Review

Opening the gate of glycobiology in the field of N-linked sugar chains

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Abstract: Based on the successful use of human milk oligosaccharide patterns for elucidation of the biochemical basis of ABO and Lewis blood types in humans, a strategy to establish reliable techniques to analyze the structures and functions of the N-linked sugar chains of glycoproteins was devised. N-Linked sugar chains were first released quantitatively as oligosaccharides by enzymatic or chemical means, and labeled by reduction with NaB^3H_4. After fractionation by gel-permeation chromatography and lectin-affinity chromatography, structure of each radioactive oligosaccharide was determined by a series of sensitive methods, which were developed for the structural study of oligosaccharides. By using these techniques, structural rules of the N-linked sugar chains were found. Furthermore, occurrence of site-specific, organ-specific and species-specific N-glycosylation of proteins, which served as important bases for the development of Glycobiology, was revealed.

Key words: Hydrazinolysis; endo-β-*N*-acetylglucosaminidases; exoglycosidases; lectins; N-linked sugar chains; ovalbumin.

Introduction. Most proteins produced by multicellular organisms contain sugar chains, and are called glycoproteins. Interest in the glycoprotein research was stimulated early in the '60s by elucidation of the antigenic determinants of human blood groups, and the molecular basis of antigenic conversion of bacteria. This research area further attracted the interest of biologists, because many studies on cell biology suggested the possibility that the sugar chains of glycoproteins play important roles as signals of cell-to-cell recognitions, which are essential to multi-cellular organisms.

However, difficulties associated with the structural study of the sugar chains of glycoproteins interrupted elucidation of the molecular basis of the functional role of the sugar chains of glycoproteins in the living organisms. A factor, which made the structural elucidation of a sugar chain difficult, is its unique structural characteristics among various chains formed in the living organisms. In contrast to nucleic acids and proteins, many different structures of sugar chains can be formed by using a small number of monosaccharide units. Let us consider the smallest unit of chains: A-B. In the case of RNA, assigning adenylic acid to A and guanylic acid to B, for example, makes only one structure. In the case of protein also, only one structure is made when valine and serine are assigned to A and B, respectively. In the case of sugar chains, however, many isomeric structures can be formed. Suppose that galactose and mannose are assigned to A and B, respectively. As shown in Fig. 1, galactose can be linked to the four hydroxyl groups of the pyranose form of mannose: C-2, C-3, C-4, and C-6. Accordingly, four isomeric structures can be formed. By virtue of the ability of the galactose residue to take two anomeric configurations, the number of possible isomeric structures becomes eight. Furthermore, galactose residues can occur in the furanose form as well as in the pyranose form shown in Fig. 1. Thus 16 isomeric structures are possible for the disaccharide: Gal-Man. When the number of units increases to three, four, and so forth, only one structure can be formed by assigning a particu-

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Abbreviations: Glc, glucose; Gal, galactose; Man, mannose; Fuc, L-fucose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; Sia, sialic acid; Neu5Ac, N-acetylneuraminic acid

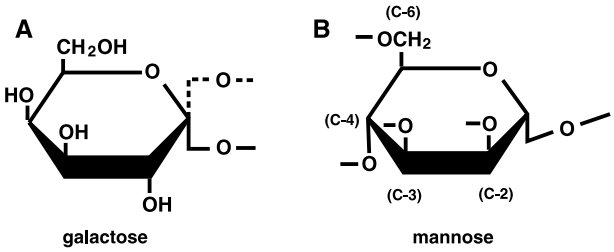


Fig. 1. Construction of Gal-Man.

lar unit at each position in the case of nucleic acids and proteins, because they are linear constructs. By contrast, the number of isomeric sugar chains increases by geometrical progression, because branching can be formed in the sugar chains larger than a disaccharide. This means that sugar chains, but not nucleic acids and proteins, can form multiple structures with a small number of units.

In addition to the structural multiplicity, another factor made the structural study of the sugar chains of glycoproteins difficult. Many glycoproteins contain more than one sugar chain in one molecule. Even in the case of a glycoprotein with only one sugar chain, there is a widespread micro-heterogeneity of the sugar chain structures as will be explained by using ovalbumin in **Section 2.1**, because the absence of a template in the biosynthetic machinery of the sugar chains makes possible the formation of incomplete chains. Accordingly, a purified glycoprotein sample is not suitable for the structural study of its sugar chains.

1. Elucidation of the biosynthetic pathway of the ABO and Lewis blood group determinants. Until 1964, 14 oligosaccharides, as listed in Table I, were found to occur in addition to lactose in human milk by the search of the chemical entities of *Bifidus* factor. Upon development of a sensitive method to finger print these oligosaccharides, Kobata *et al.*[1),2)] analyzed milk samples obtained from 50 individuals. Interesting evidence, that at least three different oligosaccharide patterns occur in individual human milk, was found by these studies.

Forty individuals gave the pattern containing all 14 oligosaccharides as shown in Fig. 2A. Eight individuals gave the pattern as shown in Fig 2B. The characteristic feature of this pattern was that four oligosaccharides were missing as shown by the spots indicated by dotted lines in the pattern. The small gray spots, detected at the positions of the three missing oligosaccharides, were minor oligosaccharides hidden under the major oligosaccharides. Important evidence was that all individuals, who gave this oligosaccharide pattern, were non-secretors of ABO blood groups. The structures of the four missing oligosaccharides indicated that they all contain the Fucα1-2Gal group. Namely, the secretory organs of non-secretor individuals lack the fucosyltransferase, which is responsible for the formation of the disaccharide group.

The remaining 2 individuals gave the oligosaccharide pattern as shown in Fig. 2C. Three oligosaccharides were missing, and three new minor oligosaccharides were found. Examination of the blood types of the donors, whose milk gave this pattern, revealed that all of them were Lewis-negative, lacking both Le^a and Le^b antigens in their secretory glycoproteins. A common feature

Table I. Structures of milk oligosaccharides found until 1964[68)]

Names of oligosaccharides	Structures
2'-Fucosyllactose (2'-FL)	Galβ1-4Glc 2 Fucα1
3-Fucosyllactose (3-FL)	Galβ1-4Glc 3 Fucα1
Lactodifucotetraose (LD)	Galβ1-4Glc 2 3 Fucα1 Fucα1
Lacto-*N*-tetraose (LNT)	Galβ1-3GlcNAcβ1-3Galβ1-4Glc
Lacto-*N*-neotetraose (LNnT)	Galβ1-4GlcNAcβ1-3Galβ1-4Glc
Lacto-*N*-fucopentaose I (LNF-I)	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 2 Fucα1
Lacto-*N*-fucopentaose II (LNF-II)	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 Fucα1
Lacto-*N*-difucohexaose I (LND-I)	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 2 4 Fucα1 Fucα1
Lacto-*N*-difucohexaose II (LND-II)	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 4 3 Fucα1 Fucα1
3'-Sialyllactose (3'-SL)	Galβ1-4Glc 3 Neu5Acα2
6'-Sialyllactose (6'-SL)	Galβ1-4Glc 6 Neu5Acα2
LST-a	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 3 Neu5Acα2
LST-b	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 6 Neu5Acα2
LST-c	Galβ1-4GlcNAcβ1-3Galβ1-4Glc 6 Neu5Acα2

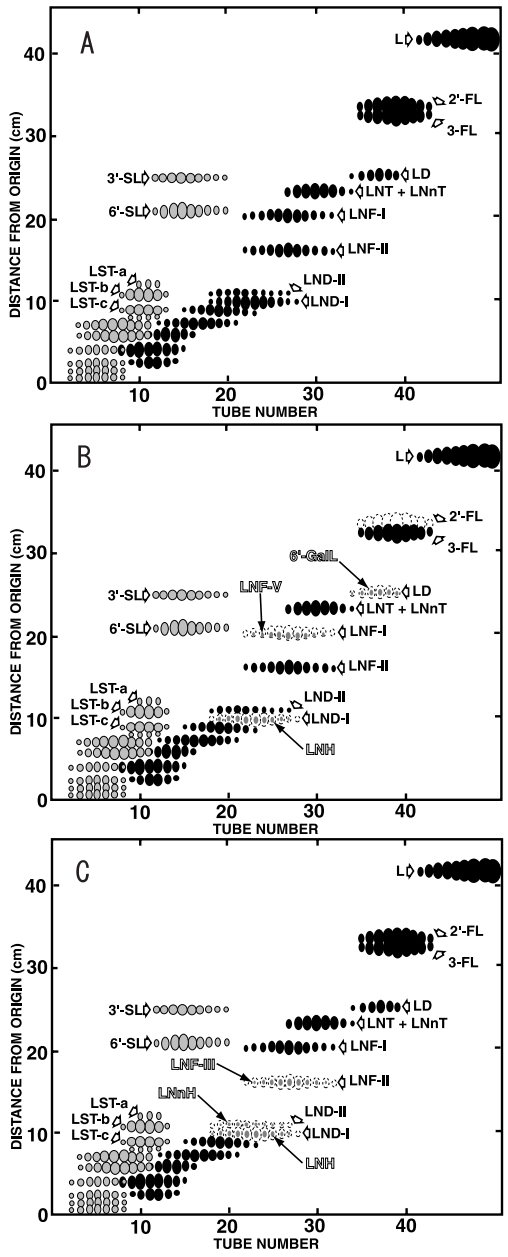


Fig. 2. Fingerprinting patterns of human milk oligosaccharides. Fraction numbers as indicated by "TUBE NUMBER" in abscissa were obtained by Sephadex G-25 column chromatography of the oligosaccharide fraction of human milk obtained from an individual donor. Aliquots of the fractions were spotted at the origin of a sheet of filter paper, and subjected to chromatography using ethylacetate/pyridine/acetic acid/ water (5:5:1:3) as a solvent. Black spots represent oligosaccharides visualized by alkaline-$AgNO_3$ reagent, and hatched ones encircled by black line represent those detected by both alkaline-$AgNO_3$ reagent and thiobarbituric acid reagent.[1] Three typical patterns are shown in A-C. Names and structures of the oligosaccharides indicated by abbreviations with white arrows are listed in Table I. The spots, indicated by dotted lines in B and C, are those missing in the fingerprints, and the grey spots detected at the position of missing oligosaccharides are minor oligosaccharides hidden under the major oligosaccharides. Written by white letters are the names assigned to these newly found minor oligosaccharides the structures of which were described in Ref. 68. Revised from the figure in Glycoconj. J. **17**, 443-464 (2000).

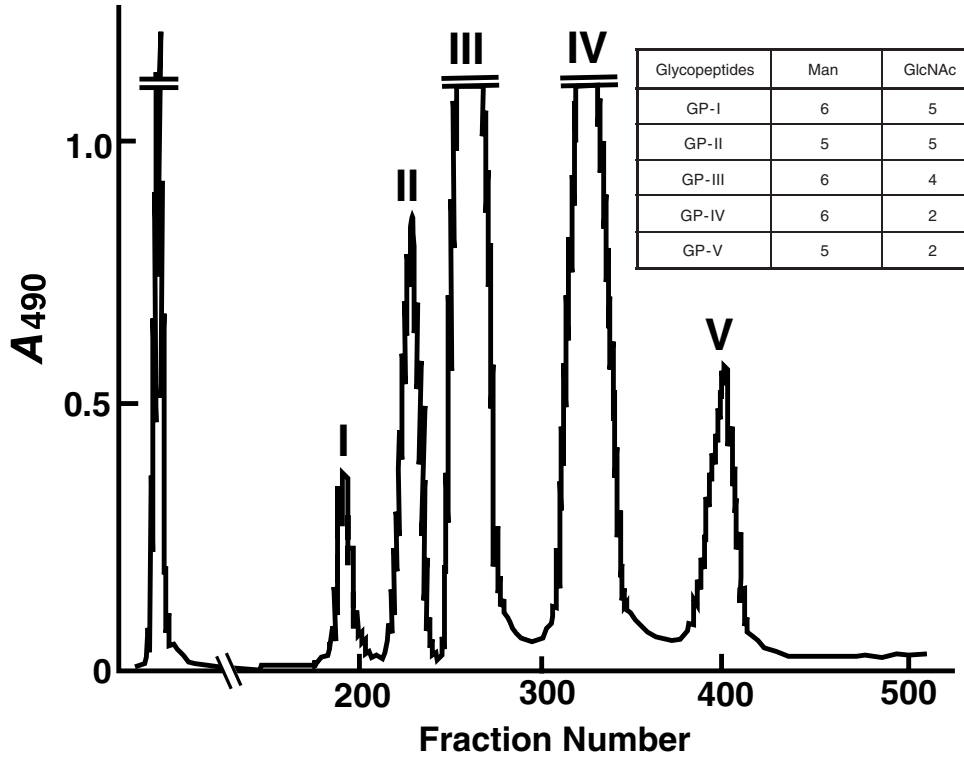
of the three missing oligosaccharides is that they all contain the Fucα1-4GlcNAc group. Based on this finding, it was concluded that another fucosyltransferase, which is responsible for the formation of the Fucα1-4GlcNAc group, is not expressed in the epithelial cells of the secretory organ of the Lewis-negative individuals.

These findings led to the elucidation of the whole biosynthetic pathway of the ABO and Lewis blood group determinants.[3]

2. A novel strategy to elucidate the structures of N-linked sugar chains of glycoproteins. Based on the successful use of milk oligosaccharide patterns for elucidating the biochemical background of the biosynthesis of blood group determinants, it was born as a fresh idea that the characteristic features of N-linked sugar chains of glycoproteins might be accurately elucidated, if an appropriate method could be established to quantitatively release the sugar chains as oligosaccharides from polypeptide backbone.

Most of the sugar chains of glycoproteins can be classified into two groups: O-linked and N-linked. An O-linked sugar chain contains at its reducing terminus an *N*-acetylgalactosamine residue, that is linked to the hydroxyl group of either serine or threonine residue in a polypeptide chain. Such sugar chains could be released as oligosaccharide alcohols by heating the glycoprotein at 48 ˚C in a solution of 1 M $NaBH_4$ and 0.05 N NaOH for 16 hours.[4]

N-Linked sugar chains are linked to the amide group of asparagine residues of a polypeptide chain. Each of these sugar chains contains an *N*-acetylglucosamine residue at its reducing terminus. Because no chemical method was available to release the N-linked sugar chains from polypeptide backbone, exhaustive pronase digestion was widely used to obtain Asn-oligosaccharides for the structural study of the N-linked sugar chains. This method, however, afforded many problems as will be also described in the next section. First of all, it was hard to fractionate the Asn-oligosaccharides containing the same number of monosaccharides as will be concretely explained by the structural studies of the sugar chains of ovalbumin in the next section. Furthermore, the samples obtained in many cases were not simple mixtures of Asn-oligosaccharides, but complicated mixtures containing heterogeneities in both peptide moieties and sugar chains, because it was hard to hydrolyze completely the polypeptide moiety. Accordingly, many of the N-linked sugar chain structures reported until 1970 contained structural errors.



Glycopeptides	Man	GlcNAc	Asn
GP-I	6	5	1
GP-II	5	5	1
GP-III	6	4	1
GP-IV	6	2	1
GP-V	5	2	1

Fig. 3. Chromatography of the Asn-oligosaccharides mixture, obtained from ovalbumin by exhaustive pronase digestion, on a Dowex 50W-X2 column (2.5 × 140 cm). Elution was performed with 1 mM sodium acetate buffer, pH 2.52, at a flow rate of 35 ml/hr, and a 15-ml fraction was collected. Elution was monitored by phenol-sulfuric acid reagent. Revised from the figure in Ref. 14.

Finding and characterization of several endo-β-*N*-acetylglucosaminidases (hereafter abbreviated to "Endo"), which hydrolytically cleave the *N*,*N*'-diacetylchitobiose moieties commonly located at the reducing termini of all N-linked sugar chains,[5)-7)] solved the problems in the structural study of the N-linked sugar chains.

2.1. Elucidation of the structures of the total sugar chains of ovalbumin – Effective use of endo-β-N-acetylglucosaminidases. Ovalbumin is a glycoprotein with a molecular weight of 45,000, and contains a little more than 3% sugars.[8)] It was confirmed by the studies of many researchers that ovalbumin contains one N-linked sugar chain, which is composed of approximately five mannoses and three *N*-acetylglucosamines. Having been obtained easily in a crystalline form, ovalbumin had served as a standard material to study the N-linked sugar chains.

By fractionating the glycopeptides mixture obtained from ovalbumin by exhaustive pronase digestion, Cunningham *et al.*[9)] found that five Asn-oligosaccharide peaks were obtained by ion-exchange column chromatography using a long column of Dowex 50 (Fig. 3). As shown in the inset of Fig. 3, each Asn-oligosaccharide contained different amounts of mannose and *N*-acetylglucosamine residues. Based on this evidence, they proposed that the sugar chain of ovalbumin has micro-heterogeneity. Since then, similar phenomenon was observed in many glycoproteins, and micro-heterogeneity is now believed to be an inherent characteristic of the sugar chains of glycoproteins. Huang *et al.*[10)] studied the structure of GP-I, the largest Asn-oligosaccharide of ovalbumin, and proposed its structure as shown in Fig. 4A. A revision that the trisaccharide structure at the reducing terminal of the sugar chain of GP-I is not Manα1-GlcNAcβ1-GlcNAc but Manβ1-GlcNAcβ1-GlcNAc was reported independently by Li and Lee,[11)] Sugahara *et al.*,[12)] and Tarentino *et al.*[13)] at the same time.

By investigating release of *N*-[^{14}C]acetylAsn-GlcNAc from *N*-[^{14}C]acetylated Asn-oligosaccharides of ovalbumin after digestion with endo-β-*N*-acetylglu-

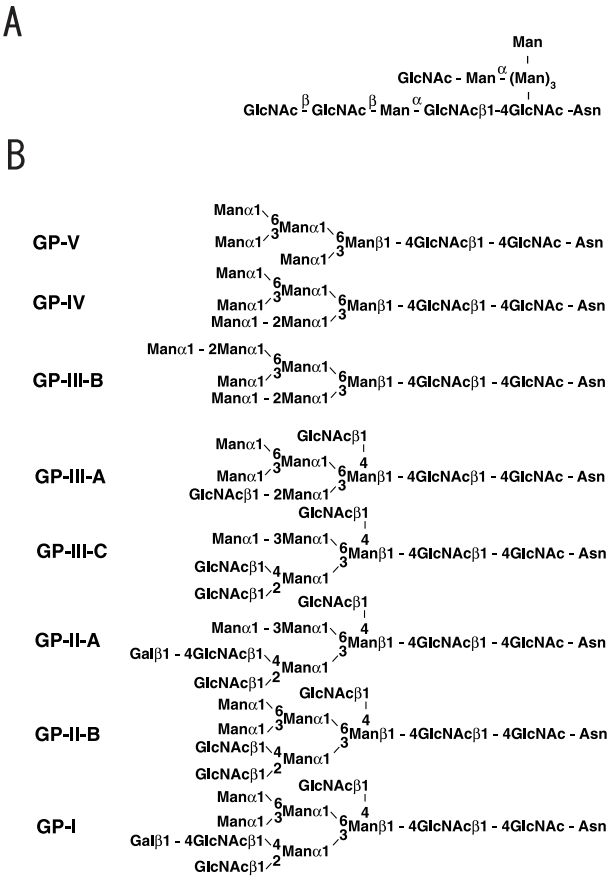


Fig. 4. Structures of the Asn-oligosaccharides obtained from ovalbumin. A, The structure proposed for GP-I by Huang *et al.*[10]; B, Structures of Asn-oligosaccharides proposed for all Asn-oligosaccharides obtained from ovalbumin.[14)-16)] Three and two Asn-oligosaccharides were included in GP-III and GP-II, respectively.

cosaminidases purified from *Diplococcus pneumoniae* (Endo-D), *Streptomyces griseus* (Endo-H), and *Clostridium perfringens* (Endo-C_{II}), we found that the three endo-β-*N*-acetylglucosaminidases show different action spectra on the five components (Fig. 5). Endo-D hydrolyzed GP-V only, while Endo-H hydrolyzed all five components to oligosaccharides and *N*-[^{14}C]acetylAsn-GlcNAc, although the hydrolytic rates of the samples with larger oligosaccharides were slower.[14)] Endo-C_{II} hydrolyzed GP-IV and V, and a part of GP-III, but neither GP-II nor GP-I at all.[15)] In order to use these interesting findings for elucidating the substrate specificities of the three enzymes, we thoroughly investigated the structures of the oligosaccharides released by the enzymes, and elucidated them as shown in Fig. 4B.[14)-16)]

The structures revealed many important new lines of evidence regarding the N-linked sugar chains of ovalbumin. First of all, the sugar chains could not be considered as incomplete biosynthetic products of the largest GP-I, but should be considered as series of two subgroups. Accordingly, GP-IV and GP-V can be considered as the incomplete sugar chains of GP-III-B, while GP-III-A, GP-III-C, GP-II-A, and GP-II-B as the incomplete chains of GP-I. The finding that the latter group could neither be classified into the high mannose-type sugar chains nor the complex-type sugar chains, which were proposed as subgroups of N-linked sugar chains at that time, confirmed the presence of the hybrid-type sugar chains as a novel subgroup. The characteristic features of these subgroups will be described later.

Plural numbers of Asn-oligosaccharides were found in peaks GP-II and GP-III. The evidence, that Asn-oligosaccharides included in the same peak in Fig. 3 contain the same number of monosaccharide units, indicated the difficulty of fractionating Asn-oligosaccharides containing the same number of monosaccharides.

That GP-V is a common core of high mannose-type sugar chains could be estimated from the structures of GP-III-B and GP-IV. This estimation was later confirmed by the structural study of the undecasaccharide, the largest high mannose-type sugar chain of glycoproteins, released from unit A of bovine thyroglobulin by Endo-H digestion.[17)]

Finding of the Asn-oligosaccharides containing one galactose residue, such as GP-II-A and GP-I, was a surprise for the people, who were involved in the study of the sugar chains of ovalbumin.

These results materially indicated the effectiveness of the new strategy of investigating the N-linked sugar chains of glycoproteins.

2.2 Substrate specificities of endo-β-N-acetylglucosaminidases. Based on the action spectra of the three endo-β-*N*-acetylglucosaminidases on various glycopeptides in addition to the Asn-oligosaccharides from ovalbumin, the substrate specificities of the three enzymes were elucidated as shown in Table II.[18),19)] Accordingly, the enzymes release many of the N-linked sugar chains of glycoproteins intact by leaving either the *N*-acetylglucosamine residue or the Fucα1-6GlcNAc group on the polypeptides. Endo-D requires the Manα1-3Manβ1-4GlcNAc group for its glycon specificity. The α-mannosyl residue of the trisaccharide group is the most important and should not be linked by other sugars except for its C-4 position. Endo-H requires for its specific glycon a tetrasaccharide structure: Manα1-3Manα1-6Manβ1-4GlcNAc. Again, the most important

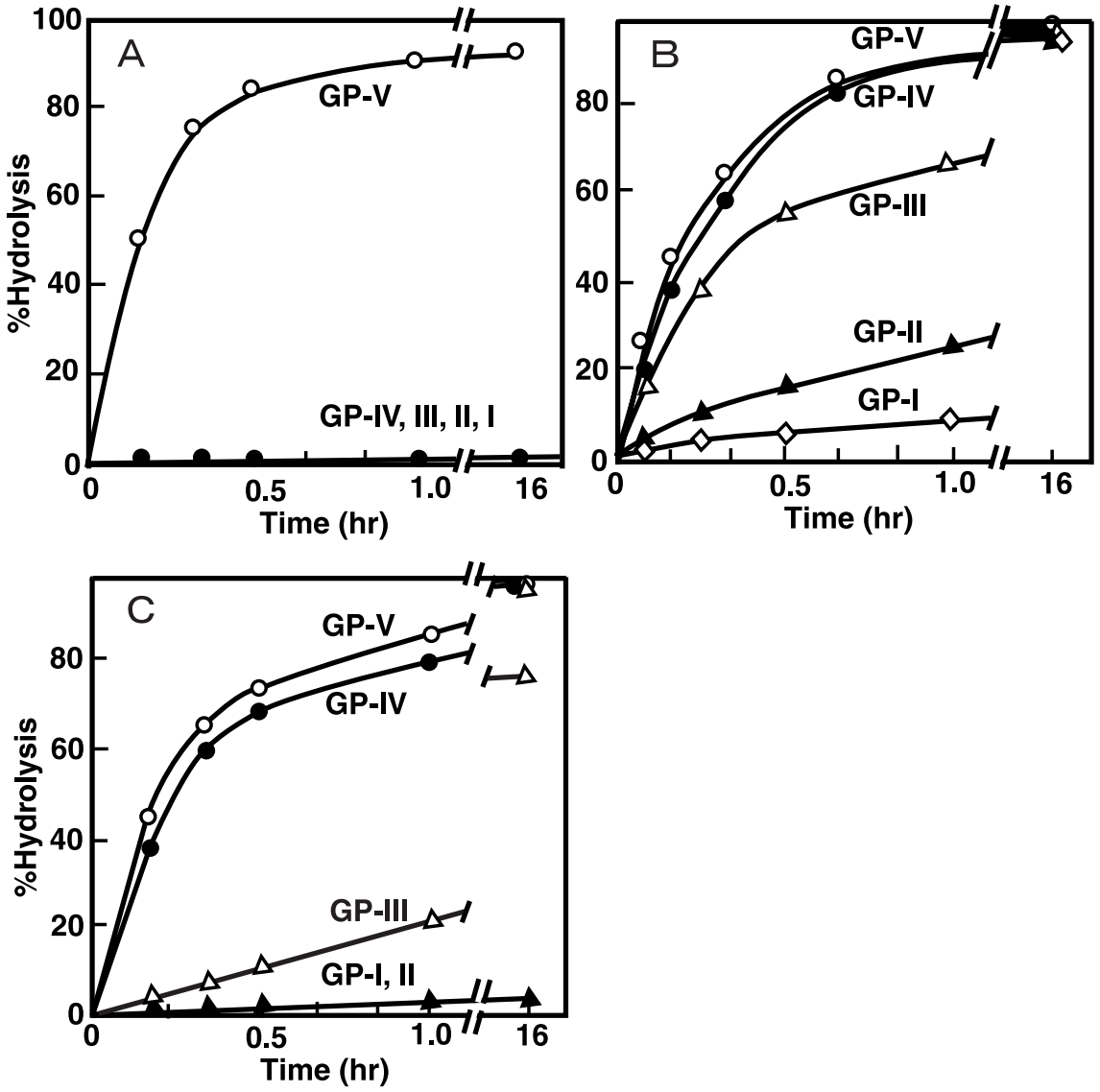


Fig. 5. Hydrolysis of the *N*-[^{14}C]acetyl Asn-oligosaccharides obtained from ovalbumin by endo-β-*N*-acetylglucosaminidases. The rates of hydrolysis by Endo-D (A), Endo-H (B), and Endo-C_{II} (C) were analyzed by the time course release of *N*-[^{14}C]acetyl Asn-GlcNAc.

part is the α-mannosyl residue at the non-reducing terminal. Addition of sugars at the hydroxyl group except for the C-2 position of this residue will make the oligosaccharide resistant to the enzyme action. Endo-C_{II} requires for its specific glycon a branched pentasaccharide: Manα1-3Manα1-6(Manα1-3)Manβ1-4GlcNAc. The two α-mannosyl residues at the non-reducing termini must be either non-substituted or replaced by other sugars only at their C-2 positions. These enzymes have rather broad aglycon specificities, since they cleave the sugar chains linked to *N*-acetylglucosamine, the Fucα1-6GlcNAc group, the GlcNAc-Asn (peptides) group, and the Fucα1-6GlcNAc-Asn (peptides) group. The enzymes except for Endo-H can also cleave the sugar chains linked to *N*-acetylglucosaminitol and the Fucα1-6*N*-acetylglucosaminitol group, but the rate of hydrolysis is much smaller than others.

Because of the strict substrate specificities, these enzymes could not be used to study complex-type sugar chains, which are the major subgroup of N-linked sugar chains. After development of hydrazinolysis,[20] however, structural studies of these sugar chains

Table II. Substrate specificities of endo-β-*N*-acetylglucosaminidases. Dotted lines indicate the hydrolytic cleavage sites. R = H or sugars

Endo enzymes	Structures of substrates
D	R-4Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc-Asn (R at 6 and 6)
H	Manα1-3Manα1- (R at 2 and 6) 6 R-4Manβ1-4GlcNAcβ1-4GlcNAc-Asn (R at 6); R at 3
CII	Manα1-3Manα1- 6 R-4Manβ1-4GlcNAcβ1-4GlcNAc-Asn; R-2Manα1- at 3

became much more reliable.

2.3. Hydrazinolysis: A useful chemical means to release quantitatively all N-linked sugar chains of glycoproteins as oligosaccharides from polypeptide backbones. Hydrazinolysis was used for the structural study of chondroitin sulfate. It was then applied for the study of the N-linked sugar chains of glycoproteins by Yoshizawa *et al.*[21] Practical application of this method for the isolation of N-linked sugar chains as oligosaccharides was reported by Bayard and Montreuil[22] in 1974. However, researchers found that no reliable result was obtained by the reported procedure. Since we already knew that all of the N-linked sugar chains of ovalbumin could be released by digestion with Endo-H, we carefully investigated the conditions of hydrazinolysis to obtain a similar (actually each oligosaccharide contained one additional *N*-acetylglucosamine residue) oligosaccharide pattern from this glycoprotein to that obtained by Endo-H digestion. This research revealed that the conditions of hydrazinolysis reported by Bayard and Montreuil (100 ˚C, 30 h) were too harsh, and destroyed a large portion of the released oligosaccharides. The research also manifested several secret measures to obtain successful results of hydrazinolysis: such as need for extensive dehydration of the hydrazine and glycoprotein samples, and complete *N*-acetylation of the released oligosaccharides in order to remove hydrazine residue from their reducing termini.[20]

Because the method can release all N-linked sugar chains as oligosaccharides from any kind of materials so long as they are successfully dehydrated, it can also be used to compare the sugar patterns of cultured cells and animal tissues. Actually, it was effectively used to find out the alterations of cell surface sugar patterns by development and by malignant transformation.[23)-27)]

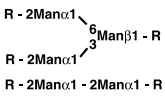
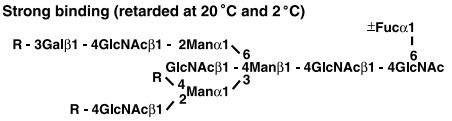
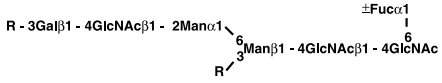
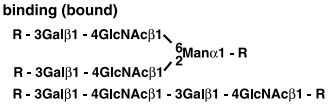
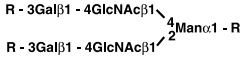
3. Development of various sensitive methods for the structural studies of oligosaccharides. The reducing termini of all oligosaccharides, thus released from glycoproteins, were occupied by an *N*-acetylglucosamine residue, which can be quantitatively labeled by reduction with NaB^3H_4,[28] or labeled by an appropriate fluorescent tag. Many novel sensitive methods to investigate the structures of the tritium-labeled oligosaccharides were developed.

Gel-permeation chromatography using a column, containing ultra-fine Bio-Gel P-4,[29),30)] was effectively used to fractionate oligosaccharides by their sizes. Oligosaccharides with sizes up to 30 glucose units can be successfully separated by using a column of 2 cm × 2 m. Since most of the N-linked sugar chains are smaller than 25 glucose units, this method was quite useful for the studies of N-linked sugar chains.

Serial lectin column chromatography was newly added as a useful fractionation method of oligosaccharides. In 1975, we investigated the behaviors of various oligosaccharides and glycopeptides through a small column containing concanavalin A (Con A)-Sepharose, and found that samples containing two mannose residues, either non-substituted or substituted only at C-2 position by other sugars, binds to the column and eluted by the buffer containing 0.1 M α-methyl mannopyranoside.[31] This finding was very useful for separating mono- and biantennary complex-type sugar chains from higher antennary sugar chains, and effectively used for elucidating the molecular basis of Warren-Glick phenomenon, which is now considered as the most important tumor related alteration of N-linked sugar chains.[32] Furthermore, the column is useful for isolating high mannose-type sugar chains from others, because the sugar chains containing more than three mannose residues, interacting with Con A, bind much more tightly to the column.[31]

Triggered by the usefulness of a Con A-Sepharose column, behaviors of oligosaccharides in many other immobilized lectin columns were investigated, and found to be useful for fractionating the sugar samples (Table III). Because many review papers have already been published on the effectiveness of these immobilized lectin columns, I will not discuss in detail here. Some lectins recognize quite a large portion of oligosaccharides.[33),34)] Accordingly, serial affinity column chro-

Table III. Binding specificities of immobilized lectin columns

Lectin	Structure Necessary for Binding
Concanavalin A	
Phytohemagglutinin E_4	
	Strong binding (retarded at 20 °C and 2 °C) 
	Weak binding (retarded at 2 °C only) 
Datura stramonium agglutinin	
	Strong binding (bound) 
	Weak binding (retarded) 
Aleuria aurantia lectin	
Allomyrina dichotoma lectin	Neu5Acα2 - 6Galβ1 - 4GlcNAcβ1 - R

R represents either hydrogen or sugar chains.

matography using a variety of immobilized lectin columns has become a useful technique to fractionate oligosaccharides and glycopeptides effectively.[35] Because N-linked sugar chains have many structural rules as will be discussed in **Section 4**, the affinity chromatography was especially useful for the analysis of these sugar chains.

Sequential exoglycosidase digestion,[36] which was devised for the structural studies of milk oligosaccharides, was also found to be an effective tool to study the structures of N-linked sugar chains. The problem, that N-linked sugar chains are much larger than milk oligosaccharides and hard to be analyzed by paper chromatography, was solved by introduction of Bio-Gel P-4 column chromatography. Furthermore, newly added exoglycosidases with unique aglycon specificities strengthened the effectiveness of the method in studying the structures of N-linked sugar chains. For example, α-fucosidase purified from *Charonia lampas*[37] has broad aglycon specificity, and hydrolyzes all α-fucosyl linkages of natural oligosaccharides. α-Fucosidase from *Bacillus fulminans*[38] hydrolyzes the Fucα1-2Gal linkage, but not the Fucα1-3GlcNAc, the Fucα1-4GlcNAc, and the Fucα1-6GlcNAc linkages. α-Fucosidase I from almond emulsin hydrolyzes the Fucα1-3GlcNAc and the Fucα1-4GlcNAc linkages, but neither the Fucα1-2Gal nor the Fucα1-6GlcNAc linkage.[39] Therefore, not only the amount of non-reducing terminal α-fucosyl residues, but also their linkages can be determined by using properly selected α-fucosidases. It must also be stressed here that even if digestion with the α-fucosidase from *Bacillus fulminans* or from almond emulsin did not release any fucose from an oligosaccharide, it does not always mean the absence of α-fucosyl residue at the non-reducing terminal of the oligosaccharide.

β-*N*-Acetylhexosaminidase purified from the culture medium of *Diplococcus pneumoniae* has very complicated but useful aglycon specificity.[40] It can cleave the GlcNAcβ1-2Man, the GlcNAcβ1-3Gal, and the GlcNAcβ1-6Gal linkages, but not the GlcNAcβ1-4Man and the GlcNAcβ1-6Man linkages. The GlcNAcβ1-2Man linkage in the GlcNAcβ1-4(GlcNAcβ1-2)Man group is cleaved, but that in the GlcNAcβ1-6(GlcNAcβ1-2)Man group is not. Furthermore, the GlcNAcβ1-2Manα1-6(GlcNAcβ1-4)(GlcNAcβ1-2Manα1-3)Man is converted to the GlcNAcβ1-2Manα1-6(GlcNAcβ1-4)(Manα1-3)Man by the enzyme digestion, indicating that bisecting GlcNAc residue is not only resistant to the enzyme, but also sterically inhibits hydrolysis of the GlcNAcβ1-2 residue on the Manα1-6 arm. With these specificities in mind, diplococcal β-*N*-acetylhexosaminidase has been effectively used for the structural analysis of N-linked sugar chains.

Methylation analysis devised by Hakomori[41] was useful, because complete methylation of oligosaccharides can be performed by a single step reaction. However, the Hakomori's method methylates also the *N*-acetyl group of aminosugars, because it is much stronger than the conventional methylation methods. The problem of poor recovery of *O*-methylated 2-*N*-methylaminosugars by gas chromatographies, which were widely used for the analysis of partially *O*-methylated monosaccharides, was solved by the successful synthesis of all partially *O*-methylated 2-*N*-methylglucosamines as standards, and by the introduction of a column packed with Gas-chrom Q coated with OV-17.[42]

4. Structural rules found in the N-linked sugar chains. Since the N-linked sugar chains found in glycoproteins usually contain more than 10 monosaccharides, the structural multiplicity generated by such

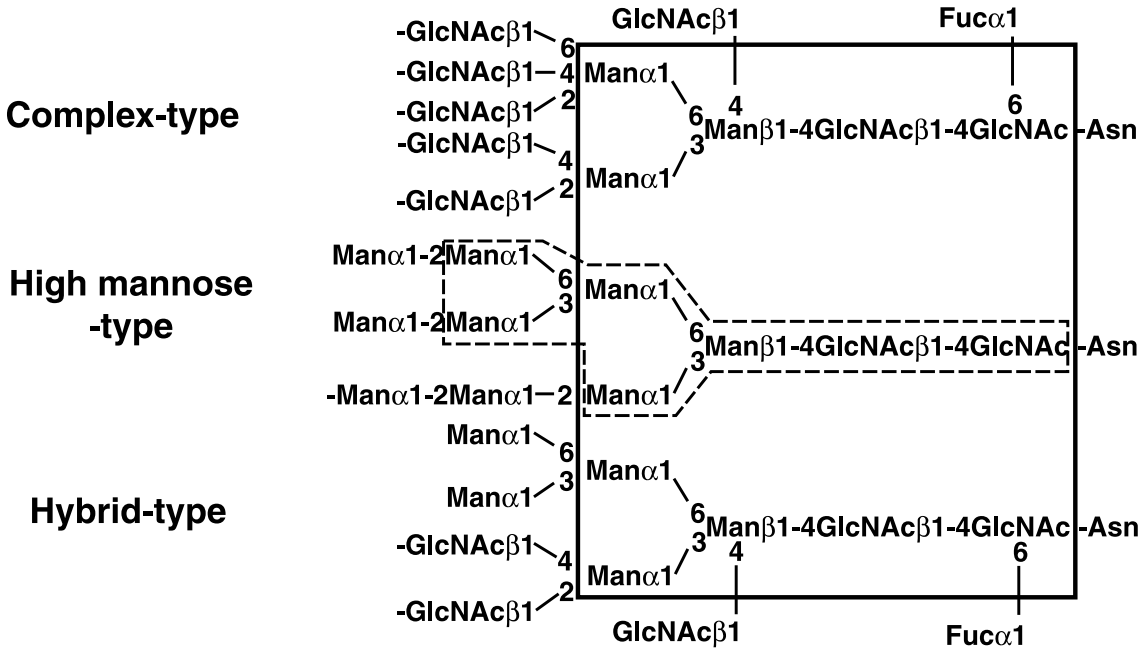


Fig. 6. Subgroups of N-linked sugar chains. Structures within the solid line are the trimannosyl core common to all N-linked sugar chains. The structure enclosed by dashed line is the common heptasaccharide core of high mannose-type sugar chains. Structures, outside the solid line and the dashed line, can vary in the subgroups. The dashes on the monosaccharide residues at the non-reducing termini indicate that the residues can further be elongated by adding monosaccharides.

size of oligosaccharide is theoretically enormous. Indeed, it might be impossible to elucidate the biological functions of the sugar chains, if we had to handle such a large number of isomers. Fortunately, studies of the sugar chain structures of various glycoproteins have revealed that a series of structural rules exists in them, and variable regions are limited to parts of their structures.

The N-linked sugar chains of glycoproteins contain as a common core the pentasaccharide: Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc, which was named "trimannosyl core". Based on the structures and locations of the sugar residues added to the trimannosyl core, N-linked sugar chains are classified into three subgroups (Fig. 6). Complex-type sugar chains contain no mannose residues other than those in the trimannosyl core. Outer chains with an *N*-acetylglucosamine residue at their reducing termini are linked to the two α-mannosyl residues of the trimannosyl core. High mannose-type sugar chains contain only α-mannosyl residues in addition to the trimannosyl core. Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc is commonly included in this type of sugar chain (enclosed by a dashed line in Fig. 6) as described already in **Section 2.1**. Hybrid-type sugar chains were so named because they have the characteristic features of both complex- and high mannose-type sugar chains. One or two α-mannosyl residues are linked to the Manα1-6 arm of the trimannosyl core, as in the case of the high mannose-type, and the outer chains found in complex-type sugar chains are linked to the Manα1-3 arm of the core. Presence or absence of the α-fucosyl residue linked to the C-6 position of the proximal *N*-acetylglucosamine residue, and the β-*N*-acetylglucosamine residue linked to the C-4 position of the β-mannosyl residue of the trimannosyl core (bisecting GlcNAc) contributes to the structural variation of complex-type and hybrid-type sugar chains.

Among the three subgroups of N-linked sugar chains, the largest structural variation resides in the complex-type sugar chains. This variation is formed by two structural factors. As shown in Fig. 7A, mono-, bi-, tri-, tetra-, and pentaantennary sugar chains are formed by adding from one to five *N*-acetylglucosamine residues to the trimannosyl core. Two isomeric triantennary sugar chains, containing either the GlcNAcβ1-4(GlcNAcβ1-2)Manα1-3 group or the GlcNAcβ1-6(GlcNAcβ1-2)Manα1-6 group, are found. These isomeric sugar

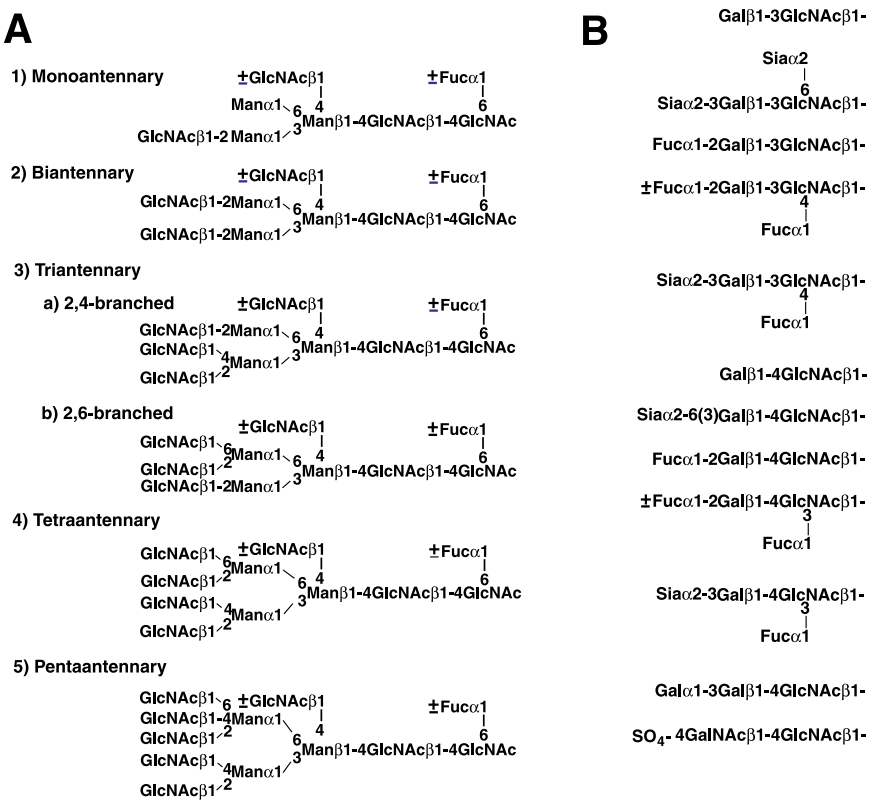


Fig. 7. Branching of the complex-type sugar chains (A), and various outer chain structures (B) found in the complex-type sugar chains.

chains are called 2,4-branched and 2,6-branched triantennary sugar chains, respectively. On these *N*-acetylglucosamine residues, various outer chains are formed (Fig. 7B) by the concerted action of many glycosyltransferases and sulfotransferases. It is important to note that bisecting GlcNAc is never elongated by the action of glycosyltransferases. Combination of the antennary and the various outer chains will form a large number of different complex-type sugar chains. In most glycoproteins, the α-fucosyl residue linked at the C-6 position of the proximal *N*-acetylglucosamine residue of the trimannosyl core is not elongated by addition of other sugars. However, the fucosyl residue of the hybrid-type sugar chain of octopus rodopsin is substituted by a β-galactosyl residue at its C-4 position.[43)]

5. Characteristic features and functional roles of the N-linked sugar chains of glycoproteins. Accumulation of information about the structural characteristics of the N-linked sugar chains of glycoproteins has enabled us to consider their functional roles in molecular terms. Glycoproteins are distributed widely inside and outside the cells of multicellular organisms. Furthermore, the molecular construction of a single glycoprotein is quite diverse.

Two different types of glycoproteins are found as extracellular components. Many serum glycoproteins have a limited number of sugar chains in one molecule. Epithelial cells lining the alimentary tracts and the respiratory tracts secrete viscous glycoproteins called mucins. These glycoproteins work to protect the surface of mucous membrane from invasion of various pathogenic bacteria and viruses. Mucins have many short sugar chains, which are distributed all over the polypeptide moiety or in clusters.

Most of the cell surface receptors are plasma membrane integrated glycoproteins. They are anchored in the phospholipid bilayer by their hydrophobic amino acid cluster portion, and the sugar chains are linked to the polypeptide portion extended outside the cells.

Although the functional roles of the sugar chains of glycoproteins are not simple because of their structural multiplicity, it is possible to classify them roughly into

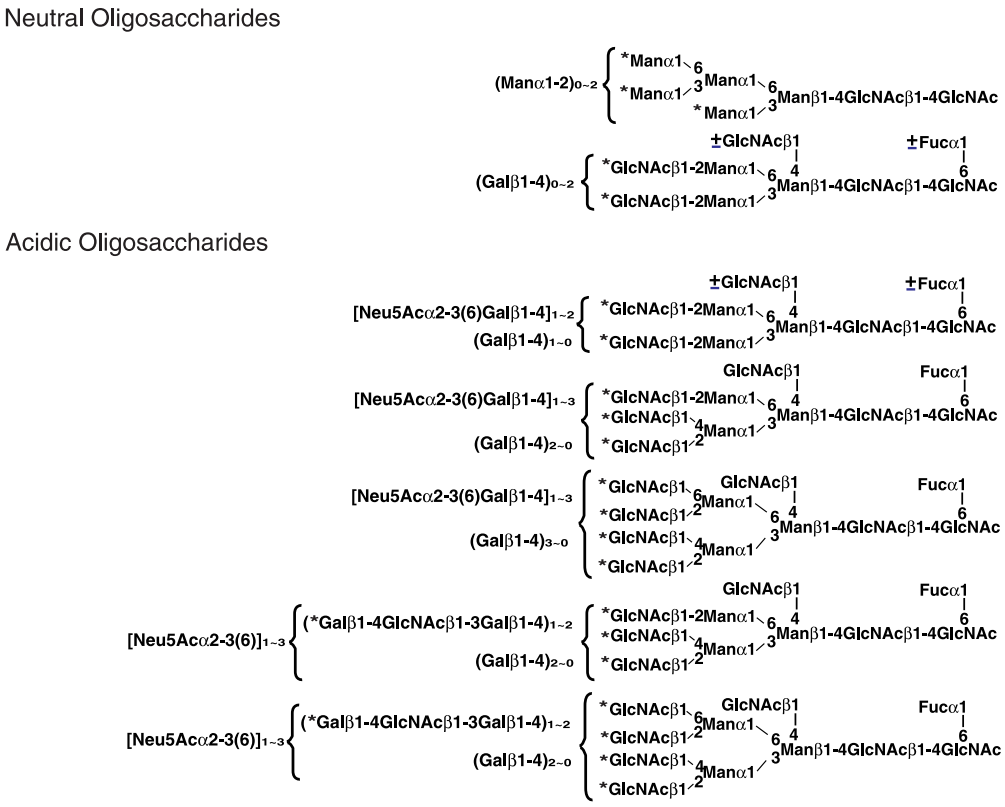


Fig. 8. Structures of the sugar chains of the γ-glutamyltranspeptidase, purified from rat kidney.[46] The sugar residues with asterisk were substituted by the outer chains.

two groups: one that acts to confer physicochemical properties on proteins, and another that acts as signals of various recognition phenomena. Some of our representative studies, which served to elucidate the characteristic features and the functional roles of the N-linked sugar chains of glycoproteins, will be introduced in the following part of this section.

5.1. Various information revealed by the study of the sugar chains of γ-glutamyltranspeptidases. γ-Glutamyltranspeptidase (γ-GTP) is a glycoprotein associated with the apical side of the epithelial cell membrane of various organs of mammals, and catalyzes the first step of glutathione catabolism.[44] It is composed of a heavy and a light subunit of glycoprotein nature, and is embedded in the plasma membrane of cells by the N-terminal portion of the heavy subunit.[45] Since glucosamine, but no galactosamine, was detected by the sugar analysis of γ-GTP, it was concluded that this enzyme contains N-linked sugar chains but not an O-linked sugar chain.

Studies of the sugar chains of γ-GTP samples purified from various sources revealed many important lines of evidence included in the N-linked sugar chains of a membrane bound glycoprotein. Analysis of the radioactive oligosaccharides, released from the rat kidney γ-GTP by hydrazinolysis, with use of paper electrophoresis and Bio-Gel P-4 column chromatography, revealed that high mannose-type and various complex-type sugar are included in this glycoprotein.[46] By a detailed study of each oligosaccharide, the whole oligosaccharides pattern of rat kidney γ-GTP was elucidated as shown in Fig. 8. A very similar oligosaccharides pattern was obtained from the study of bovine kidney γ-GTP.[47]

Comparative study of the sugar patterns of the two subunits of rat kidney γ-GTP revealed that high mannose-type sugar chains are included only in the heavy subunit, and the non-sialylated and non-fucosylated biantennary sugar chains are included only in the light subunit,[48] indicating the presence of site specific glycosylation.

By ion-exchange column chromatography, rat kid-

ney γ-GTP was separated into several fractions with different iso-electric points.[49] Comparative study of the sugar patterns of four pure isozymic forms, obtained by DE-52 column chromatography of rat kidney γ-GTP, revealed that all these isozymic forms contain 2 mol of neutral oligosaccharides but different numbers of acidic sugar chains. The total numbers of sialic acid residues showed a reciprocal relationship to the isoelectric point of each isozymic form.[48]

Comparative studies of the sugar chains of γ-GTP samples, purified from the kidney and the liver of various mammals, unraveled additional important lines of evidence regarding the N-linked sugar chains of glycoproteins.[46),47),50)-54] As is evident from the structures of the major oligosaccharides of various γ-GTP samples in Fig. 9, occurrence of both organ- and species-specific glycosylation of γ-GTPs was observed. An interesting observation is that the bisecting GlcNAc is detected in the sugar chains of all kidney enzymes, but not in those of liver enzymes. This evidence, together with the fact that none of the glycoproteins produced by the liver, so far studied, contain bisected sugar chains, indicated that expression of *N*-acetylglucosaminyltransferase-III, which catalyzes the addition of the bisecting GlcNAc residue, is suppressed by differentiation to hepatocytes. In contrast, the enzyme should be strongly expressed in the kidney cells of all mammals.

It was reported by Fiala *et al*. that γ-GTP activity was prominently elevated in rat hepatoma induced by oral administration of 3'-methyl-4-dimethylaminoazobenzene.[55] Since the elevation had been observed in the preneoplastic nodules of the liver,[56] the enzyme was expected to be a good marker for the diagnosis of hepatoma. γ-GTPs, produced in azo dye-induced rat hepatoma,[57] rat malignant mammary tissue,[58] and human renal carcinoma,[59] had more acidic pI values than those in the respective normal tissues. Since the differences mostly disappeared after sialidase digestion,[59] the transformational changes of γ-GTPs were estimated to be mainly induced in the sugar moieties of the enzyme molecule.

Comparative study of the sugar chains of γ-GTP samples, purified from rat AH-66 hepatoma cells and from rat liver, revealed that the two enzymes contain very different sets of sugar chains.[50] The sugar chains of the liver enzymes were all acidic, while 28% of those of the hepatoma enzyme were neutral. The neutral oligosaccharides of the hepatoma enzyme were composed of both high mannose-type and complex-type sugar chains. Three prominent structural differences

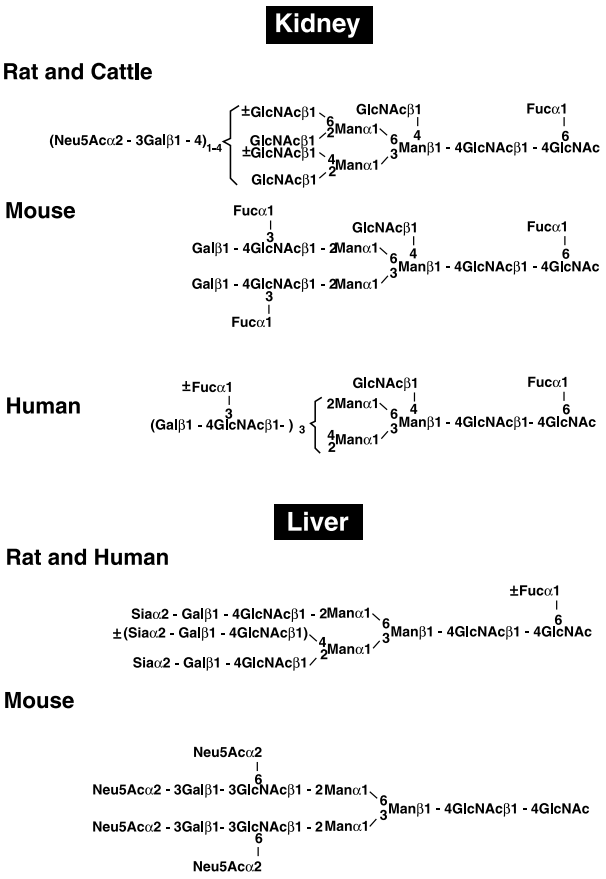


Fig. 9. Major sugar chain structures of γ-glutamyltranspeptidases purified from the kidney and the liver of various mammals. Taken from Glycoconj. J. **17**, 443-464 (2000).

were found in the acidic sugar chains of the two enzymes. The sugar chains of the liver enzyme have complete outer chains, Galβ1-4GlcNAcβ1-, while many of those of the hepatoma enzyme have incomplete outer chains lacking the galactose residues. Galβ1-4GlcNAc tandem repeats were found in the sugar chains of the liver enzyme, but not in those of the hepatoma enzyme. More than 40% of the sugar chains of the hepatoma enzyme contain bisecting GlcNAc, which is not found in those of the liver enzyme.

Among the various alterations found in the tumor enzyme, occurrence of the bisecting GlcNAc residue was considered to be the most interesting, because the expression of GnT-III should be suppressed in the normal livers of all mammals as described already. Therefore, this phenomenon seems to indicate that GnT-III is ectopically expressed in hepatoma.

However, this interesting finding could not be

applied to diagnose the human hepatoma, because bisected complex-type sugar chains could not be prominently detected in the human hepatoma γ-GTP.[54] This evidence indicated that the structural alteration of the sugar chains of glycoproteins, induced by malignant transformation, could be species-specific.

5.2. Recombinant glycoproteins. Many recombinant glycoproteins have been obtained by using various animal cell lines as hosts. However, as introduced in **Section 5.1**, both organ- and species-specific differences occur in the N-linked sugar chains of glycoproteins. In addition, an altered glycosylation phenomenon, found widely in malignant cell lines is reflected in the sugar chains of recombinant glycoproteins, because many of the cell lines used as hosts are somewhat malignant cells. Accordingly, the sugar chains of recombinant glycoproteins may display structural variations according to the type of the cells used, even though the polypeptide structures are the same. Actually, comparative study of the N-linked sugar chains of natural human interferon-β1 (IFN-β1) and three recombinant IFN-β1 samples, produced by different cell lines transfected with the gene coding for human IFN-β1, revealed that their sugar patterns were different in spite of containing the same number of complex-type sugar chains.[60] The differences occur both in the antennary structures, and in the structures of outer chain moieties. This phenomenon affords us a way to elucidate the function of the sugar chains by investigating the biological activities of several preparations of recombinant glycoproteins. Comparative study of the sugar patterns and the *in vivo* activities of several preparations of recombinant human erythropoietin revealed that the activity was proportional to the ratio of tetraantennary to biantennary oligosaccharides.[61]

6. Future prospects. By recent development of the methods for the structural studies of N-linked sugar chains, many of the techniques introduced in this article have now become classical ones. However, I must add that most of the important lines of evidence, such as structural rules, species- and organ-specificities, site-directed glycosylation on the polypeptides, and the metabolic pathways of the N-linked sugar chains[62] were elucidated by using these classical techniques.

Based on the accumulation of these information, the functional roles of the N-linked sugar chains have been elucidated with far more accuracy. By the finding of pathological alterations of the N-linked sugar chains of IgG[63] and tumor glycoproteins,[64] a new field, which might be called glycopathology, was created.

Investigations of structural changes of sugar chains that are caused by aging have already started to afford some useful information pertaining to the elucidation of diseases induced by aging.[65] We also noticed that glycobiology has been making big developments in the fields of cellular immunology and brain-nervous system. In the development of the brain-nervous system, glycans have been implicated as important mediators of adhesive interactions among neural cells.[66),67]

Among the recent developments of instrumental analysis of the structures of the N-linked sugar chains, NMR and mass spectrometry are making tremendous developments. Especially, introduction of tandem mass spectrometry is offering a very sensitive and reliable method to determine the structures of N-linked sugar chains. Therefore, future development of labeling and fractionation methods of N-linked sugar chains must be directed to afford suitable samples for applying this useful physical method.

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Profile

Akira Kobata was born in 1933, and graduated from Faculty of Pharmaceutical Science, School of Medicine, University of Tokyo in 1956. He received Ph. D. degree from University of Tokyo in 1962. In 1967, he joined the Section of Biochemistry (Chief, Victor Ginsburg), Laboratory of Biochemical Pharmacology, National Institute of Arthritis and Metabolic Diseases, NIH as a visiting scientist. During that time, he was involved in elucidation of the whole biosynthetic pathway of ABH and Lewis antigenic determinants. In 1971, he moved to Kobe University as the Professor of the First Department of Biochemistry, School of Medicine. There, he developed a series of sensitive methods to investigate the structures of the N-linked sugar chains of glycoproteins. In 1982, he moved to University of Tokyo as the Professor and Chairman of the Department of Biochemistry, Institute of Medical Science, and expanded his research to the functions and pathology of the N-linked sugar chains of glycoproteins. He was awarded the Prize for the Promotion of Young Scientists for 1963 from the Pharmaceutical Society of Japan, Science and Technology Prize for 1985 from Toray Science Foundation, PSJ Award for 1992 from the Pharmaceutical Society of Japan, Claude S. Hudson Award for 1992 from American Chemical Society, and also the 1992 Japan Academy Prize. He was a Fogarty Scholar-in-Residence in NIH from 1985 to 1987, Auckland Foundation Visiting Professor in New Zealand in 1988, and also served as the Director of Institute of Medical Science from 1990 to 1992. In 1993, he was appointed as the Director of Tokyo Metropolitan Institute of Gerontology, and became a Professor Emeritus of University of Tokyo. In this last carrier as a scientist, he developed a new glycobiology area in the field of aging research. From 2000, he has been the Director Emeritus of Tokyo Metropolitan Institute of Gerontology, and served as the advisor of Seikagaku Kogyo Co., LTD until 2003. Currently, he is the scientific advisor of the Noguchi Institute, a non-profit institution established for the study of carbohydrate chemistry in Japan.