of endoglycosidases that release oligosaccharides from glycoproteins and glycolipids is opening a new era in the structural study of glycoconjugates (*23,32*).

Exoglycosidases are hydrolases, that cleave monosaccharide units from the nonreducing terminus of oligosaccharides and from the sugar chains of glycopeptides and glycolipids. Since exoglycosidases cleave only monosaccharide residues that are located at the nonreducing terminus, they can be useful tools for sequencing the sugar chain. In addition, information about the anomeric configuration of glycosidic linkage can be obtained by using the known specificity of glycosidases. Enzymatic hydrolysis can be carried out on very small quantities of a radiolabeled sample, making it possible to analyze the structure of only a few micrograms of an oligosaccharide or glycopeptide. The oligosaccharide is first reduced with $NaB^{3}H_{4}$, and the oligosaccharide, having its reducing end labeled with tritium, is subjected to an enzymatic digestion. The radioactive oligosaccharide in the reaction mixture is then analyzed by either paper

chromatography (*45,54,74,132*) or gel permeation chromatography (*43, 60–62*). Since the tritium label is located at the reducing end of the sugar chain, the digestion products are a monosaccharide and smaller radiolabeled oligosaccharide. The oligosaccharide recovered from paper, or from the column effluent, can be used for the next enzymatic digestion. For example, if the carbohydrate chain terminates with the sequence *N*-acetylneuraminicacid-α-(2→6)-Galactose-β-(1→4)-*N*-acetylglucosamine β-(1→2)-Mannose-α-(1→), treatment with neuraminidase (*8,18,75,76*), will release *N*-acetylneuraminic (sialic) acid, but other exoglycosidases will be without effect. Subsequent sequential digestion with β-galactosidase, β-*N*-acetylhexosaminidase and α-mannosidase can establish the sequence as *N*-acetylneuraminic acid-α-(2→6)-Galactose β-(1→4)-*N*-acetylglucosamine β-(1→2)-Mannose.

There are, however, some pitfalls in this approach, particularly when the exact specificity of the enzyme is unknown or when the enzyme fails to behave in a predictable manner when challenged by a new substrate. The complete structures of many glycoprotein carbohydrate units can be deduced with greater certainty by combining the information obtained using sequential digestion by exoglycosidases having different specificity, with linkage analysis by methylation and periodate oxidation (*17,50*). The problem of contamination of the other glycosidases activities must also be considered, because prolonged digestion with relatively high concentrations of enzyme are often necessary to bring about the complete release of a terminal monosaccharide. Under these conditions, even levels of contaminating activities that may appear low when assayed for a few minutes with synthetic substrates can have a major effect on oligosaccharide substrates (*30*). An enzyme preparation that has been confirmed as being free from other glycosidases using *p*-nitrophenylglycoside as a substrate may still not be safe. Many exoglycosidases that hydrolyze sugar chains are nearly inactive against synthetic glycosides (*133*). A careful interpretation of positive results obtained by glycosidase digestion, therefore, is required. Multi-point enzyme digestions should be performed using time course studies to discriminate the side reactions resulting from contaminating enzymes (*30*).

## Sequencing by GC-MS

Oligosaccharide and glycopeptide analysis has been successfully performed on derivatized (i.e., peracetylated and/or permethylated) samples using GC-MS (*134,135*). Recently, underivatized glycopeptides have been successfully analyzed by fast atom bombardment mass spectrometry (FAB-MS) (*136*). FAB-MS and electron-impact mass spectrometry (EI-MS) each provide valuable information on the monosaccharide sequences within oligosaccharide chains, as well as on their molecular weight (*46, 68,137,138*). The carbohydrate structure of human thrombin has been determined by direct probe insertion mass spectrometry of the oligosac-

charides released by trifluoroacetolysis from the asialoglycoprotein (*90*). The linkage between the monosaccharides were determined by methylation analysis of thrombin glycopeptides prepared by pronase digestion.

## Sequence Analysis by LC-MS

Liquid chromatography-mass spectrometry (LC-MS) has the distinct advantage of combining a separation and analysis technique for a nonvolatile, underivatized sample. We have recently begun using LC-MS in conjunction with specific endoglycosidases (*139*) and exoglycosidases (*30*) in our laboratory to establish oligosaccharide and glycopeptide structures. Newly available MS/MS capabilities should facilitate the fragmentation of these neutral oligosaccharides at glycosidic linkages, providing sequence information where previously only molecular ion peaks were observed.

## Sequencing Using Exoglycosidase, a Pellicular Resin Column and PAD

Chen et al. (*84*) has proposed that a mixture of oligo-mannose and hybrid-type glycans can be separated and characterized with a pulsed amperometric detector (PAD) system combined with the use of exoglycosidases and endoglycosidases. The PAD system permits the detection of pmol amounts of underivatized reducing and nonreducing carbohydrates. One caution on the approach is that it exposes the oligosaccharides being analyzed to very high pHs, at which degradative reactions may occur. If a database can be assembled for standardized oligosaccharides and glycopeptides using this technique, it might become a valuable technique in determining the structure of the oligosaccharides chains of glycoproteins (*140*).

## Sequencing Using Monoclonal Antibodies and Exoglycosidases

One of the major problems in determining the carbohydrate structure of glycoproteins is the considerable difficulty in preparing sufficient quantities of purified glycoproteins. Therefore, any method that is able to provide structure information about the carbohydrate chains on glycoproteins, without requiring the purification of large quantities of a glycoprotein, would be extremely useful. One such approach is the exploitation of specific and sensitive antigen–antibody reactions (*141*). The determination of antibody and lectin reactivities before and after specific deglycosylation procedures make it possible to obtain structural information about the carbohydrate chains on glycoproteins (*142*). Recently, the structure of several carbohydrate antigens have been determined using these methods.

The increased availability of well-characterized monoclonal antibodies prepared against specific oligosaccharide structures should make it possible to determine the structure of carbohydrate chains of unknown glycoproteins (*143,144*).

## Integrated Methods to Determine Sequence

Webb et al. (*93*) have reported a mass-spectrometric-based strategy for determining the detailed structural features of *N*-linked oligosaccharides from glycoproteins. This comprehensive strategy includes: enzymatic release of *N*-linked oligosaccharides using Endo H or PNGase F (*see* Table 2), derivatization of the oligosaccharides with ethyl *p*-aminobenzoate, separation of the products by HPLC, and structural characterization by cesium ion liquid matrix ion mass spectrometry. This strategy was used to characterize a series of intact high mannose oligosaccharides isolated from human immunoglobulin M (IgM).

Tomiya et al. (*70*) recently proposed a two-dimensional map method for the analysis of the structures of *N*-linked oligosaccharides. The structures of unknown oligosaccharide can be characterized from its position on the map. This strategy involves: (1) preparation of carbohydrate chains from glycopeptides by *N*-oligosaccharide glycopeptidase digestion (Table 2); (2) derivatization of the reducing ends of carbohydrate chains with a fluorescent reagent, 2-aminopyridine, using sodium cyanoborohydride; (3) separation of oligosaccharide derivatives by reverse-phase HPLC; (4) analysis of the size of each separated oligosaccharides on an amide silica HPLC column; (5) preparation of the two-dimensional map (RP-HPLC retention time vs amide silica HPLC retention time); and (6) structural analysis of oligosaccharides by exoglycosidase digestion.

Integrated approaches, such as the two described above, offer the best hope that, one day, it will be possible to rapidly and accurately determine the composition and sequence of the carbohydrate portion of a glycoprotein available in only microgram quantities.

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