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A journey to the world of glycobiology

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Finding of the deletion phenomenon of certain oligosaccharides in human milk and its correlation to the blood types of the donors opened a way to elucidate the biochemical basis of blood types in man. This success led to the idea of establishing reliable techniques to elucidate the structures and functions of the *N*-linked sugar chains of glycoproteins. *N*-Linked sugar chains were first released quantitatively as oligosaccharides by enzymatic and chemical means, and labelled by reduction with NaB^3H_4. After fractionation, structures of the radioactive oligosaccharides were determined by a series of methods developed for the studies of milk oligosaccharides. By using such techniques, structural rules hidden in the *N*-linked sugar chains, and organ- and species-specific *N*-glycosylation of glycoproteins, which afforded a firm basis to the development of glycobiology, were elucidated. Finding of galactose deficiency in the *N*-linked sugar chains of serum IgG from patients with rheumatoid arthritis, and malignant alteration of *N*-glycosylation in various tumors opened a new research world called glycopathology.

However, recent studies revealed that several structural exceptions occur in the sugar chains of particular glycoproteins. Finding of the occurrence of the Galβ1-4Fucα1- group linked at the C-6 position of the proximal *N*-acetylglucosamine residue of the hybrid type sugar chains of octopus rhodopsin is one of such examples. This finding indicated that the fucosyl residue of the fucosylated trimannosyl core should no more be considered as a stop signal as has long been believed. Furthermore, recent studies on dystroglycan revealed that the sugar chains, which do not fall into the current classification of *N*- and *O*-linked sugar chains, are essential for the expression of the functional role of this glycoprotein.

It was found that expression of many glycoproteins is altered by aging. Among the alterations of the glycoprotein patterns found in the brain nervous system, the most prominent evidence was found in P_0. This protein is produced in non-glycosylated form in the spinal cord of young mammals. However, it starts to be *N*-glycosylated in the spinal cord of aged animals.

These evidences indicate that various unusual sugar chains occur as minor components in mammals, and play important roles in particular tissues.

Keywords: **milk oligosaccharide, *N*-linked sugar chain, glycopathology, rhodopsin, dystroglycan, P_0, blood types.**

I was invited by Dr. Turner to write a retrospective and prospective article for a special issue of the Glycoconjugate Journal, commemorating glycobiology at the millennium. Because so many top glycobiologists and sugar chemists in a variety of areas of the glycoconjugate research were included in the list of invited people, I thoroughly considered this proposal, and decided to write a history of my own work, including some future prospects for the field. My expectation is that young scientists, who are planning to enter this rather new field of life science, may learn something by reading the story of a biochemist who stood at the dawn of the glycobiology.

After finishing a post-graduate course at the University of Tokyo in 1958, I joined the Department of Nutrition in the Research Institute of Takeda Chemical Co. Ltd in Osaka. The Department was headed by Dr. Jiro Suzuoki, and was just newly separated from the Department of Biochemistry in the Institute. We discussed thoroughly in what field we should develop our research. Because we were not planning simply to contribute to the promotion of the health of the whole nation, there were two possibilities: one was to focus on the nutrition of senior people, and the other was to consider the nutrition of babies. Being a graduate of the Faculty of Pharmaceutical Science of the University of Tokyo, which was leading research in the chemistry of natural resources at that time in Japan, my interest was directed to the materials rather than to the biological phenomena. Therefore, I decided to focus my research on baby nutrition, because many answers were expected to be obtained by the comparative study of the ingredients in human milk and cows' milk. That was exactly the time when various oligosaccharides were being found in

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human milk through a search to find the chemical entity of the *Bifidus* factor (Table 1) [1–13].

About that time, Takeda Chemical Co. was starting to expand a new business to produce a novel flavor, a mixture of inosinic acid and 5′-guanylic acid obtained by enzymatic degradation of yeast RNA. It was also the time when Van R. Potter [14] reported a method for the systematic analysis of nucleotides in animal tissues by using the newly introduced Dowex 1 ion-exchange column. In order to find the ingredients that occurred in human milk but not in cows' milk, we analyzed comparatively the nucleotides in human and cow's milk by using Potter's analytical method [15]. This research led us to an interesting finding that human milk contains various pyrimidine nucleotides including large amounts of 5′-cytidylic acid and 5′-uridylic acid, regardless of the time of lactation of mothers. In contrast, cows' milk contains negligible amounts of nucleotides. Based on this finding, 5′-cytidylic acid and 5′-uridylic acid, which were by-products of the flavor manufacture, could be used as additives for powdered milk.

Furthermore, this study led us to finding several new nucleotides, among which two very interesting UDP-oligosaccharides were discovered [16–18]. Since no such nucleotide had been found previously, we investigated in detail the

Table 1. Structures of human milk oligosaccharides reported by 1965.

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

Table 2. Nucleotide sugars containing an oligosaccharide as their sugar moieties.

Nucleotide sugars	*Sources*
Neu5Acα2-3(6) Galβ1-4GlcNAc-UDP	goat colostrum [19]
Fucα1-2Galβ1-4GlcNAc-UDP	human milk [16–18]
Galβ1-4GlcNAc-UDP	human milk, pig milk [16,17] [20]
Fucα1-4GlcNAc-UDP	hen oviduct [21]

Numbers in the brackets indicate references.

structures of these nucleotides. As shown in Table 2, they contained either a disaccharide or trisaccharide as their sugar components. When we submitted our results for publication, we noticed that another series of UDP-trisaccharides, shown in Table 2, had also been found in goat colostrum by Jourdian *et al.* [19].

The pattern of milk oligosaccharides is related to blood types

Arround the mid 1960s, I received post-doctoral offers from several laboratories. In 1967, I resigned Takeda Chemical Co.,

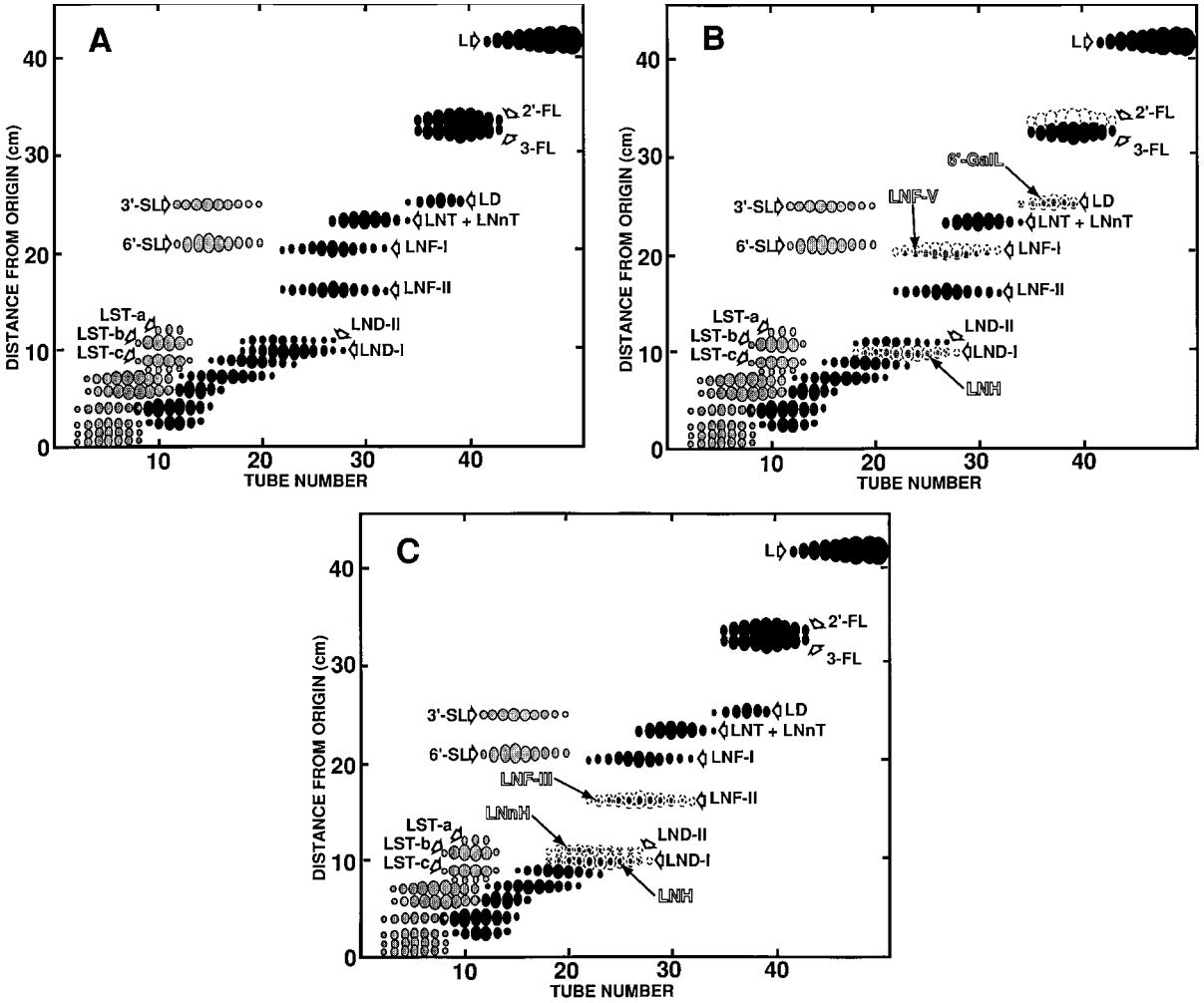


Figure 1. Fingerprintings of human milk oligosaccharides. Fraction numbers as indicated by "tube number" in abscissa were obtained by Sephadex G-25 column chromatography of human milk oligosaccharide fraction of an individual donor. Aliquots of the fractions were spotted at the origin of a sheet of a filter paper, and subjected to chromatography using ethyl acetate/pyridine/acetic acid/water (5:5:1:3) as solvent. Black spots represent oligosaccharides visualized by alkaline-$AgNO_3$ reagent [173], and hatched ones encircled by black line represent those detected by both alkaline-$AgNO_3$ reagent and thiobarbituric acid reagent [24]. Three typical patterns are shown in A–C. Names and structures of the oligosaccharides indicated by abbreviations with white arrows are listed in Table 1. Spots indicated by dotted lines in B and C are those missing in the fingerprints.

and joined Dr. Victor Ginsburg's laboratory in NIH as a visiting scientist. The main reason that I chose Dr. Ginsburg's laboratory was that he had elucidated the biosynthetic mechanism of GDP-Fuc [22]. Since one of my UDP-oligosaccharides contains fucose, I decided that Dr. Ginsburg's laboratory would be the most appropriate research group to investigate the biological meaning of our UDP-oligosaccharides.

Initially, I assumed that the blood group H determinant may be synthesized via our UDP-trisaccharide, since this determinant is included as the sugar portion of this sugar nucleotide. However, all of our experimental results indicated that blood group determinants of sugar nature were synthesized by adding monosaccharides one by one.

In 1967, Shen and Grollman [23] in Dr. Ginsburg's group found that the milk samples obtained from women with non-secretor status of ABO-blood group lacked 2′-fucosyllactose. In order to expand this important finding further, we established a new paper chromatography method to fingerprint all milk oligosaccharides listed in Table 1 [24]. Application of this method to the analysis of milk samples, obtained from individual donors, revealed that three different patterns can be obtained. The first group, which occupied approximately 80% of the samples, contain all fourteen oligosaccharides as shown in Figure 1A. The second group, which is approximately 15% of the samples, gave the oligosaccharide pattern as shown in Figure lB. The dotted lines indicate the missing oligosaccharides, and the dotted spots, shown by arrows with the white second coated letters, indicate minor oligosaccharides which were hidden under the major oligosaccharides in Figure 1A. An important finding was that all individuals, who gave this oligosaccharide pattern (Figure 1B), were non-secretors with Le(a+ b−) blood type. A common feature of the four missing oligosaccharides was that they all contained the Fucαl-2Gal group, in agreement with the finding of Shen and Grollman. Based on this evidence, we concluded that non-secretor individuals do not express the following fucosyltransferase in their secretory organs.

$$\text{GDP-Fuc} + \text{Gal}\beta 1\text{---} \rightarrow \text{Fuc}\alpha 1\text{-2Gal}\beta 1\text{---} + \text{GDP}$$

Approximately 5% of the milk samples investigated gave the oligosaccharide pattern as shown in Figure 1C [25,32]. Three oligosaccharides were missing in this pattern, and three minor oligosaccharides were detected. A search for the blood group status of the donors giving this pattern revealed that they are all Le(a−b−). The three missing oligosaccharides all contain the Fucα1-4GlcNAc group. Accordingly, we concluded that Lewis negative individuals lacked another fucosyltransferase catalyzing the following reaction.

$$\text{GDP-Fuc} + \text{Gal}\beta 1\text{-3GlcNAc}\beta 1\text{---} \rightarrow \text{Gal}\beta 1\text{-3(Fuc}\alpha 1\text{-4)GlcNAc}\beta 1\text{---} + \text{GDP}$$

By adding enzymatic studies to the data described above, we reported that the biosynthetic pathway for the human ABO and Lewis blood group determinants were as shown in Figure 2 [26]. In the mucous epithelial cells of non-secretor individuals, the Fucα1-2Gal group is not formed on the sugar chains of their secretory glycoproteins, because the fucosyltransferase, shown as FucT(I) in Figure 2, is not expressed. Since the Fucα1-2Gal group, formed by the action of the fucosyltransferase, is the substrate of A and B enzymes [27–30], blood group A and B determinants could not be expressed in the sugar chains of the secretory glycoproteins of non-secretor individuals, even if they have *A* and *B* genes. Expression of *H* gene, the structural gene for FucT(I), in mucous epithelial cells was originally considered to be regulated by *Se* gene [26]. However, recent gene cloning studies indicated that *Se* gene is

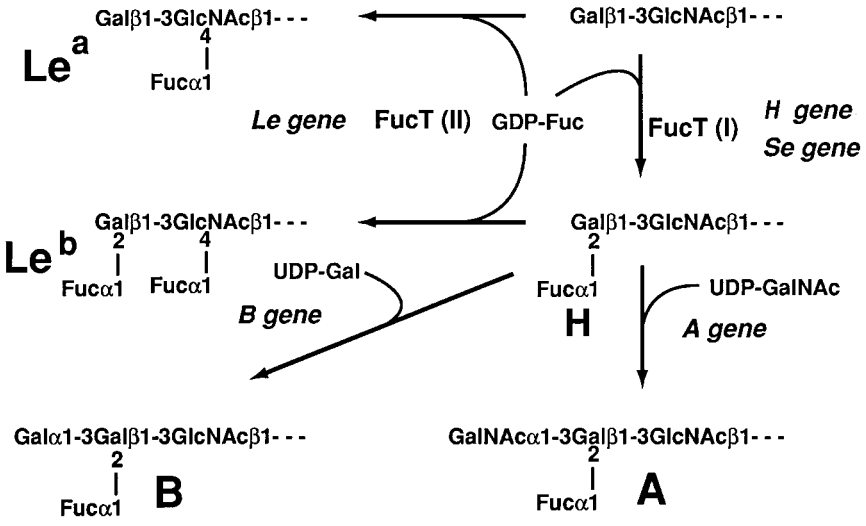


Figure 2. Biosynthetic pathway of ABO and Lewis blood group determinants.

also a structural gene that codes for the FucT(I) specifically in the secretory organs [31].

The *Le* gene is the structural gene of FucT(II) in Figure 2. Therefore, individuals, who are of *le*,*le* genotype, lack both Le^a and Le^b antigenic determinants.

Structures of the three novel minor oligosaccharides found in nonsecretors, and two additional novel minor oligosaccharides found in the milk of Lewis negative individuals were elucidated as shown in Table 3 [32–36]. Lacto-*N*-fucopentaose III later served as an important haptenic oligosaccharide to investigate the functional role of Le^x antigen [37]. Lacto-*N*-hexaose and lacto-*N*-*neo*hexaose were used as the models of the core portions for the later studies of many blood group related antigens [38].

As in the case of other milk oligosaccharides already reported, all these oligosaccharides contain lactose at their reducing termini. This evidence, together with the deletion phenomena found in various blood type individuals, suggested that human milk oligosaccharides are formed by the concerted action of glycosyltransferases, which are responsible for the formation of the sugar chains of glycoproteins. The elongation may start by the action of the *N*-acetylglucosaminyltransferase VII (Figure 5), which is responsible for the addition of a *β*-*N*-acetylglucosamine residue to the *N*-acetyllactosamine group of the sugar chains of glycoproteins, on lactose by mistake because of their structural similarity. Therefore, oligosaccharides in human milk may include many structures, starting from the *N*-acetyllactosamine residues in the sugar chains of various glycoconjugates, and possibly working as the receptors on the epithelial cell surface. A recent finding of very minor human milk oligosaccharides, which inhibit the binding of virulent enteric bacteria [39], is considered as a proof of such an hypothesis.

For the structural studies of these novel milk oligosaccharides, many new sensitive analytical techniques, such as tritium-labelling [40], sequential exoglycosidase digestion [41], and sensitive methylation analysis suitable for the aminosugar-containing oligosaccharides [42], have been developed.

Enzymatic release of the *N*-linked sugar chains as oligosaccharides

In 1971, I left Dr. Ginsburg's laboratory and became a Professor in the Department of Biochemistry, Kobe University School of Medicine. In fact this was the start of my own laboratory, and I decided to begin research on the sugar chains of glycoproteins.

At that time, the development of cell biology revealed a possible role for sugar chains of glycoproteins as signals of cell-to-cell recognition, but studies of the structures and functional roles of the sugar moieties of glycoproteins had been hampered by their structural complexity. For example, a disaccharide: Man-Gal can theoretically form 16 isomeric structures [43]. Because branching can be formed, the number of the isomeric structures of a trisaccharide composed of three different monosaccharides reaches several hundreds. In addition to the structural multiplicity of the sugar chains, many glycoproteins contain more than one sugar chain in one molecule. Even in the case of a glycoprotein containing only one sugar chain, microheterogeneity of the sugar moiety still occurs as an inherent characteristic [44]. This is because the absence of a template in the biosynthetic mechanism of the sugar chains affords the chance of incomplete sugar chain formation. Therefore, a thoroughly purified glycoprotein is not a suitable sample to elucidate the structures of its sugar chains. Each sugar chain must be separated for its structural study.

Table 3. Novel oligosaccharides isolated from milk of non-secretor and *lele* individuals.

Names	*Structures*
6′-Galactosyllactose (6′-GalL)	Galβ1-6Galβ1-4Glc
Lacto-*N*-fucopentaose V (LNF-V)	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 3 Fucα1
Lacto-*N*-fucopentaose III (LNF-III)	Galβ1-4GlcNAcβ1-3Galβ1-4Glc 3 Fucα1
Lacto-*N*-hexaose (LNH)	Galβ1-4GlcNAcβ1 6 3Galβ1-4Glc Galβ1-3GlcNAcβ1
Lacto-*N*-neohexaose (LNnH)	Galβ1-4GlcNAcβ1 6 3Galβ1-4Glc Galβ1-4GlcNAcβ1

Effective use of the milk oligosaccharides pattern for the elucidation of the blood group determinants indicated that the characteristic feature of sugar chains of a glycoprotein can be elucidated, if an appropriate method to release them quantitatively as oligosaccharides was established. In addition, the techniques developed for the study of milk oligosaccharides can be effectively used for the structural study of the sugar chains of glycoproteins, if they are converted to tritium-labelled oligosaccharides.

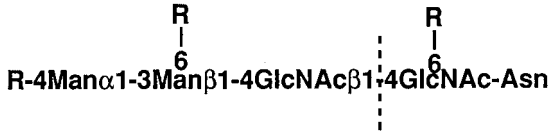
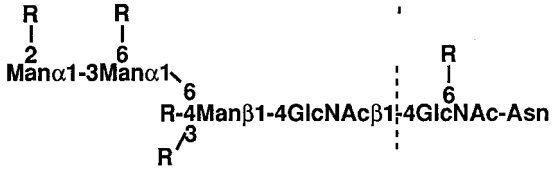
As was well recognized, the sugar chains of glycoproteins can be classified into two groups: *N*-linked and *O*-linked. Since the method to release the *O*-linked sugar chains quantitatively as oligosaccharides had already been established by Carlson [45], I decided to establish a method to release quantitatively the *N*-linked sugar chains from glycoproteins. Fortunately, Takashi Muramatsu, who joined my laboratory at Kobe University, had discovered a new glycosidase activity in the culture medium of *Streptococcus pneumoniae* [46]. This enzyme released oligosaccharides from human immunoglobulin G. Koide and Muramatsu purified this enzyme, and named it endo-*β*-*N*-acetylglucosaminidase D (abbreviated as endo D) [47]. Similar enzyme from *Streptomyces plicatus* was found by Maley's group and named endo-*β*-*N*-acetylglucosaminidase H (endo H) [48]. Additional endo-type enzymes named endo-*β*-*N*-acetylglucosaminidases C_I and C_{II} (endo C_I and endo C_{II}) were found by Ito in our laboratory from the culture medium of *Clostridium perfringens* [49]. Since these enzymes were expected to become useful tools to perform our research, we decided to start investigating in detail the substrate specificities of these enzymes in order to use them as the reagents to release the *N*-linked sugar chains as oligosaccharides [50,51]. As summarized in Table 4, endo D and endo C_I were found to have the same substrate specificity, requiring the tetrasaccharide structure as their substrate. Endo H requires the pentasaccharide as its substrate. In contrast, endo C_{II} requires the branched hexasaccharide as its substrate.

As will be described later, these enzymes, especially endo H served very effectively for the structural elucidation of high mannose-type and hybrid-type sugar chains (Figure 3). However, we met with an obstacle, none of the enzymes could cleave the complex type sugar chains, which is the most abundant subgroup of the *N*-linked sugar chains.

Chemical release of the *N*-linked sugar chains

In order to overcome the limitations of the enzymatic methods, we looked for several other techniques to release quantitatively the *N*-linked sugar chains from glycoproteins, and finally came upon hydrazinolysis [52]. Hydrazinolysis, which was originally used for the structural study of chondroitin sulfate [53], was first applied for the study of the *N*-linked sugar chains of glycoproteins in 1966 by Yoshizawa *et al.* [54]. Practical application of this method for the isolation of *N*-linked sugar chains as oligosaccharides was reported by Bayard and Montreuil [55] in 1974. Because we already knew that all of the *N*-linked sugar chains of ovalbumin can be quantitatively released by digestion with endo H, we carefully investigated the conditions for hydrazinolysis to obtain a similar (actually each oligosaccharide should contain 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 extreme, and destroyed a large portion of the released oligosaccharides. The research also added several important methodological details required to obtain successful hydrazinolysis results: such as the need for extensive dehydration of the hydrazine and glycoprotein samples, and complete *N*-acetylation of the released oligosaccharides to remove hydrazine

Table 4. Substrate specificities of endo-*β*-*N*-acetylglucosaminidases.

Endo enzymes	*Structures of substrates*
D and C_I	
H	
C_{II}	

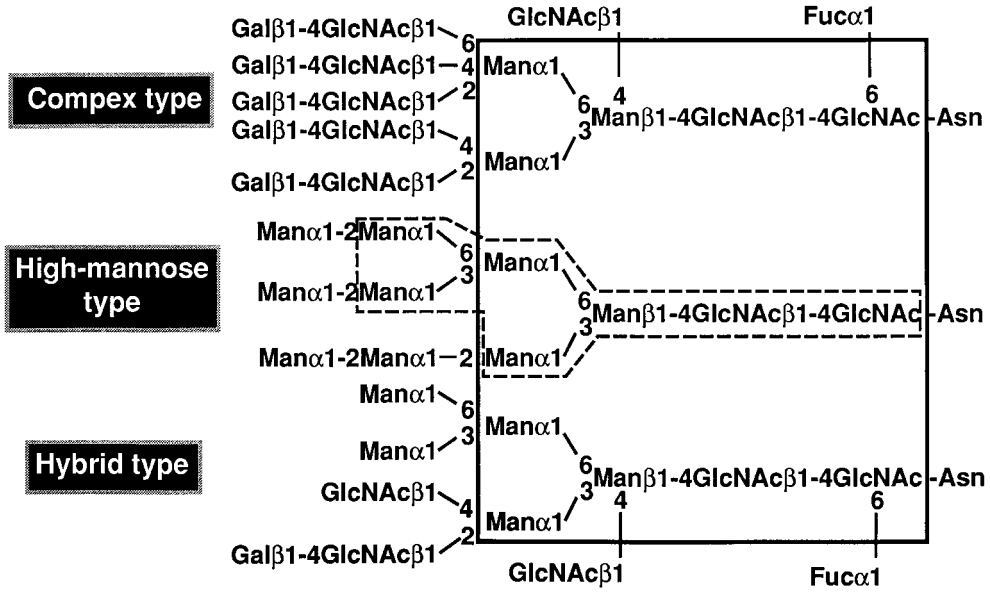


Figure 3. The subgroups of *N*-linked sugar chains. Structures within the solid line are the trimannosyl core common to all *N*-linked sugar chains. Structures outside the solid line can vary in their sugar chains.

residue from their reducing termini. In the beginning, the hydrazinolysis method we reported caused suspicion among many glycoprotein researchers, because they met with unsuccessful results. In 1981, Christian Derappe (Paris), Hans Kamerling (Utrecht), Tom Rademacher (Oxford), and Ed Kean (Cleveland) came to visit my laboratory in Kobe University, and learnt in detail the procedure for the new method. Since then, hydrazinolysis has been widely accepted as a reliable method that is very useful for the study of glycoproteins.

Structural rules found in the *N*-linked sugar chains

Establishment of enzymatic and chemical methods to release quantitatively all the *N*-linked sugar chains as oligosaccharides afforded us much important structural information concerning *N*-linked sugar chains.

As described already, even a trisaccharide containing three different monosaccharide units can form several hundred different isomeric structures. Therefore, a massive number of isomeric sugar chains can be formed in the case of the *N*-linked sugar chain containing more than 10 monosaccharides. It was feared that it might be impossible to elucidate the biological information for these 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 can describe them, and variable regions are limited to a part of their structures.

N-Linked sugar chains may be classified into three subgroups as shown in Figure 3. All three subgroups contain the pentasaccharide consisting of three mannoses and two *N*-acetylglucosamines [56]. This pentasaccharide has been called the "trimannosyl core".

Sugar chains of the complex-type 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. Presence or absence of the α-fucosyl residue at the C-6 position of the proximal *N*-acetylglucosamine residue, and the *β*-*N*-acetylglucosamine residue, linked at the C-4 position of the *β*-mannosyl residue of the trimannosyl core, contributes to the structural variation of the complex-type sugar chains. This latter *N*-acetylglucosamine residue was named bisecting *N*-acetylglucosamine.

High mannose-type sugar chains contain only α-mannosyl residues in addition to the trimannosyl core. By investigating the structures of various high mannose-type sugar chains, released from glycoproteins by endo H digestion, it was elucidated that the largest high mannose-type sugar chain has the structure as shown in Figure 3 [57], and the heptasaccharide with two branch structures, as shown by the dotted line in Figure 3, is commonly included in all of this type of sugar chain [58,59].

The third group is called hybrid-type, because the sugar chains have the characteristic features of both complex-type and high mannose-type sugar chains [59,60]. 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 the complex-type sugar chains are linked to the Manα1-3 arm of the core. Presence or absence of the fucose and the bisecting GlcNAc linked to the trimannosyl core also produces structural variations of the sugar chains of this subgroup.

Discovery of the structural rule of high mannose-type sugar chains was linked up with the interesting evidence found by

Summer's group [61] and Robbins' group at that time. They studied the biosynthetic mechanism of the *N*-linked sugar chain of G protein, a capsular glycoprotein of vesicular stomatitis virus (VSV).

By label-chase experiment, they found that the *N*-linked sugar chains of G protein at early stage are larger than those at the later stage. This finding was hard to interpret, because the sugar chains of G protein should be synthesized by the biosynthetic machinery of host cells, and the biosynthesis of *N*-linked sugar chains in animal cells were considered at that time to proceed by the addition of monosaccharide units from sugar nucleotide one by one. Kornfeld's group and Robbins' group [63–65] linked this peculiar phenomenon to the structural rules of high mannose-type and hybrid-type sugar chains, and proposed the processing pathway as shown in Figure 4. Endo C_{II} was used effectively in the studies of Kornfeld's group to discriminate a series of high mannose-type sugar chains from complex-type sugar chains [63].

This pathway was confirmed to be working both in animal and plant kingdoms. In this biosynthetic pathway, the high mannose-type sugar chains are the precursors of the biosynthesis of the complex-type sugar chains. The enzymatic background to form the hybrid-type sugar chains is the lack of GnT-II in Figure 5. Contribution of GnT-III in Figure 5 for the formation of the hybrid-type sugar chains was also proposed by Narasimhan *et al.* [66]. They found that the addition of bisecting GlcNAc by GnT-III to the processing intermediates I and II in Figure 4 prevents removal of α-mannosyl residues by α-mannosidase II in Figure 4, resulting in a stop in the processing at the hybrid-type stage.

Among the three subgroups of *N*-linked sugar chains, the complex-type has the largest structural variation. This variation is formed mainly by two factors. As shown in Figure 6A, from one to five outer chains are linked to the trimannosyl core by different linkages, resulting in the formation of mono-, bi-, tri-, tetra-, and pentaantennary sugar

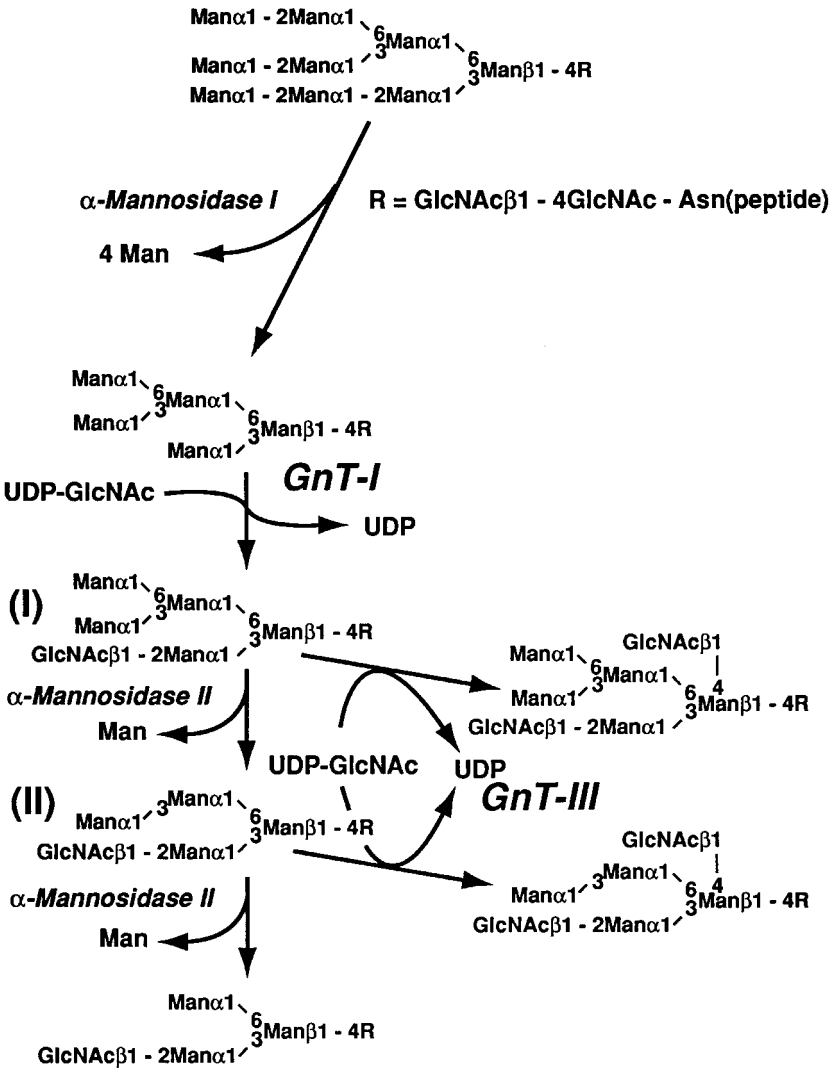


Figure 4. Processing in the biosynthesis of the *N*-linked sugar chains.

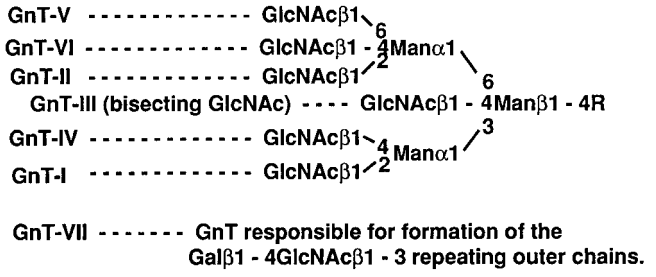


Figure 5. *β-N*-Acetylglucosaminyltransferases (GnTs) responsible for the addition of *N*-acetylglucosamine residues to the trimannosyl core [67]. R = GlcNAc*β*1-4GlcNAc-protein.

chains. Two isomeric triantennary sugar chains containing either the 2,4-branch or 2,6-branch are found. These *N*-acetylglucosamine residues linked to the trimannosyl core are added by the six *β-N*-acetylglucosaminyltransferases (GnT-I ~ GnT-VI) as shown in Figure 5.

Another factor, which contributes to the structural variation of the complex-type sugar chains, is the variation in the structures of outer chains. A very large number of different structures are found in the outer chain moieties of the complex-type sugar chains. In Figure 6B, only a part of such outer chains are shown, and the numbers of different outer chains are increasing year by year.

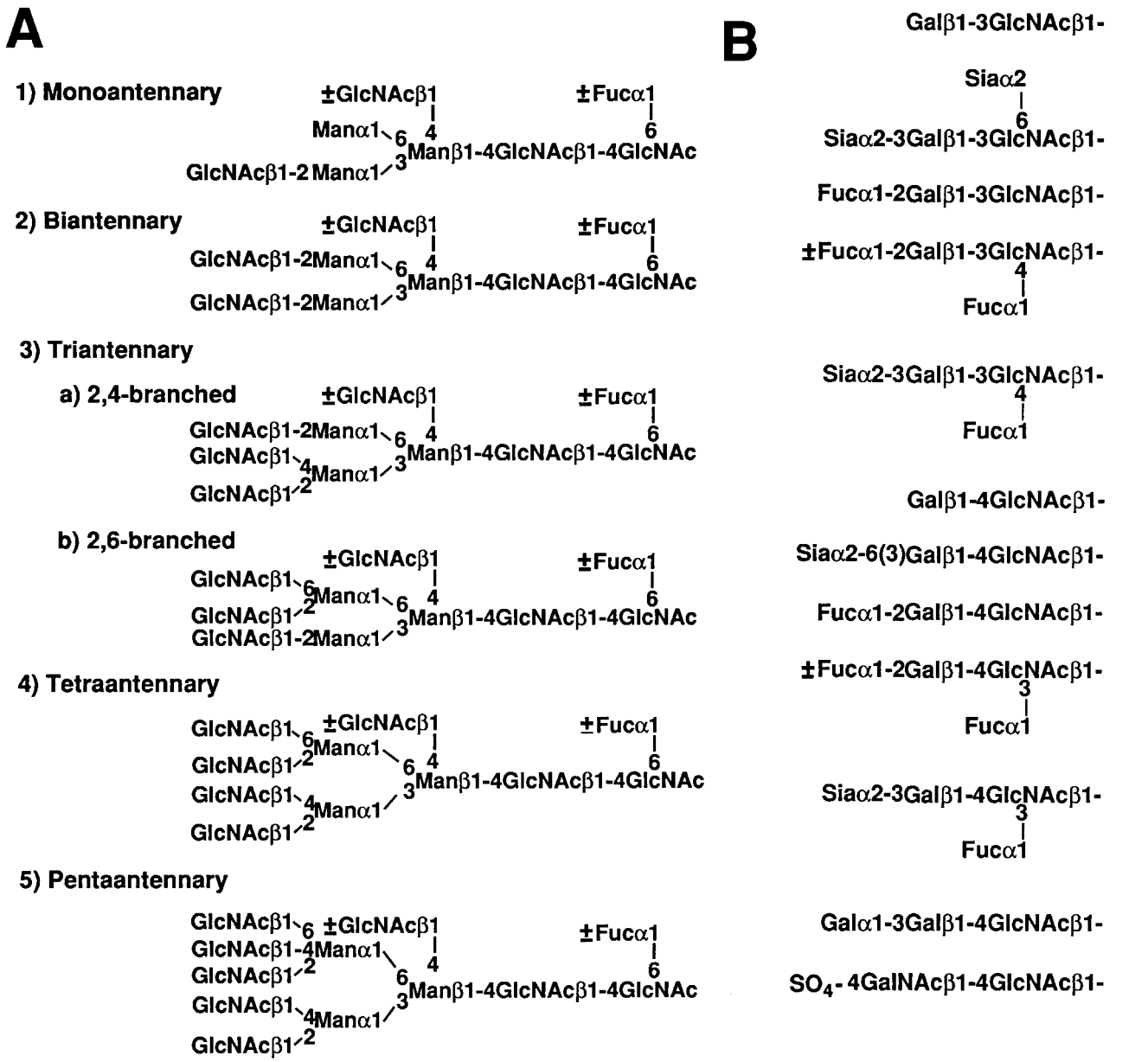


Figure 6. Branching of the complex-type sugar chains (A), and various other chain structures (B) found in the omplex-type sugar chains.

Combination of different antennary structures and various outer chains forms a large number of different complex-type sugar chains. With such rules in mind, elucidation of the sugar chain structure related to a particular biological function comes within the range of laboratory investigation. Actually, many important functional roles of the *N*-linked sugar chains have been revealed based on these structural rules.

Establishment of new methods for the structural study of the *N*-linked sugar chains

Although the structural rules were found in the *N*-linked sugar chains, variety in the structures of the *N*-linked sugar chains is still large, and several cutting-edge techniques had to be established for the development of glycobiology. Introduction of hydrazinolysis enabled us to obtain many oligosaccharides in their tritiated form. These oligosaccharides served as effective probes in developing such new techniques, which have been useful for the structural study of the *N*-linked sugar chains.

Bio-gel P-4 column chromatography

Each time we reported finding a new oligosaccharide, we were asked from many researchers to provide a small amount of the sample as the standard for their own research. Because of this tedious business, we wanted to establish a method that gave a standard number for each oligosaccharide, which can be used by everybody to assign to their oligosaccharide structure. For this purpose, we measured precisely the elution volume of each oligosaccharide with gel permeation chromatography using a Bio-gel P-4 column [68,69]. In order to standardize the method, we used a mixture of glucose oligomers obtained from the acid hydrolysate of dextran as an internal standards. The effective size of each oligosaccharide, expressed in glucose units, could then be used everywhere in the world. From this study, we noticed that each monosaccharide residue contributes to an oligosaccharide to give a different effective size. Accordingly, the size of an oligosaccharide can be estimated by adding the effective sizes of monosaccharides, constructing the oligosaccharide. The data also indicated that not too many oligosaccharides give the same mobility in the column. Effective sizes of oligosaccharides that originated from *O*-linked sugar chains were also reported [70].

Sequential exoglycosidase digestion

Bio-gel P-4 column chromatography was also found to be a useful method for determining the monosaccharide sequence of an oligosaccharide, especially when combined with sequential exoglycosidase digestion [41], originally developed for the structural studies of milk oligosaccharides.

Exoglycosidases hydrolytically release monosaccharides located at the non-reducing termini of oligosaccharides. Each exoglycosidase has a strict **glycon specificity**, which is directed to the structure of monosaccharide to be released, including its anomeric configuration. For example, α-mannosidase cleaves only α-mannose residues, but not *β*-mannose residues at the non-reducing termini of an oligosaccharide. So far, no exoglycosidase have been reported that cleave both α- and *β*-glycosidic linkages. It must be remembered, however, that *β*-*N*-acetylhexosaminidases are so named because they cleave both *β*-*N*-acetylglucosaminyl and *β*-*N*-acetylgalactosaminyl linkages.

Many exoglycosidases also show **aglycon specificities**, which are directed to the structures of inner oligosaccharide portions. Because of this specificity, many exoglycosidases act only on a particular positional isomer of oligosaccharides with the same monosaccharides sequence. For example, α-fucosidase purified from *Charonia lampas* [71,72] has a broad aglycon specificity, and hydrolyzes all α-fucosyl linkages of natural oligosaccharides. α-Fucosidase from *Bacillus fulminans* [73] 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 not the Fucα1-2Gal linkage [74]. Therefore, even if digestion with the α-fucosidase from *Bacillus fulminans* or almond emulsin did not release any fucose, it does not always mean the absence of α-fucosyl residue at the non-reducing terminal of the sugar chain. Because of the strict aglycon specificity, many exoglycosidases, useful for the sequential exoglycosidase digestion, do not act on synthetic glycosides such as *p*-nitrophenyl glycosides, which are widely used for the assay of the activities of glycosidases. With these important characteristics in mind, many useful exoglycosidases have been isolated by using natural oligosaccharides as substrates, and their specificities have been determined [75].

For actual sequencing of an oligosaccharide, the amount of a monosaccharide released by each exoglycosidase digestion can be determined by comparing the effective sizes of a tritium-labelled oligosaccharide in a Bio-gel P-4 column before and after the enzyme digestion.

Serial lectin affinity chromatography

In 1975, we found that oligosaccharides with two α-mannosyl residues, either at non-reducing terminal or substituted only at the C-2 position by another sugar, bind strongly to an concanavalin A (Con A)-Sepharose column and eluted with the buffer containing α-methylglucopyranoside, while oligosaccharides with only one such α-mannosyl residue are slightly retarded in the column [76]. Because of this characteristic, a Con A-Sepharose column has been used as an effective tool to fractionate or characterize the structures of many *N*-linked sugar chains [77,78]. This was actually the start of development of a series of immobilized lectin column chromatographies.

Many lectins recognize quite a large portion of oligosaccharides [79]. Accordingly, serial affinity column chromato-

graphy using immobilized lectin columns has become a useful technique to fractionate effectively oligosaccharides and glycopeptides [80].

Development of glycobiology

In 1983, I was appointed as the Professor in the Department of Biochemistry, which was newly founded in the Institute of Medical Science, at the University of Tokyo. This was a good chance for me to expand my research into many biological phenomena, because the Department was surrounded by many research groups investigating various biomedical problems.

Establishment of hydrazinolysis opened a way to obtain a reliable oligosaccharides pattern from a glycoprotein, because release of the sugar chain moieties from the *N*-linked sugar chains is not affected by the structure of the polypeptide moieties like in the case of enzymatic methods. Furthermore, the method can also be used to obtain the oligosaccharide patterns of cells and tissues, if an appropriate method is available to dehydrate the samples. In the following part of this article, I would like to describe the development of glycobiology, mainly made by using this chemical technique.

Accumulation of structural information of *N*-linked sugar chains of various glycoproteins revealed important evidence, which have become critical in the development of glycobiology and glycotechnology.

Site-directed *N*-glycosylation

Occurrence of microheterogeneity is widely distributed in the *N*-linked sugar chains of glycoproteins. These different sugar chains are not evenly distributed at each *N*-glycosylation site.

The first case of site-directed *N*-glycosylation was found by a comparative study of the oligosaccharides patterns of the two subunits of human chorionic gonadotropin (hCG) [81]. HCG is a glycoprotein hormone produced by trophoblasts of placenta. It is a heterodimer composed of α- and *β*-subunits. Both subunits contain two *N*-linked sugar chains [82,83]. By investigation of the structures of oligosaccharides, released from urinary hCG samples by hydrazinolysis, it was found that five *N*-linked sugar chains as listed in Figure 7 were included in hCG [84,85]. Studies of the sugar chains of α- and *β*-subunits revealed that oligosaccharide A-5 is detected only in α-subunit, while oligosaccharides A-1 and A-2 are detected mainly in *β*-subunit. Furthermore, the molar ratio of the oligosaccharides in the two subunits suggested that the four potential *N*-glycosylation sites of hCG gave different ratios of each oligosaccharide [81], indicating the presence of quite strict site-directed *N*-glycosylation. This interesting phenomenon, together with the functional role of the *N*-linked sugar chains of hCG, was described in our recent review [86].

Occurrence of a specific distribution of different sugar chains at different potential *N*-glycosylation sites of polypeptides indicated that polypeptide portions other than the Asn-X-Ser(Thr) sequence affect the formation of the sugar chain structures. Such a role for the polypeptide moiety was later suggested by the studies of the sugar chains of lysosomal enzymes of fibroblasts [87]. These sugar chains have mannose-6-phosphate residues linked to the high mannose type sugar chains, and act as signals to transport the enzymes to lysosomes. Although many glycoproteins produced by fibroblasts contain high mannose type sugar chains, only the sugar chains of lysosomal enzymes are phosphorylated.

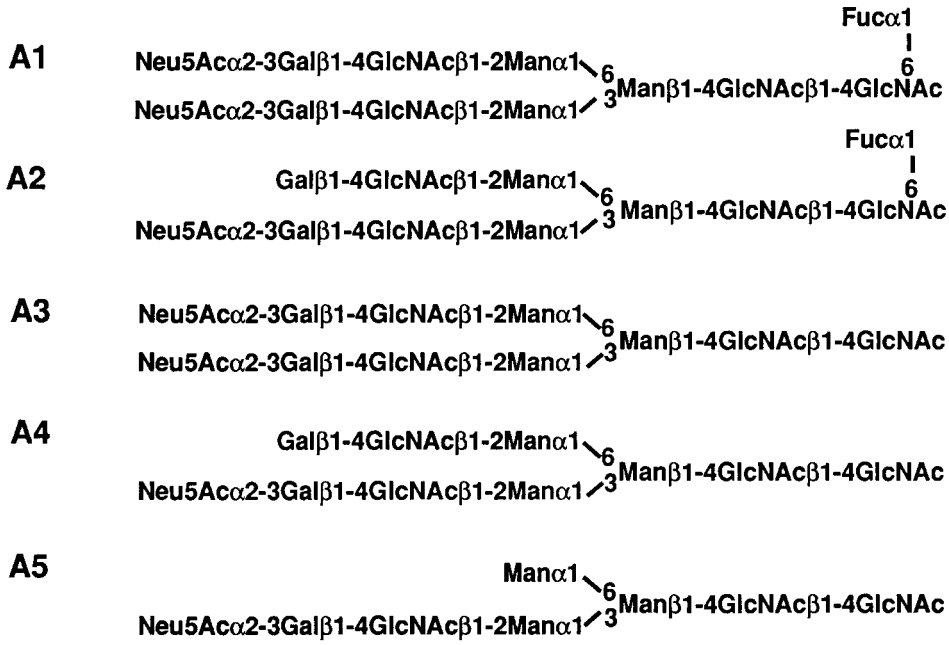


Figure 7. Structures of the *N*-linked sugar chains found in the hCG sample purified from the urine of pregnant women.

Occurrence of particular amino acid sequence to be recognized by the kinase was suggested later [88].

From the standpoint of the functional role of the sugar chains, localization of a specific sugar chain at definite loci of a glycoprotein is of particular interest, because it indicates the possibility that each sugar chain in a glycoprotein may be playing a different role, such as a recognition signal.

Organ- and species specific *N*-glycosylation

By comparative studies of the sugar patterns of the same glycoproteins produced in various organs of an animal, and those produced in the same organ of different animals, occurrence of organ- and species-specific *N*-glycosylation were found. The most clear examples of these phenomena were obtained by the studies of the sugar chains of γ-glutamyltranspeptidases (γ-GTPs), which contain only *N*-linked sugar chains [89–94]. This enzyme occurs as a membrane-integrated glycoprotein of the epithelial cells of various organs in all mammals. In Figure 8, structures of the major sugar chains of γ-GTPs purified from kidneys and livers of various mammals are shown. The data clearly indicated the occurrence of both organ- and species-specific *N*-glycosylation of glycoproteins. 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 liver glycoproteins contain bisected sugar chain, indicated that expression of GnT-III is suppressed by hepatocyte differentiation. In contrast, the enzyme is strongly expressed in the kidney cells of all mammals. This finding was supported by the later molecular biological studies of GnT-III [95].

Afterwards, another example of species-specific *N*-glycosylation was shown in rhodopsin [96–100]. As shown in Figure 9, various *N*-linked sugar chains were found to occur in the rhodopsin obtained from the eyes of various animals. An interesting observation is that all these sugar chains are to be classified as hybrid-type subgroup containing the hexasaccharide as enclosed by dotted lines in Figure 9. This evidence may indicate that GnT-II is not expressed in the retina of various animals.

Presence of species- and organ-specific differences in the glycosylation of proteins created many problems in the production of glycoproteins by recombinant techniques. Comparative study of the *N*-linked sugar chains of natural human interferon-β1 (IFN-β1) and three recombinant IFN-β1's produced by different mammalian cell lines transfected with the gene coding for human IFN-β1 revealed that they all contain the same number of complex-type sugar chains. However, their sugar patterns were different [101]. The differences occur both in the antennary structures, and in the structures of outer chain moieties. Organ-specific and species-specific expression of the sets of glycosyltransferases must be the enzymatic basis of these interesting phenomena. Another factor, transformational change in the expression of glycosyltransferases, as will be described in the next section, should also be considered as the background of this phenomena, because most host cell lines used for the production of recombinant glycoproteins are tumor cells.

Malignant alteration of *N*-glycosylation

It has been known from early '70s that altered glycosylation occurs in the glycoproteins produced by tumor cells. This information was obtained by using various indirect methods, such as monosaccharide analysis, comparison of the sizes of the glycopeptides obtained after exhaustive pronase digestion, and the study of the reactivities of glycoproteins with several lectins. This phenomenon was considered to be very important for the diagnosis of tumors, because the sugar chains of glycoproteins on the surface of cells, and in the intercellular matrices were found to play important roles in controlling the social behavior of cells in multi-cellular organisms. Alteration in the carbohydrate structures of glycoproteins found in various tumors were, therefore, considered to be the basis of the abnormal social behavior of tumor cells, such as invasion into the surrounding tissues and metastasis.

Discovery by Robbins' group [102] of the 'Warren-Glick' phenomenon, the expression of large *N*-linked sugar chains on the surface of malignant cells, should be considered as the first solid evidence to have indicated the malignant alteration of the *N*-linked sugar chains of glycoproteins. Extensive analyses have been performed to elucidate the molecular basis of 'Warren-Glick' phenomenon [103–107]. By analyzing the behavior of glycopeptides, obtained by exhaustive pronase digestion of the plasma membrane of normal and malignant cells on a Con A-Sepharose column, Ogata *et al.* [78] proposed that the molecular basis of 'Warren-Glick' phenomenon is the increase of tri- and tetraantennary complex-type *N*-linked sugar chains in malignant cells. This finding was confirmed by the structural study of the radioactive oligosaccharides released from the glycoproteins mixture by hydrazinolysis [108]. Oligosaccharides released from the glycoproteins of polyoma (Py) transformant of baby hamster kidney (BHK) cells were more enriched in the complex type sugar chains containing the Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ1-2)Man group as well as the elongated outer chains, than those from BHK cells.

Increase of the complex-type sugar chains with the 2,6-branched outer chain and of those with the elongated outer chains was also found to occur in RSV transformed BHK cells [109]. Therefore, increase of these sugar chains might well be considered as the molecular basis of 'Warren-Glick' phenomenon.

By comparative study of the six GnTs in the homogenates of BHK and Py-BHK cells [110], it was found that GnT-V activity was increased approximately three-fold in Py-BHK cells. In contrast, no changes in levels of GnT-I, -II, and -IV, and GnT-VII, which are responsible for the extention of outer chain moieties, were detected in both cells. No GnT-III activity was detected in the two cell types. This result agreed with the fact that no bisected *N*-linked sugar chain was detected in the

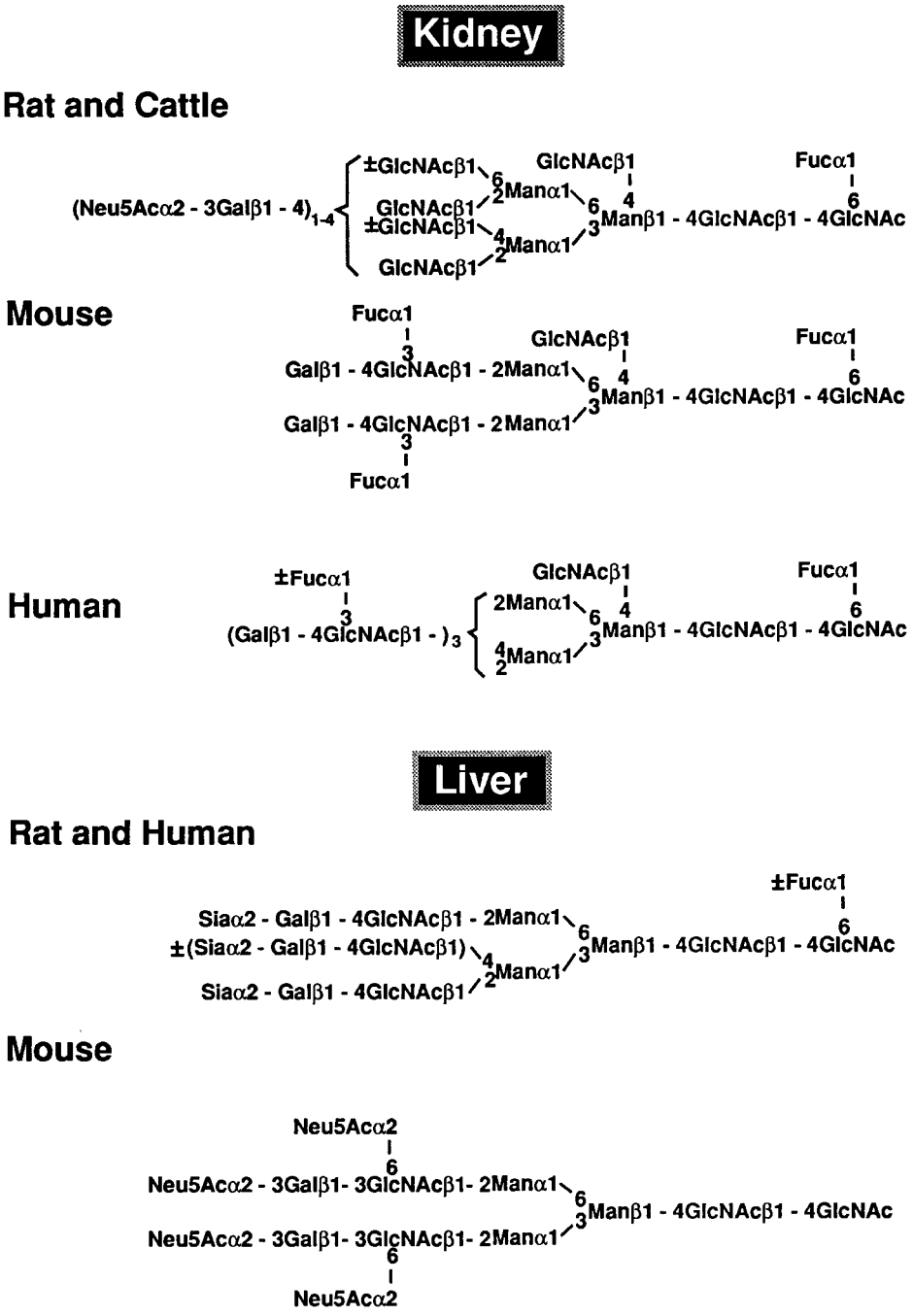


Figure 8. Major sugar chain structures of γ-GTPs from the kidney and the liver of various mammals.

membrane glycoproteins of both cells. Since it was reported that GnT-VII works most favorably on the sugar chains containing the Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ1-2)Manα1-6 group [111], increase of the sugar chains with elongated outer chains can also be explained by the increase of GnT-V activity in Py-BHK cells. For further development of studies related to the 'Warren-Glick' phenomenon, please consult a review [112] published previously.

More clear-cut data of altered *N*-glycosylation in the glycoproteins produced by tumor cells were obtained from the studies of hCG. High levels of hCG are also detected in the blood and the urine of patients with a variety of trophoblastic diseases as well as pregnant women. Therefore, urinary and serum hCG levels have been used as a useful marker for the diagnosis and prognosis of trophoblastic diseases as well as normal pregnancy [113]. Many sensitive methods to determine

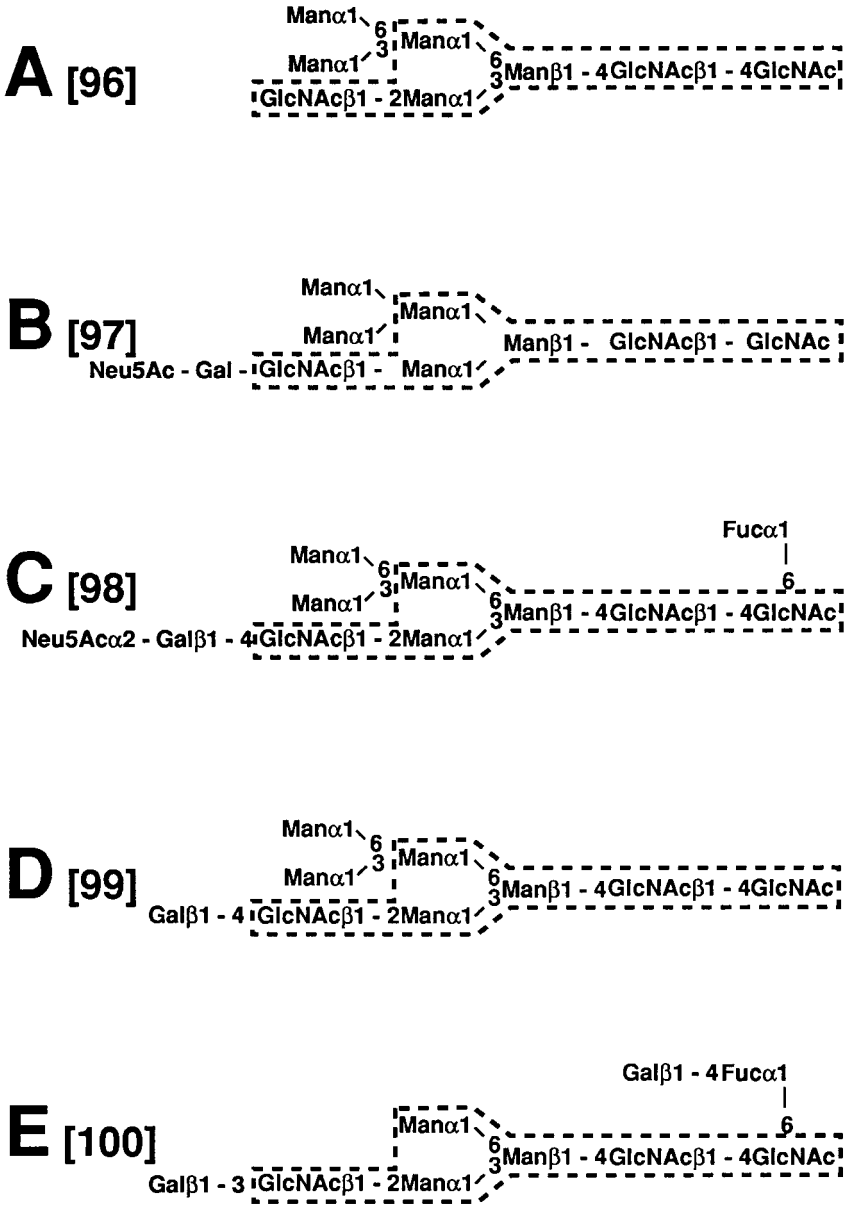


Figure 9. Major sugar chain structures of rhodopsin from various animals. A, cattle; B, frog; C, human; D, rat; E, octopus. Numbers in the brackets indicate references. The hexasaccharides enclosed by dotted lines are the common structures found in all rhodopsin molecules.

the level of hCG in the body fluids have been developed. Although these methods have solved the problems to eliminate interfering materials coexisting with hCG in the serum and the urine, none of them provided a way to discriminate hCGs from various trophoblastic diseases.

By comparative studies of the oligosaccharides, released from hCGs purified from the urine of pregnant women and patients with trophoblastic diseases, we have revealed the whole feature of the altered *N*-glycosylation of hCG in malignant cells [114–116]. Among various complex-type sugar chains detected in the malignant hCGs, expression of the abnormal biantennary sugar chains, in which two outer chains are linked to the Manα1-3 residue of the trimannosyl core (Figure 10), was of particular interest, because they could not be detected in normal glycoproteins. A novel lectin column chromatography to effectively discriminate the hCGs of malignant cells from those of normal cells was developed [117]. More details of this line of study, together with the malignant alteration of *O*-linked sugar chains of hCG, were reviewed recently [118].

As already described, the glycoproteins produced by normal liver of mammals do not contain bisected sugar chains, because GnT-III is not expressed in mammalian hepatocytes. Ectopic expression of bisected sugar chains in hepatoma was found by the comparative study of the *N*-linked sugar chains of γ-GTP, purified from rat liver and rat AH-66 hepatoma [93].

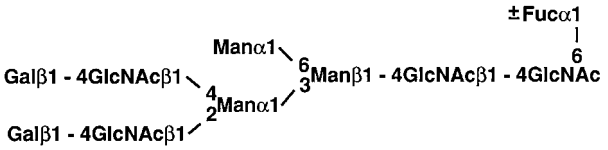


Figure 10. Structures of the abnormal biantennary complex-type sugar chains.

However, this interesting finding could not be applied for the diagnosis of human hepatoma, because the expression of bisected sugar chains was not prominent in the γ-GTP produced by human hepatoma [94]. This is a good example indicating that some of the altered glycosylation of proteins produced by tumor cells are species-specific. The γ-GTP, purified from human hepatoma, was found to be more enriched in the tri- and tetra-antennary sugar chains, indicating the expression of 'Warren-Glick' phenomenon in this glycoprotein. Expression of the abnormal biantennary sugar chains (Figure 10), found in the choriocarcinoma hCG, was also observed in this glycoprotein.

Comparative studies of the sugar chains of carcinoembryonic antigen (CEA) and its normal counterparts revealed a structural difference. The data could be used for the development of a novel technique to discriminate the malignant CEA from its normal counterparts. This interesting subject was reviewed previously [119].

Birth and quick development of glycopathology

Tom Rademacher brought several human immunoglobulin G (IgG) samples, when he came to Kobe University to learn hydrazinolysis. After he went back to Oxford, Tsuguo Mizuochi in my laboratory analyzed the oligosaccharide fractions, which Dr. Rademacher left behind. By that time, we already knew that the sugar patterns obtained by gel-permeation column chromatography are quite constant [120]. Mizuochi, however, found that for IgG the oligosaccharide pattern of one sample was different from others. We were intrigued by this result, and asked the Oxford group about the physiological background of the donor. It was found that the donor was a patient with rheumatoid arthritis. This was the beginning of the story of galactose-deficiency in the serum IgG molecule of patients with rheumatoid arthritis [121]. Since the details of the researches on this interesting phenomenon were reviewed previously [122,123], I will not repeat them here.

Various lysosomal exoglycosidase deficiencies have been known for a long time as congenital diseases related to the abnormal turnover of the sugar chains of glycoconjugates [124]. However, finding a galactose deficiency in the *N*-linked sugar chains of serum IgG from patients with rheumatoid arthritis [121], and the malignant alteration of the *N*-linked sugar chains, as was described in the previous section, opened a new world for studying the diseases caused by abnormalities in the sugar chains. A distinguishing trait of this newly expanding field is that the diseases are related to abnormalities in the biosynthesis of the sugar chains. Accumulation of our knowledge in the biosynthesis and the functional roles of the sugar chains of glycoproteins, and the advance of methods to study the sugar chain structures, are contributing to the development of this new genre, which might be called glycopathology.

As described in the previous section, comparative studies of the sugar chains of glycoproteins produced in various tumors and their normal counterparts indicated that the malignant alteration in the sugar chains is quite variable. However, it is also true that a quite reproducible alteration was induced in a particular glycoprotein produced by a particular tumor. Therefore, it is possible to develop novel methods for the diagnosis and prognosis of various tumors by investigating the malignant alteration of the sugar chains of their glycoproteins. In relation to this, I would like to emphasize here that many tumor markers, so far reported, are glycoproteins. As exemplified by the study of hCG, search for the tumor specific alteration in their sugar chains may reveal the evidence useful for increasing the values of these tumor markers.

As in the case of the abnormal biantennary sugar chains, abnormal sugar chains produced by cancer cells can include those which have never been detected in normal human glycoproteins. Detection of the abnormal biantennary sugar chains (Figure 10) in γ-GTP from human hepatoma [94] and CEA [125] as well as hCG, suggested that some of these alterations may act as new antigens induced in many tumors. Therefore, elucidation of the mechanism to prevent formation of the abnormal biantennary sugar chains in normal cells, and its depression in tumor cells, is expected to be an interesting target for future study.

Many studies, which will contribute to the development of glycopathology, have been reported. These include genetic disorders caused by the defect in *N*-linked sugar chain formation, such as HEMPAS [126] and carbohydrate-deficiency glycoprotein syndrome [127]. Wiskott-Aldorich syndrome was found to be related to the abnormality of the *O*-linked sugar chains [128]. Recently, leukocyte adhesion deficiency Type II (LAD II), which was discovered by Etzioni and Frydman [129], was found to be a general fucose deficiency. The LAD II patients lack ABO-blood groups containing the common Fucα1-2Gal group. These patients are also Le^a and Le^b negative indicating that they cannot form the Fucα1-4GlcNAc group [129,130]. Since they cannot form sialyl-Le^x, and other fucosylated oligosaccharide groups, which are the important signals for recruitment of neutrophils to sites of inflammation, and lymphocyte recirculation [131–135], the deficiency causes disease. It was assumed that a step in fucose metabolism is defective in LAD II patients [130]. Sturla *et al.* found that the conversion of GDP-mannose to GDP-fucose is impaired in LAD II patients due to the inactivity of GDP-D-mannose-4,6-dehydratase [136]. Quite recently, Lübke *et al.* reported that their LAD II patients cannot add a fucose residue to the sugar chains because they

lack GDP-Fuc transporter [137]. Therefore, LAD II should be considered as a syndrome. Because of the complex nature of biosynthetic mechanisms for the formation of sugar chains of glycoproteins, many other diseases are expected to be found in the near future.

In contrast to proteins, sugar chains are not formed by the direct transfer of the information stored in genes, but are formed by the concerted action of glycosyltransferases coded by their structural genes. Therefore, the structures of the sugar chains of glycoproteins are apt to be modified by changes in the physiological condition of cells.

Novel sugar chain structures

Recently, several novel sugar chains, containing the GIc-Ser [138] and the Fuc-Ser/Thr [139] groups at their linkage regions, were detected. However, no functional evidence is reported for these sugar chains, except for *O*-GlcNAc found by Hart's group [140]*[1].

Recent finding of a galactose cap on the fucosyl residue linked to the trimannosyl core of the hybrid-type sugar chain of octopus rhodopsin [100], as shown in Figure 9, indicated that some *N*-linked sugar chains are not limited to the structural rules described in the previous section. Cloning of the β-galactosyltransferase to form the Galβ1-6Fuc group could be important, because such enzyme will become an interesting tool for glycobiology by converting the *N*-linked sugar chains of various glycoproteins with the fucosylated trimannosyl core to abnormal β-galactosylated ones.

Our recent studies of the sugar chains of α-dystroglycan, as will be described in the next section, indicate that a novel minor group of sugar chains, which do not fall into the *N*-linked and *O*-linked sugar chains, plays an important role in organogenesis.

The functional role of the sugar chains of α-dystroglycan in the peripheral nerve

In 1993, I retired from the University of Tokyo, and moved to the Tokyo Metropolitan Institute of Gerontology (TMIG) as the Director. That the sugar chain structures of some glycoproteins could be modified by aging was shown by the finding that the galactose content of human serum IgG decreases in the aged persons [141,142]. It was reported that galactose deficiency decreases the complement binding potential of IgG [143]. Hence the alteration induced in the sugar chains of IgG can partly explain the phenomenon of immunodeficiency observed in aged persons.

Dementia is one of the most important targets of aging research because it severely lowers the quality of life of an aged person. By pathological study of the brains of patients with Alzheimer's disease, deposition of β-amyloid was observed as a highly correlated event with the disease. Structural study of β-amyloid [144] and subsequent cloning of the gene, producing its polypeptide portion, revealed that a protein called amyloid precursor protein (APP) is a precursor of β-amyloid [145]. Since a large amount of APP is also produced in a healthy brain, elucidation of the mechanism to induce an abnormal cleavage of APP, which leads to the production of β-amyloid, is considered as a key step in this line of study. In view of the fact that APP is a glycoprotein, study of its sugar chains and age related alteration of their structures could be important.

Because the biosynthesis of sugar chains is not controlled by the translation of a template, the structures of sugar chains are much less rigidly defined than those of proteins and nucleic acids. Accordingly, age related alteration of the sugar chains of various glycoproteins is an important target to solve various pathological problems found in elderly individuals. Based on such idea, I founded the Department of Glycobiology in TMIG, and installed Tamao Endo, who was my research associate in the University of Tokyo, as the chief of the Department. Although the history of this Department is short, it already issued several important results on the functional aspects of the sugar chains of glycoproteins in brain and nervous system. I would like to introduce one of them in the following part of this section.

Linkages between the extracellular matrix and the cytoskeleton play key roles in forming and maintaining specialized membrane domains, ensuring the structural integrity of the plasma membrane, attaching cells to the extracellular matrix, and also supporting cell signaling mechanisms.

Dystroglycan-complex is a membrane integrated glycoprotein, that plays an important role as such a linking molecule. It is a heterodimer composed of two glycoproteins: α-dystroglycan and β-dystroglycan [146]. α-Dystroglycan is a highly glycosylated extrinsic peripheral membrane protein that binds the basal lamina elements such as agrin and laminins. β-Dystroglycan is a transmembrane protein whose cytoplasmic domain binds cytoskeletal proteins such as dystrophin detected in skeletal muscle cells, and utrophin or Dp116 found in Schwann cells [147].

That α- and β-dystroglycans are encoded by a single gene, and cleaved into the two proteins by post-translational processing was confirmed by cloning the gene. In 1992, lbraghimov-Beskrovnaya *et al.* [148] reported the primary sequence of dystroglycan based on the data of cDNA cloning from Igt11 cDNA expression library obtained from rabbit skeletal muscle (Figure 11). A polypeptide composed of 895 amino acids is produced from the 5.8 K base mRNA. The polypeptide is then cleaved at the Gly_{653} and Ser_{654} region, resulting in the formation of α- and β-dystroglycans.

Much experimental evidence suggested that the interaction of dystroglycan with laminin in the extracellular matrices is important in peripheral myelinogenesis [149–151]. In order to elucidate the molecular basis of this interesting phenomenon, Yamada *et al*. [152] have investigated in detail the dystroglycan–laminin interaction in peripheral nerves.

By confocal laser scanning microscopic analysis of rabbit sciatic nerve samples, which were stained by various

monoclonal antibodies, it was found that α- and β-dystroglycans and laminins αl chain and β1 chain were localized surrounding the outermost layer of myelin sheath of nerve fibers. On the other hand, immunoreactivities for utrophin and Dp116, a dystrophin gene product specific to peripheral nerve, resembled that for Schwann cell marker S-100 and appeared to be localized in the Schwann cell cytoplasm [152].

The amino acid sequence of dystroglycan-complex indicated that it contains many potential glycosylation sites. Blotting studies using peroxidase conjugated lectins indicated

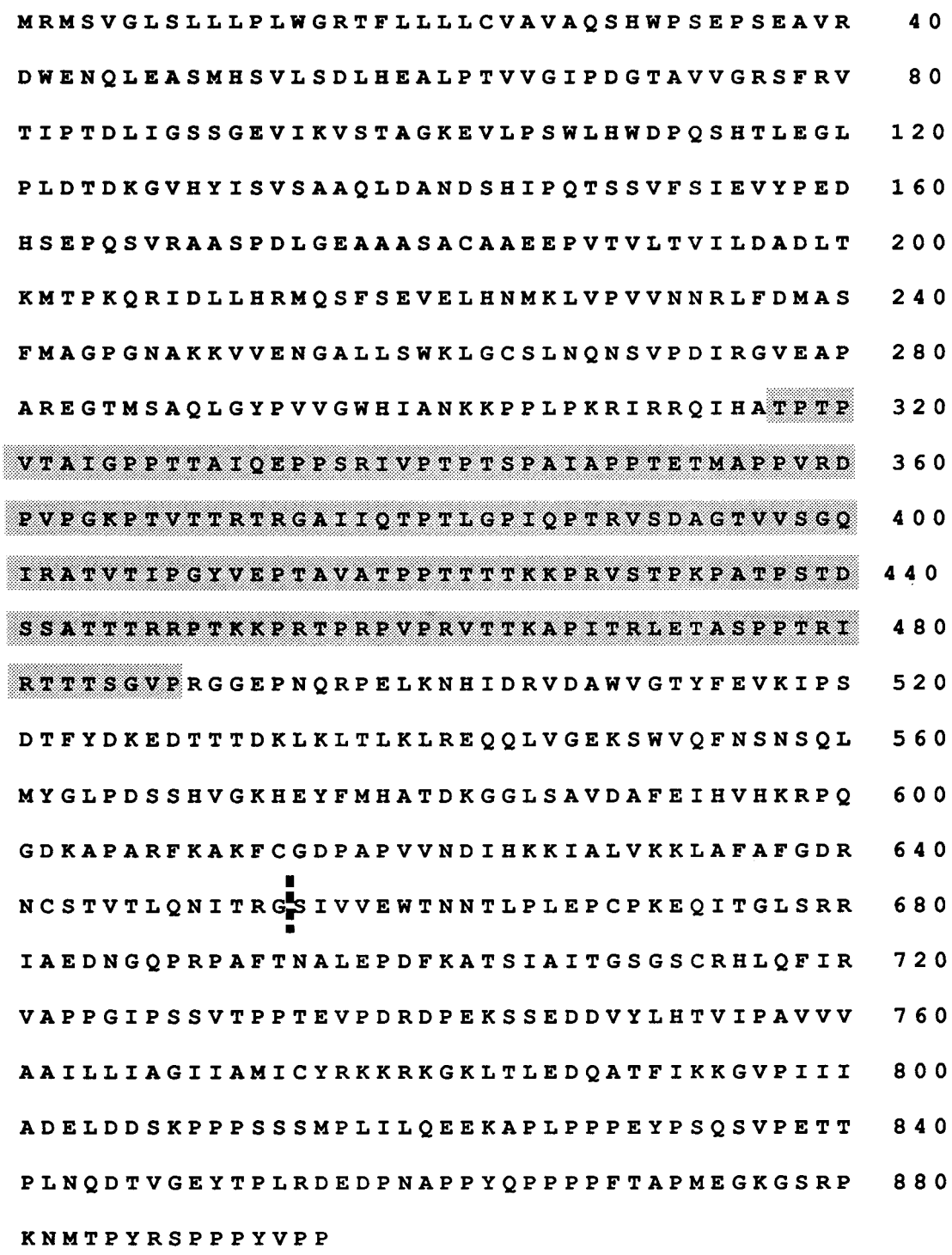


Figure 11. Amino acid sequence of bovine dystroglycan complex [148]. Polypeptide portion covered by a grey zone is the area, where clusters of *O*-linked sugar chains are supposed to be linked. The dotted line indicate the position, which is cleaved to form α- and β-dystroglycans.

that α-dystroglycan contains various types of sugar chains. In order to find out if these carbohydrate residues are involved in laminin binding, the effects of glycosidase digestion of α-dystroglycan on its laminin binding capacity were investigated by using blot-overlay assay.

α-Dystroglycan on a nitrocellulose membrane was positively stained with ^{125}I-laminin-1 and ^{125}I-laminin-2. Treatment of α-dystroglycan with *Arthrobacter ureafaciens* sialidase greatly reduced the binding to laminins. The binding to laminins was also diminished by treatment of a nitrocellulose transfer of α-dystroglycan with Newcastle disease virus sialidase. Neu5Ac, NeuGc, and colominic acid inhibited the binding of laminin. However, addition of GlcNAc or NaCl did not inhibit the binding.

By investigating bovine peripheral nerve α-dystroglycan, Chiba *et al*. [153] found that a cluster of sialylated *O*-linked sugar chains of the α-dystroglycan is responsible for its specific binding to laminin. Structural study of the *O*-linked oligosaccharides, released by either mild hydrazinolysis [154] or alkaline borotritide treatment, revealed that the Neu5Ac(and NeuGc)α2-3Galβ1-4GlcNAcβ1-2Man groups linked to the serine and threonine residues, possibly clustered between the 317th and the 488th amino acid residues (Figure 11), specifically bind to the laminin molecule [153]. Haptenic inhibition studies indicated that the interaction between laminin and sialylated oligosaccharides of α-dystroglycan is not simply dependent on the anionic charge of sialic acid residues, but on the structure comprising the neutral sugar portion and the sialic acid linkage.

Prospects for the future studies of sugar chains

Based on the strategy to perform reliable structural studies of the *N*-linked sugar chains of glycoproteins as described in this review, many cutting edge methods were reported later.

Finding and the successful purification of endo-β-*N*-acetylglucosaminidase M [155] and amidase (glycopeptidase A) [156] afforded enzymatic methods to cleave most of the *N*-linked sugar chains of glycoproteins. Development of several fluorescent tags-labelling of sugar chains [157,158] also contributed to this line of study.

It is obvious that development of auto-sequencers contributed tremendously for the expansion of molecular biology in the field of DNA and proteins research. Therefore, development of auto-sequencer for the analysis of the sugar chains will be essential for the future expansion of the glycobiology. However, it is not so easy to develop such a machine, because of the inherent complexity of the sugar chains, described already. Our results for the use of serial affinity chromatography with immobilized lectin columns [80] indicated that the structure of more than 80% of the radioactive oligosaccharides mixtures, obtained by hydrazinolysis of most glycoproteins, could be elucidated by choosing an appropriate set of immobilized lectin columns followed by Bio-gel P-4 column chromatography. Therefore, this method would be most suitable for the development of the sugar chain autoanalyzer in the future.

At the Glyco XV, held in Tokyo last year, an important report was presented by Hirabayashi and Kasai [159]. Based on the elucidation of the whole genome of *Caenorhabditis elegans*, they proposed a "glycome*2" project of this organism. In their proposal, they planned to analyze the whole range of glycopeptides, which will be obtained from the glycoprotein mixture of *C. elegans* by digestion with *Achromobacter* lysine-specific protease. For the analysis of glycopeptides, they plan to use immobilized lectin columns followed by reversed-phase chromatography. They expected that glycopeptides will be categorized into several structural glycans groups based on the binding specificity of each lectin column. They then plan to elucidate the structure of each glycopeptide by peptide sequencing, mass spectrometry, and frontal affinity chromatography developed by them. Although this procedure seems to be tedious, I consider, from our own experience in using the serial lectin column chromatography for the studies of *N*-linked sugar chains of glycoproteins, that it can be performed rather easily after accumulating several basic key data. The most promising point of their proposal is that the structural information of each glycopeptide can be linked to the *C. elegans* genome data base. In view of the recent important findings, that the three proteins involved in *C. elegans* vulval invagination similar in their glycosylation components [160], this proposal could afford a revolutionary development for the study of the glycobiology of this organism.

Many participants at Glyco XV obtained much useful information. Especially, we noticed that glycobiology has been making big development 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 [161,162].

For the purpose of investigating the alteration of *N*-linked sugar chains by ageing, Sato *et al.* [163] analyzed comparatively the glycoproteins in the soluble fractions and the membrane fractions of various portions of brains and spinal cords, obtained from 9 weeks-old rats and 29 months-old rats, by sodium dodecylsulfate-polyacrylamide gel electrophoresis. By staining with lectins, selected to detect representative groups of *N*-linked sugar chains, many alterations of the sugar moieties by the age of donors were found. The most prominent difference was detected by staining with *Lens culinaris* agglutinin (LCA) of the membrane glycoprotein fraction of the spinal cord. A 30 KDa band (gp30) was detected in the membranes of the spinal cords of aged rats, but not in those of young-adult rats. Based on the amino acid sequence of the glycopeptide obtained from gp30 by lysylendopeptidase digestion, gp30 was identified as P_0. This glycoprotein had been recognized as a member of immunoglobulin superfamily, which occurs as a major component in the mammalian peripheral nerve [164]. We were interested in this finding, because it had been widely accepted that P_0 does not occur in

the central nervous system [165–167]. Further investigation revealed more important evidence that P_0 also occurs in the spinal cords of young adult rats in a non-glycosylated form [164]. In view of the reports indicating the important role of the sugar chains of P_0 in the homophilic binding of this glycoprotein [168], which is essential for normal spiraling, compaction and maintenance of the peripheral myelin sheath and continued integrity of associated axons [169], further studies on the function of P_0 in the spinal cord and its age related glycosylation may unravel a new field of glycobiology in the brain nervous system.

The sugar chains, which are linked to the polypeptide moiety through Man-Ser and Thr linkages, as described for the α-dystroglycan in the previous section, are found widely in yeast, but are extremely rare in mammals. The only paper reporting the occurrence of this group of sugar chains was that of Krusius *et al.* [170], indicating their presence in rat brain proteoglycans. Quite recently, Yuen *et al.* [171] reported that the HNK-1 epitope (the SO_4-3GlcAβ1-3Galβ1-4GlcNAc group) in rat brain is located on the sugar chains, which are linked to polypeptides through Man-Ser or Thr linkages. It was also found that the Le^x determinant detected in cranin, purified from sheep brain, resides in the sugar chains starting by *O*-linked mannose [172]. Therefore, finding such unusual sugar chains as an important signal in the function of peripheral nerve dystroglycan may indicate that various unusual sugar chains occur as minor components in mammals and play important roles in particular tissues.

In contrast to the complex type sugar chains, variation of the *O*-linked sugar chains found in the α-dystroglycan are formed by the arrangement of Ser and Thr residues in a polypeptide chain, together with the structural variation of the sugar chains linked to the mannose moiety. Therefore, the sugar chain signal will be expanded tremendously by the addition of this new group.

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Notes

*1) After this manuscript was submitted, a series of fascinating papers related to the function of the Fucα1-Ser/Thr groups was reported [174-177].

Inductive signalling through the Notch receptors was shown to play a central role in cell-fate decisions in metazoans during organogenesis and morphogenesis. It has been known that a molecule named Fringe controls the binding of Notch receptors to various ligands, such as Delta and Serrate/Jagged protein families.

Many data were presented indicating that Fringe is an *β-N*-acetylglucosaminyltransferase and modifies the affinities of Notch receptors to the ligands by elongating the Fucα1-Ser/Thr/Hyl groups, which are present in the EGF-like domains at the extracellular portion of Notch, to the Siaα2-3Galβ1-4GlcNAcβ1-3Fucα1-Ser/Thr/Hyl groups. The enzyme is highly specific to the fucosyl residue and does not transfer an *N*-acetylglucosamine residue onto other acceptor sugars.

This series of works presented the first example of how an important signalling pathway can be modulated by differential receptor glycosylation.

A *β-N*-acetylglucosaminyltransferase, which converts Man1-Ser/Thr residues of dystroglycan to GlcNAcβ1-2Man1-Ser/Thr residues was found recently [178]. This enzyme is also strictly specific to the Man-Ser/Thr groups, and could not work on other acceptor sugars. These results may indicate that cell recognitions through sugar chain signal are widely working as essential processes in the multi-cellular organisms.

*2) The name Glycome was originally proposed by Vernon Reinhold.

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