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SPATIAL STRUCTURES OF GLYCAN CHAINS OF GLYCOPROTEINS IN RELATION TO METABOLISM AND FUNCTION. SURVEY OF A DECADE OF RESEARCH

Jean Montreuil

Université des Sciences et Techniques de Lille I, Laboratoire de Chimie Biologique et Laboratoire Associé au C.N.R.S. n° 217, 59655 - Villeneuve d'Ascq Cédex, France

Abstract - Thanks to the progress in chemical and enzymatic methodologies, and to the use of high-resolution NMR spectroscopy the primary structure of hundreds of glycoprotein glycans has been determined. The knowledge acquired in this field has been decisive in the elucidation of the mechanisms of glycoprotein biosynthesis and of their regulation, and in a better understanding of their biological roles. In this connection, the determination of the spatial configuration became a necessity. Ten years ago, future results were anticipated by building the Y-shaped molecular model of a biantennary glycan. Little by little, this structure has been refined and modified thanks to experimental data obtained by using physical methods : X-ray diffraction, EPR, NMR, including two-dimensional NMR and one-dimensional ^{1}H-nuclear Overhauser effect (NOE) experiments, neutron scattering and hard-sphere *exo*-anomeric (HSEA) calculations. So, the concept evolved successively from the Y-, to the T-, the bird- and the "broken wing"- conformation, until the demonstration that these conformers are interconvertible. The bird-conformation as well as the concept of the mobility of antennae are in a good agreement with the reactivity of lectins, including membrane lectins, by rendering accessible any specific sugar structure, and with the activity of glycosyltransferases by making attainable the substituable hydroxyl groups even in the case of pentaantennary structures. In the same way, we know now that the tetraantennary glycans adopt an "umbrella conformation" in which the four antennae are disposed parallely to the protein surface and act as protective shields. So could be explained the resistance towards proteases and the weak antigenicity of numerous glycoproteins as well as the peculiar behaviour and resistance of metastatic cancerous cells since it has been recently demonstrated that membrane glycoproteins and fibronectin of this kind of cells are significantly enriched in tri- and tetraantennary glycans.

INTRODUCTION

Ten years ago, in a lecture delivered at the 7th International Symposium on Carbohydrate Chemistry organized at Bratislava by Stefan Bauer, I reviewed (Ref. 1) our knowledge concerning the primary structure, the metabolism and the biological roles of the carbohydrate moiety of glycoproteins. At that time, the situation was as follows : the primary structure of about a dozen of glycans had just been determined, the first informations about the biosynthesis and the catabolism of glycans of N-glycosylproteins were available and nothing was known of the spatial conformation of glycans. However, the concept of glycans as recognition signals was already firmly established thanks to the discovery by Ashwell's group of the membrane lectins.

Ten years later, it is of interest to draw the general features of our knowledge in the field of glycoprotein structure and conformation, and to look backward the way which has been followed during the past decade.

For a long time, carbohydrates have been considered as reserve substances, energy-storage compounds and support structures and regarded as entirely devoided of any "biological intelligence". But, during the last 15 years, the chemistry and biochemistry of glycoconjugates -which result from the covalent linkage of a sugar moiety called glycan with a protein (glycoprotein) or a lipid (glycolipid)- have acquired an importance as great as that of proteins and nucleic acid because of the tenacity and trust of a few scientists who were firmly convinced that the knowledge of the metabolism and of the molecular biology of glycoconjugates was based on the knowledge of the primary structure of glycans. Thanks to the improvement of chemical, physical and enzymic approaches they developed, the chemistry of glycoproteins progressed rapidly, leading to the determination of the primary structure of several hundreds of glycans which made possible the knowledge in detail of the mechanisms of glycan biosynthesis and catabolism (for reviews, see Ref. 2 to 18).

We have now crossed "the shores of the great unexplored continent" announced by A. Gottschalk in 1973 : that of the molecular biology of glycoproteins. We have now to understand the biological message the glycans carry and to answer two questions. First : why are proteins glycosylated ?. Second : how act the glycans *i*) in the protection of the peptide chain against proteolytic attack, *ii*) in the induction and maintenance of protein conformation, *iii*) in the decrease of immunogenicity of proteins, *iv*) in the mechanism of recognition and association with viruses, with enzymes during the glycan biosynthesis, and with lectins -particularly with membrane lectins, *v*) in the intercellular adhesion and recognition and in cell-contact inhibition ? Moreover, we know now that the glycan structure of the cell membrane glycoconjugates is profoundly altered in cancer cells, as revealed by the use of lectins. This molecular transformation could be related to the appearance of surface neo-antigens and could be a factor of cancer induction and metastatic diffusion.

These problems cannot be solved only on the basis of primary structure of the glycans but also on the basis of their spatial conformation. Unfortunately,up to the 1980's, our ignorance about this latter was immense and our knowledge was essentially restricted to the structure of the human IgG crystallisable Fc fragment explored by X-ray diffraction by Deisenhofer and Huber (Ref. 18 to 23). This situation was due to the difficulties encountered in obtaining crystals of glycoproteins and of glycans, the X-ray diffraction being at that time the absolute arm for determining the spatial conformation of molecules. However, in the last few years, our knowledge rapidly progressed thanks to the improvements of the organic synthesis of glycans which solved the problem of quantity and of purity of these compounds, on the one hand, and thanks to the development of the physical methodologies, in particular, of the n.m.r. techniques, on the other hand.

Thus, after a long period of slow development, the research on glycoconjugates suddenly explosed during the last decade, simultaneously in the fields of primary structure, spatial conformation, normal and pathological metabolism and molecular biology. In this connection, the following title of a review of Nathan Sharon and Halina Lis (Ref. 10) perfectly reflects the situation : "Glycoproteins : research booming on long-ignored, ubiquitous compounds".

CONFORMATION OF THE PROTEIN MOIETY

Our knowledge of the spatial conformation of glycoproteins as a whole is still too limited to draw general laws. However, fundamental informations have been brought about the configuration of the peptide chain around the glycan-amino acid linkage to which I shall focus this short review.

It is now well established that the existence of key peptide sequences called *sequons* (Ref. 24), around the glycan receptor amino-acid, is a prerequisite feature for glycosylation of a protein. For example, the tripeptide sequence Asn-X-Ser(Thr), where X can be almost any amino-acid, except proline, is the prerequisite for N-glycosylation of proteins. However, as sequons of certain peptide chains are sometimes not glycosylated, their presence is not a sufficient condition for glycosylation. Recently, a second requirement for carbohydrate attachment to proteins has been defined which is associated with a specific secondary structure of the polypeptide chain : the β-*turn*. In fact, by applying predictive methods (Ref. 25) to amino acid sequences adjacent to the glycosylated sites of numerous glycoproteins, Aubert and Loucheux-Lefebvre (Ref. 26 & 27) and Beeley (Ref. 28 & 29) demonstrated that glycans are located in amino-acid sequences favouring turn or loop or hairpin structures (Fig. 1). Most are in tetrapeptide β-turns. These turns consist of only 4 amino-acids enabling a polypeptide chain to reverse by nearly 180°, this conformation been maintained by a hydrogen bond between the CO function of the first amino-acid of the tetrapeptide and the NH function of the fourth amino-acid. Asparagine, serine and threonine are especially abundant in these regions.

Thus, the glycosylation by enzymes may be favoured as turn and loop conformations are generally located at the surface of globular proteins, making the receptor amino-acids readily accessible to glycosyltransferases (Ref. 30). In addition, proofs have been obtained that the carbohydrate moieties are located on the outside of glycoprotein molecules. This result is in a good agreement with the role of recognition signals played by numerous glycans. Moreover, it is possible that the protection against proteolytic attack may be due to the masking of turns and loops by the glycans acting as protective shields. The poor immunogenic activity often observed with glycosylated proteins could be also related to this masking. This opinion is reinforced by the molecular model given in Fig. 19.

However, it seems that the presence of the required sequons located in β-turns is not a sufficient condition for glycosylation. In fact, the knowledge of the complete primary peptide sequence of some glycoproteins leads to the conclusion that the double concept of the sequon and of the β-turn must be completed by an additional one, because certain glycoproteins possess residual unglycosylated sequons located in β-turns. For example, even though the human lactotransferrin peptide chain contains three Asn-X-Ser(Thr) sequons in

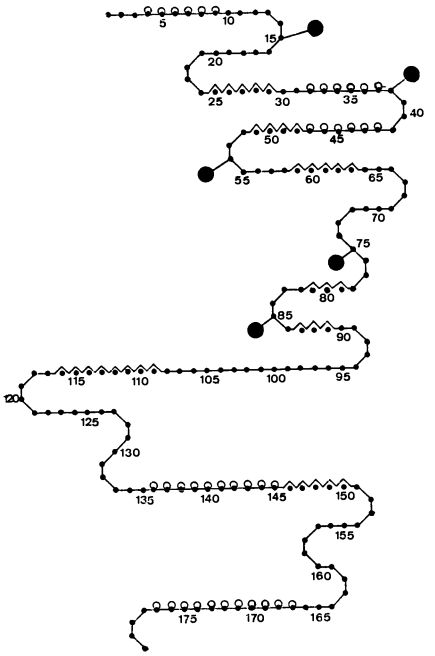

Fig. 1. Suggested secondary structure of α_1-acid glycoprotein (Ref. 26) according to the predictive method of Chou and Fasman (Ref. 25). Residues are represented in their respective conformational state : helical (o), β-sheet (⋀), coil (-), β-turn tetrapeptide ( ). Asn 15, 38, 54, 75 and 85 carry a carbohydrate moiety.

β-turns, only two asparagine residues (Asn 137 and Asn 488) are glycosylated (Ref. 31). Thus, at least a third condition must be envisaged. We hypothesize that the protein could carry specific peptide sequences recognized by and associating with the glycosyltransferases.

STRUCTURE OF GLYCOPROTEIN GLYCANS

The knowledge we now possess of the structure of numerous glycans raises a very exciting problem from a comparative biochemical point of view and entirely confirms the concepts I developed since 1962 (Ref. 1, 32 & 33). In fact, glycan structures are not randomly constructed. On the contrary, they are subject to laws the bases of which are found in the specificity of glycosyltransferases and in a conservative evolution of these enzymes. In this connection, the survey of all the glycan structures determined up to now shows that they may be divided into families, within each of which, structures are very similar and present common oligosaccharide sequences,whether they originate from animals, plants, microorganisms, or viruses. Consequently a series of classes, concepts and rules is now firmly established, including the sugar-protein linkage.

Nature of linkages between glycans and proteins

Glycans are conjugated to peptide chains through two types of primary covalent linkages : N-glycosyl and O-glycosyl linkages, leading to the definition of two classes of glycoproteins : N-glycosylproteins and O-glycosylproteins (Table 1).

TABLE 1. Classification of glycoproteins

Types of glycoproteins	Types of linkages	
	Monosaccharide	Amino acid
N-glycosylproteins	GlcNAc	Asn
O-glycosylproteins		
Mucin type	GalNAc	Ser,Thr
Proteoglycan type	Xyl	Ser
Collagen type	Gal	OH-Lys
Extensin type	ʃAra	OH-Pro

Until now, the only N-glycosidic bond characterized in glycoproteins is the N-acetylglucosaminyl-asparagine : GlcNAc(β1-N)Asn, which is considered as the ancestral linkage (Ref. 34). On the contrary, the O-glycosidic type offers a wide variety of linkages the most distributed ones being the following :

i - linkage between N-acetyl-D-galactosamine and L-serine or L-threonine : GalNAc(α-1,3)Ser or GalNAc(α-1,3)Thr, alkali-labile, found in very numerous glycoproteins which are said of the "mucin type" ;

ii - linkage between D-xylose and serine : Xyl(β1-3)Ser, also alkali-labile involved in the acidic mucopolysaccharide-protein bond of proteoglycans ;

iii - linkage between D-galactose and 5-hydroxy-L-lysine : Gal(β1-5)OH-Lys, alkali-stable, characterized in collagens ;

iv - linkage between L-arabofuranose and 4-hydroxy-L-proline : L-Ara(β1-4)OH-Pro, alkali-stable, identified in plant glycoproteins.

Primary structure of glycoprotein glycans

1. The concept of the common inner-core. The carbohydrate moiety of N- and O-glycosylproteins derive from the substitution of oligosaccharide structures common to all glycans of a given class of glycoproteins. These non-specific and invariant structures are conjugated to the peptide chain and constitute the *inner-core*. The most current inner-cores characterized until now in glycans are described in Fig. 2. Cores A and B exist in the O-glycosylproteins

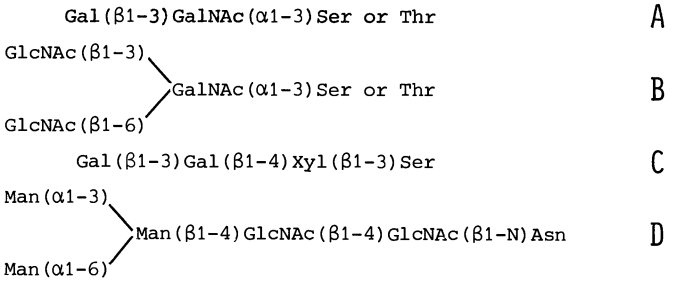

Fig. 2. Oligosaccharide inner-cores of glycoprotein glycans.

of the mucin-type. Core C constitutes the terminal sequence of almost all the glycosaminoglycans of proteoglycans. Core D is common to all N-glycosylproteins.

2. The concept of the antenna. The glycan structures derive from the substitution of the invariant inner-cores by a wide variety of oligosaccharidic structures which carry the specificity of the glycans and constitute the variable fraction of these latter. On the basis of their morphology, their flexibility and their property of being recognition signals the term *antennae* has been proposed, from 1974, for the outer arms substituting the inner-cores (Ref. 1).

3. Glycan structures present in O-glycosylproteins

Glycans conjugated through a Xyl(β1-3)Ser linkage. This group of glycans consists of the acid mucopolysaccharides or glycosaminoglycans. All are linear polymers made up of disaccharide repeating units (Fig. 3) and O-glycosidically linked to the peptide chain in the

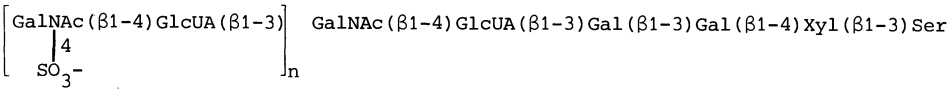

Fig. 3. Primary structure of seryl-chondroitin 4-sulfate.

so called proteoglycans, except in two cases : *i*) that of hyaluronic acid which does not seem to exist as a proteoglycan ; *ii*) that of keratan sulfate I which is conjugated to the protein through an N-acetylglucosaminyl-asparagine linkage.

Glycans conjugated through a GalNAc(α1-3)Ser or Thr linkage. This group mainly comprises structures consisting of mucins and these glycans are often designated as *mucin-like* structures. Some of these structures are described in Fig. 4. The main observation which could be formulated about these structures is the lack in structural specificity of numerous of them. In fact, the same structures can be found in glycoproteins of very different origins and roles. So, they could be regarded as structures playing essentially a role of a physicochemical order and intervening essentially in the conformation of the peptide chain. In this connection, it is worthwhile to note that this kind of glycans is often located in "strategic" domains of the protein moiety, for example, in the hinge region of immunoglobulins.

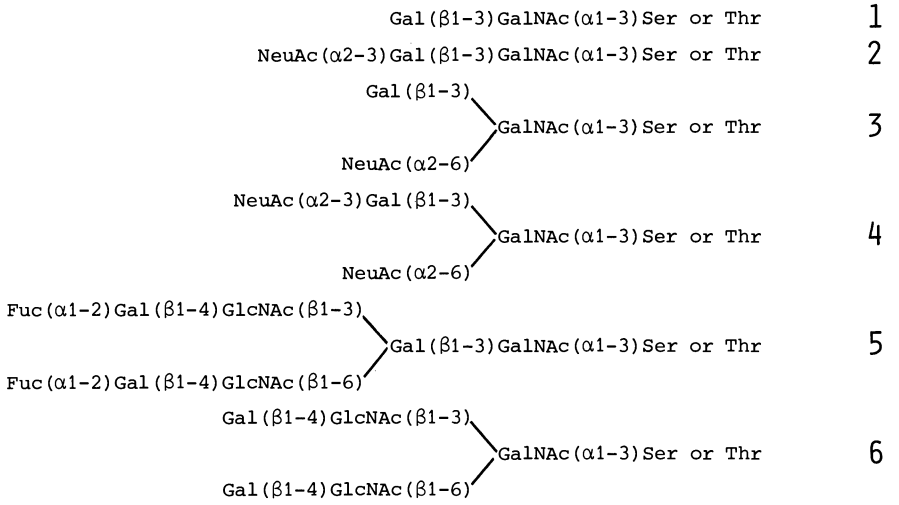

Fig. 4. Some examples of structures of glycans O-glycosidically conjugated to protein through the linkage GalNAc(α1-3)Ser or Thr. These glycans are distributed in the following glycoproteins : Structure 1 : anti-freeze glycoprotein from antarctic fish, human chorionic gonadotropin β-subunit, human serum IgA_1, epiglycanin of TA3-Ha cells, T-reactive erythrocytes, rat brain glycoproteins. Structure 2 : human glycophorin, bovine kappa-casein, fetuin, human milk secretory IgA, N-blood group. Structure 3 : submaxillary mucins, rat brain glycoproteins, trout and herring eggs. Structure 4 : human glycophorin and gonadotropin ß-subunit, fetuin, lymphocyte plasma membrane, M-blood group. Structure 5 : porcine blood-groop H substance. Structure 6 : bronchial mucin of patients suffering from cystic fibrosis. For reviews, see Ref. 9 & 14.

4. Glycan structures present in N-glycosylproteins. The N-glycosylproteins are divided into three families according to the nature of the carbohydrate linked to the pentasaccharidic inner-core D (Fig. 2). In the first family, the glycans contain mannose and N-acetylglucosamine only. They are called glycans of the *oligomannosidic type* or *high-mannose type* glycans. In the second family, the sugar composition of glycans is more complex. In fact, these glycans contain galactose, fucose and sialic acids in addition to mannose and N-acetylglucosamine. They derive fundamentally from the addition to the pentasaccharidic inner-core D of a variable number of N-acetyllactosamine residues : Gal(β1-4)GlcNAc. These structures have been called glycans of the *N-acetyllactosamine type* or of the *complex type*. In the third family, the glycans simultaneously have structures of the oligomannosidic and of the N-acetyllactosaminic type. They belong to the *oligomannosidic-N-acetyllactosaminic type* or *hybrid type* glycans.

Glycans of the oligomannosidic type (high-mannose type). The glycans of the oligomannosidic type constitute a very homogeneous group. In Fig. 5 are given the structures of the two

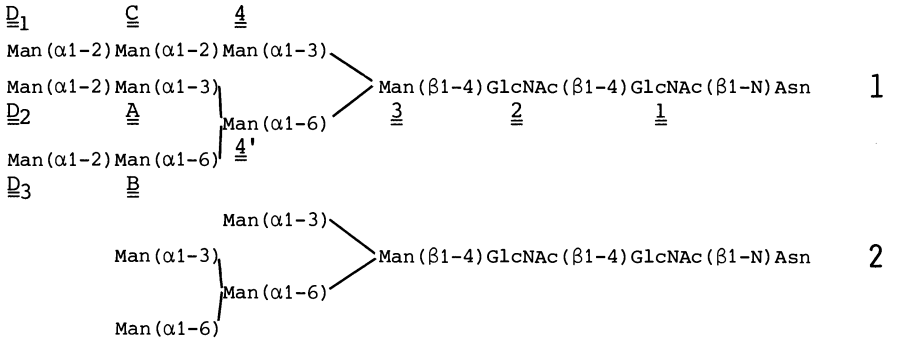

Fig. 5. Structure of glycans of the oligomannosidic type (high-mannose type). Structure 1 is present in calf thyroglobulin unit A, human IgD and myeloma IgM, Chinese hamster ovary cell glycoproteins, bovine lactotransferrin, soybean agglutinin, Newcastle virus, scorpion hemocyanin. Structure 2 has been found in Taka-amylase, in hen ovalbumin and in human myeloma IgM. For reviews, see Ref. 9 & 14.

glycans, the most widely distributed in plants and animals. These structures are common to numerous glycoproteins of different origins and roles and, thus, do not present any structural specificity. On the basis of the findings concerning the biosynthesis of the N-glycosidically linked glycans, they could be regarded as metabolic intermediates in the maturation of the glycans of the N-acetyllactosaminic type as shown in Fig. 6 (For reviews, see Ref. 7, 9, 10, 12, 14-16 & 35). However, this cannot be considered as their only role.

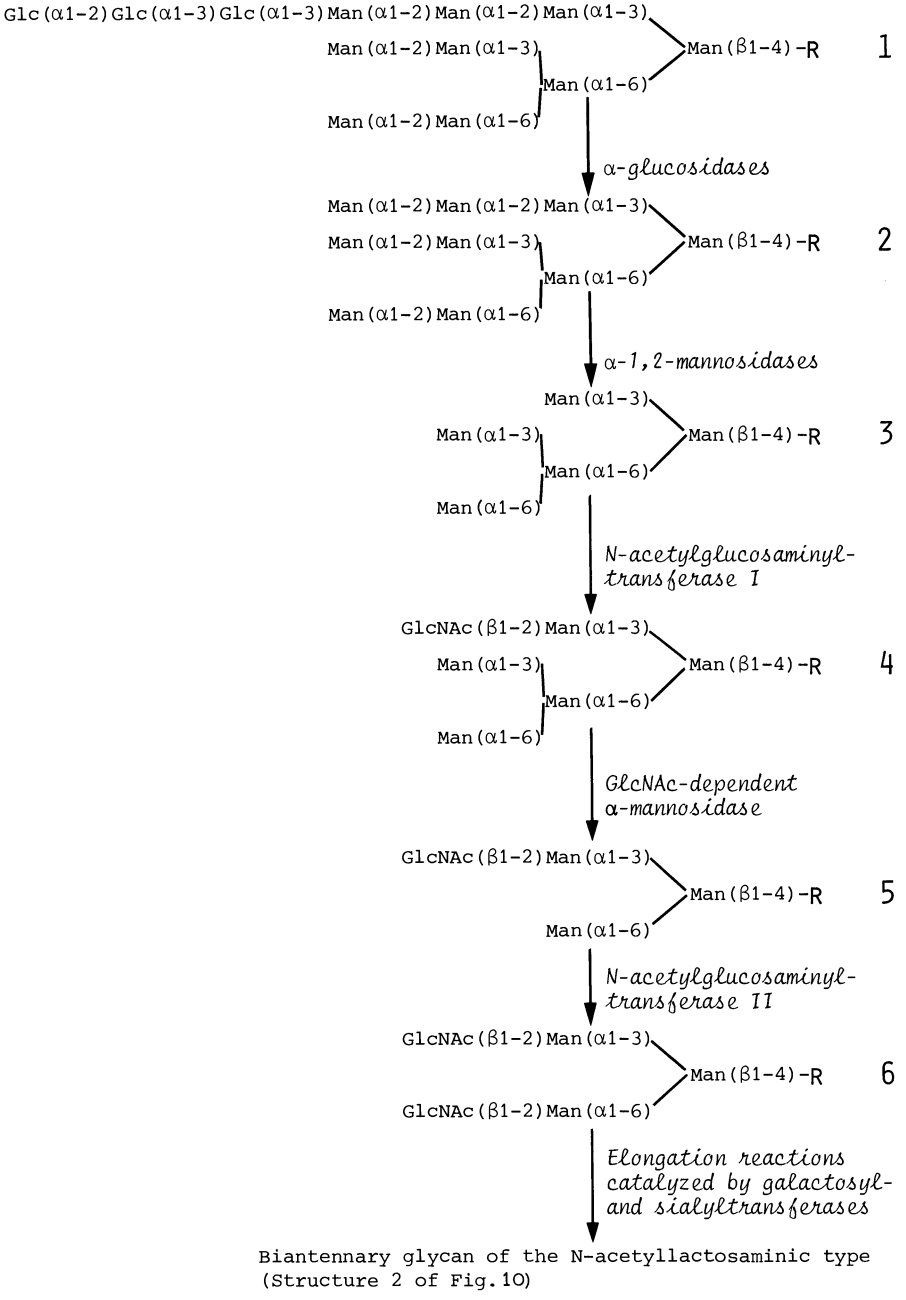

Fig. 6. Biosynthesis pathway of a glycan of the N-acetyllactosaminic type. R = GlcNAc(β1-4)GlcNAc(β1-N)Peptide. Glycan 1 is transferred en bloc from a dolichol pyrophosphate intermediate onto the nascent peptide chain in the granular endoplasmic reticulum. The newly formed glycoprotein is further trimmed in the Golgi apparatus by specific α-glucosidases and α-mannosidases leading to glycans of the oligomannosidic type : first to the glycan 2 and then to the glycan 3. This latter is the acceptor for the transfer of the N-acetylglucosaminyl residue on the α-1,3-linked mannose (glycan 4). The two α-1,3 and α-1,6 "extra" mannose residues are then cleaved off leading to the glycan 5 which is the acceptor of the N-acetyl-glucosaminyl residue on the α-1,6-linked mannose (glycan 6). The completion

of the chain is achieved by adding galactosyl, fucosyl and sialyl residues. If the maturation process stops at the level of glycans 2 or 3, the glycoprotein is of the oligomannosidic type (see structures A and B of Fig. 5). If it develops only on the α-1,3-Man branch of the glycan 4, the glycoprotein is of the hybrid type (see structures of Fig. 9).

In fact, some observations show that oligomannosidic structures act as recognition signals. For example, *Escherichia coli* binds to human epithelial cells, a prerequisite for colonization and infection, by means of a lectin present on the *pili* of the bacteria with a specificity for oligo- and polymannosides. Moreover, phosphorylated glycans of the oligomannosidic type target newly synthesized lysosomal enzymes to lysosomes by specific recognition of receptors present in Golgi and plasma membrane (For reviews, see Ref. 36 & 37).

Glycans of the N-acetyllactosaminic type (complex type). In contrast to the above described glycans, glycans of the N-acetyllactosaminic type present a wide variety of structures and this observation favours the hypothesis that they act essentially as recognition signals. In this connection, the structures described in Fig.10 underline the particular importance of two monosaccharides whose systematically external position designates them as recognition signal, i.e. sialic acids (reviews in Ref. 38 & 39) and fucose (review in Ref. 40).

Occasionally, the branchings are incomplete and the formation of the N-acetyllactosamine residues is only started in outline, as in the glycans of hen-ovotransferrin (Fig. 7),

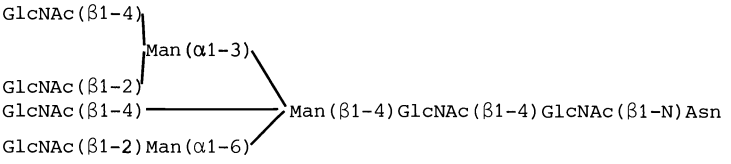

Fig. 7. Structure of hen ovotransferrin glycan.

probably due to a lack in galactosyltransferases or to a signal of non-galactosylation. Moreover, structures containing linear oligo- or poly-N-acetyllactosaminyl sequences (Fig. 8),

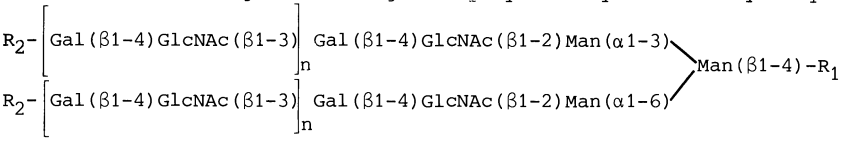

Fig. 8. General basic structure of the biantennary glycans of the poly (glycosyl)-peptides in which n varies from 1 to 15. R_1 = GlcNAc(β1-4) GlcNAc(β1-N)Asn. In human erythrocyte membrane, R_2 = NeuAc(α2-3)Gal(β1-4) GlcNAc ; NeuAc(α2-6)Gal(β1-4)GlcNAc, Fuc(α1-2)Gal(β1-4)GlcNAc (Group O) or GalNAc(α1-3) { Fuc(α1-2)} Gal(β1-4)GlcNAc (Group A) or Gal(α1-3) { Fuc (α1-2) } Gal(β1-4)GlcNAc (Group B).

named poly (glycosyl)-peptides have been characterized in erythrocyte membrane (Ref. 41), human lactotransferrin (Ref. 42) and Chinese Hamster ovary cell glycoprotein (Ref. 43).

Glycans of the hybrid type. Findings on ovalbumin glycopeptides led to the definition of a third group of N-glycosylproteins in which the glycans simultaneously present structure of the oligomannosidic and of the N-acetyllactosaminic type (Fig. 9) and thus belongs to the

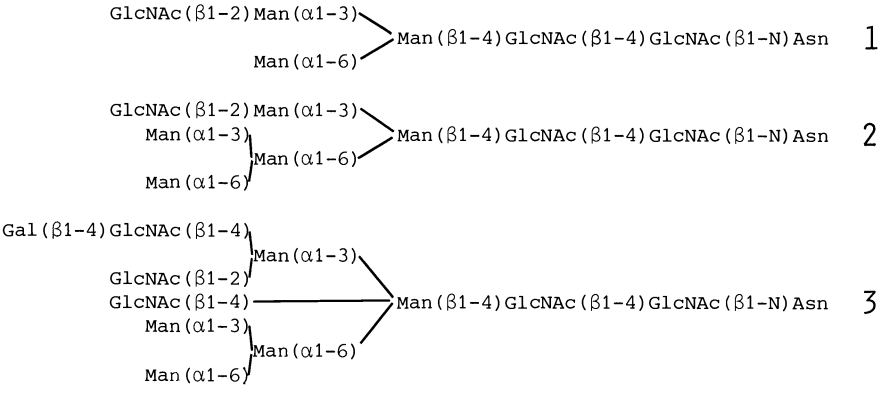

Fig. 9. Structure of glycans of the hybrid type characterized : 1 : in bovine rhodopsin ; 2 : in bovine rhodopsin and human myeloma IgM ; 3 : in hen ovalbumin. For reviews, see Ref. 9 & 14.

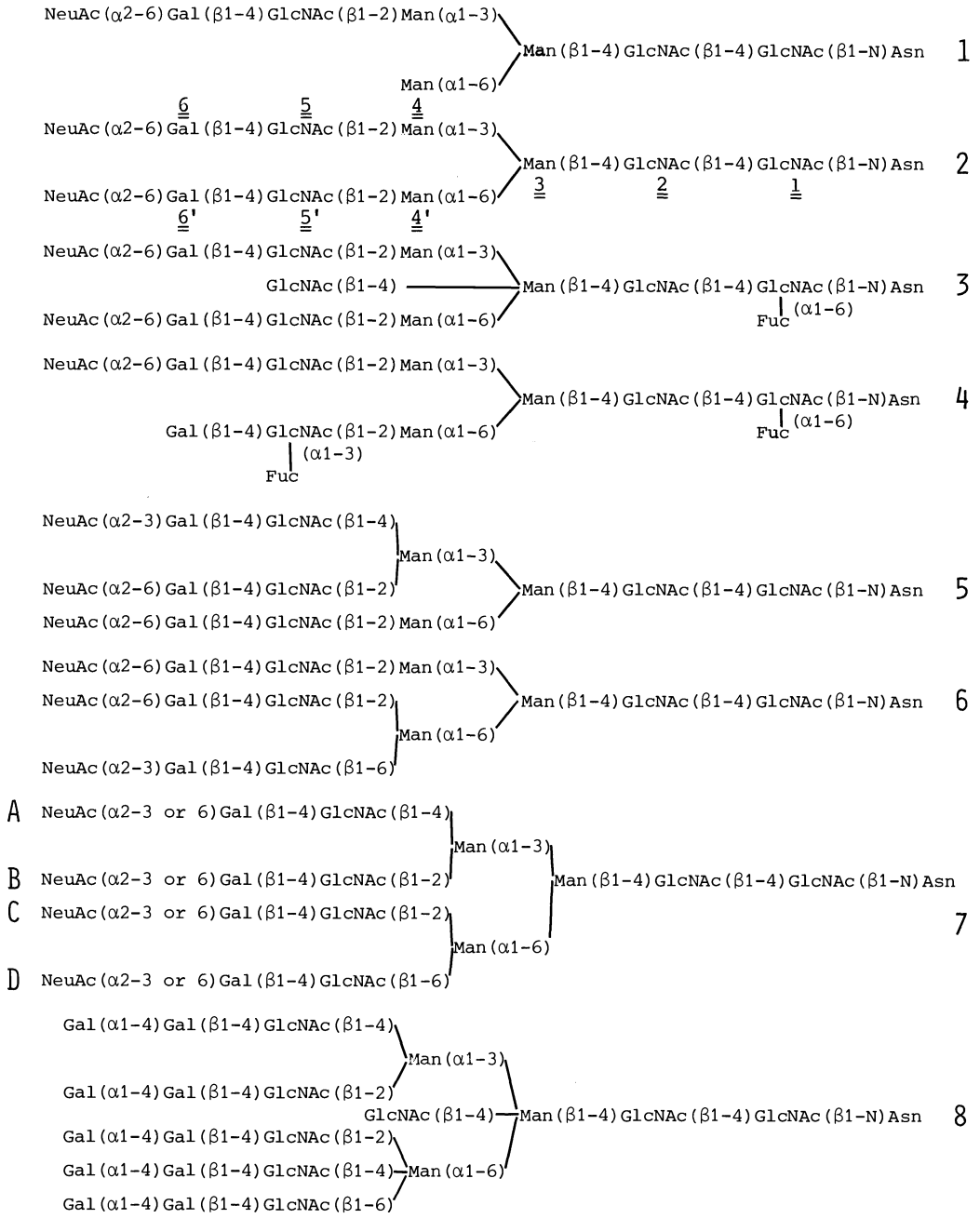

Fig.10. Structure of glycans of the N-acetyllactosaminic type (complex type). 1 : monoantennary glycan of secretory component from human milk and of human chorionic gonadotropin; 2: biantennary glycan of human serum transferrin ; 3 : monofucosylated and bisected (presence of a bisecting N-acetylglucosamine residue) biantennary glycan of human IgG ; 4 : difucosylated biantennary glycan of human lactotransferrin ; 5 and 6 : triantennary glycans of human serum transferrin ; 7 ; tetraantennary glycan of human α_1-acid glycoprotein ; 8 : pentaantennary glycan of turtle-dove ovomucoid possessing a P_1-serologic activity. For reviews, see Ref. 9 & 14.

hybrid type. These structures are considered as intermediates in the biosynthesis of the glycans of the N-acetyllactosaminic type (see Fig. 6).

Conclusion

The survey of the structures we described shows that the substitution of monosaccharides taking part of a glycan structure conforms to a certain orthodoxy which is very restrictive, the basis of which is found in the specificity of glycosyltransferases and in a conservative evolution of these enzymes. In fact, the possibilities of substitution of a given sugar

are limited to 1,2 or 3 well-defined monosaccharides, generally conjugated by a unique type of glycosidic linkage. So could be established the concept of "one-glycosidic bond-one glycosyltransferase", the action of which is, in addition, guided by substitution rules which depend on the primary structure and conformation of the nascent glycan (For reviews, see Ref. 14 & 35). Moreover, the substitution rules raise very interesting questions of comparative biochemistry and of phylogeny and, in addition, allow the foundations to be laid for genetics of glycosyltransferases.

SPATIAL CONFORMATION OF GLYCANS

Glycans of O-glycosylproteins

Glycosaminoglycans and proteoglycans. Conformation of acidic mucopolysaccharides and assembly of proteoglycans are pretty well known due to their relative simplicity (for recent reviews, see Ref. 44 & 45). X-ray diffraction studies have shown that the polysaccharides are generally in a helical conformation. For example, X-ray diffraction of highly crystalline oriented fibers of hyaluronic acid demonstrates that this compound possesses a double helical structure in which two identical left-handed strands having four disaccharide units per turn, are antiparallel to one another and maintained in this conformation by hydrogen bonds. In the intercellular matrix of cartilage, most of the proteoglycans exist in the form of aggregates which are constituted of the association of proteoglycan monomers with hyaluronic acid. The structure of the monomer molecules is that of a central core to which glycosaminoglycans are covalently bound. A low molecular weight (43-48 Kd) glycoprotein, called *link protein* is responsible for promoting proteoglycans-hyaluronic acid aggregation.

Whether this kind of architecture could be generalized and would exist in the intercellular matrix of any tissue remains to be demonstrated.

Mucin-like glycans. In contrast to proteoglycans, very little is known at the moment about the conformation of O-glycosidically linked glycans and of O-glycosylproteins, except in the case of submaxillary mucins. In fact, mucins have an elevated axial ratio and are in a rod-conformation maintained as a result of repulsive electronegative charges carried by the sialic acid residues present in the 800 disaccharide moieties NeuAc(α2-6)GalNAc(α1-3)Ser or Thr which are almost regularly distributed along the long peptide chain (Ref. 46). This extended conformation is responsible of the high viscosity of mucin solutions and of secretions of mucous cells. Removal of sialic acid makes the glycoprotein globular, the solutions of which become weakly viscous. This example clearly illustrates the role the glycans could play in maintaining the protein in a biologically active conformation.

Glycans of N-glycosylproteins

1. From the Y- to the "broken-wing" conformation

The Y-conformation. Until recently the only image we had of the spatial conformation of glycans N-glycosidically conjugated was speculative because it was obtained by the construction of molecular models (for review see Ref. 1, 9, 14, 17 & 47). The first one was presented (Ref. 1) in 1974 at the 7th International Symposium on Carbohydrate Chemistry in Bratislava. The construction of the biantennary glycan molecule of human serotransferrin (Fig. 10 ; structure 2), in creating thermodynamically possible hydrogen-bonds, led to the Y-shaped conformation illustrated in Fig. 11A. This structure may be divided into two parts. The first is compact and constituted of the *inv* pentasaccharidic innercore of mannotriosido-di-N-acetylchitobiose. The terminal trisaccharide Man(β1-4)GlcNAc(β1-4)GlcNAc(β1-N) is flat (Fig. 12) and rigid due to hydrogen bonds. The second part is looser and made up of the two antennae constituted of the trisaccharide NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2). It corresponds to the *var* structure attached to the inner-core and carries the biological activity.

Of course, when I proposed the Y-shaped model, I had the choice between different conformations, but I preferred the Y-conformation because, at that time, one still believed that lectins, including membrane lectins discovered in 1968 by Morell and Ashwell (for reviews, see Ref. 48), were able to recognize and to bind only the monosaccharides in a terminal non-reducting position. Thus, the Y-conformation was in a good agreement with this concept.

The T-conformation. During the past 10 years, the Y-conformation was revisited and little by little refined on the basis of experimental data . For example, using X-ray diffraction studies, we brought in 1978 (Ref. 49) the first modification and proposed the *T-conformation* (Fig. 11B) in which the α-1,3 antenna is disposed perpendicularly in relation to the α-1,6 antenna. In fact, the X-ray diffraction pattern of crystals of the trisaccharide Man(α1-3) Man(β1-4)GlcNAc isolated from urine of mannosidosis showed that the α-1,3 linked mannose residue was perpendicular to the plane of the disaccharide Man(β1-4)GlcNAc (Ref. 50). Moreover, the obtained results confirmed that the latterdisaccharide was planar.

This particular position in space of the α-1,3 mannose residue has been experimentally confirmed (Ref. 51 to 54). Moreover, according to Sutton and Phillips (Ref. 54),

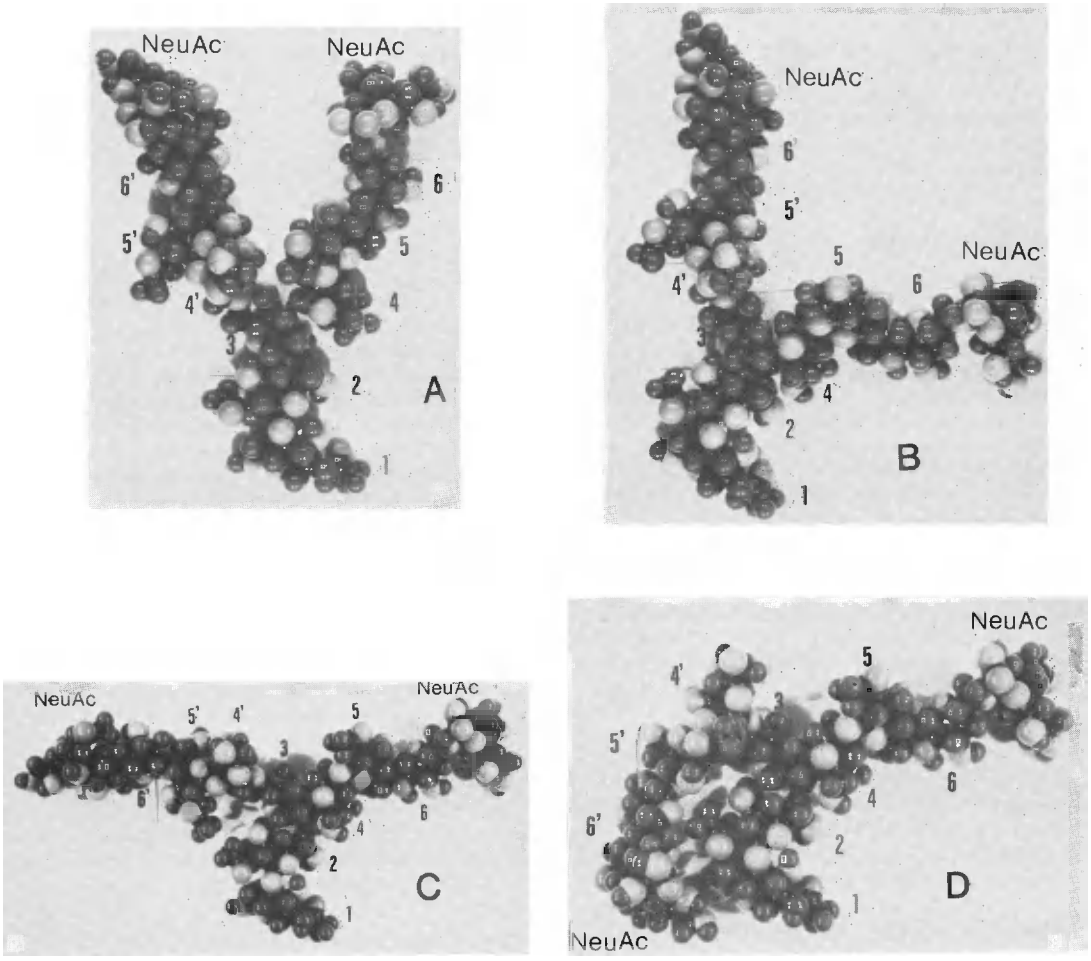

Fig. 11. Spatial conformation of a biantennary glycan of the N-acetyllactosaminic type. A : Y-conformation (Ref. 1) ; B : T-conformation (Ref. 59) ; C : bird-conformation (Ref. 8, 14, 47 & 58) ; D : "broken wing"-conformation (Ref. 8 & 58).

Numbers correspond to the numbering used in structure 2 of Fig. 10.

Rademacher *et al.* (Ref. 55) and CARVER *et al.* (Ref. 53, 56 & 57), the relative orientation of the α-1,3 mannose linkage is invariant and, consequently, that of the α-1,3 antenna.

The bird-conformation. More recently, I suggested to call *bird-conformation* (Ref. 8, 14, 47 & 58) an other kind of conformation (Fig. 11C) obtained by a large rotation around the glycosidic bond of the α-1,6 linked mannose residue, on the basis of the following experimental results :

1°) in 1980, B. Gallot *et al.* realized a very fine work which seems to be ignored by the Biochemists probably because the obtained results were published in specialized reviews (Ref. 59). The authors synthesized amphipatic block polymers by coupling glycopeptides of the N-acetyllactosamine type with hydrophobic peptide block : the poly (γ-benzoyl-L-glutamate) block. The obtained copolymers were studied by freeze fracture electron microscopy and by low angle X-ray scattering. Gallot *et al.* demonstrated that amphipatic carbohydrate-peptide block polymers exhibit lamellar structure, the conformation of which was determined. Two classes, L_1 and L_2, were characterized : the structure L_2 was T-shaped

2°) In the same time, Pérez in Grenoble and Warin in Lille, in collaboration with our Laboratory, used computer calculations to examine which structures are sterically feasible and which conformation is energetically the most favourable. The programme was based *i)* on crystallographic data concerning the following compounds : GlcNAc(β1-N)Asn (Ref. 60), Gal(β1-4)GlcNAc (Ref. 61), Man(α1-3)Man(β1-4)GlcNAc (Ref. 50), GlcNAc(β1-4)GlcNAc (Ref. 62) ; *ii)* on compilation data related to β-1,2 and α-1,6 linkages. Answer of computer was the drawing given in Fig. 13, that of the bird-conformation (Ref. 63).

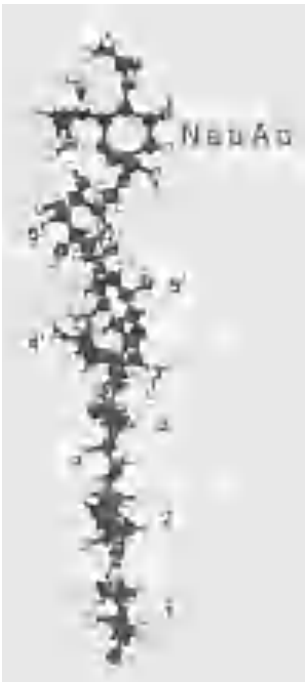

Fig. 12. Lateral view of the molecular model of human serotransferrin biantennary glycan. Only the α-1,6 antenna is visible. Numbers correspond to the numbering used in structure 2 of Fig. 10.

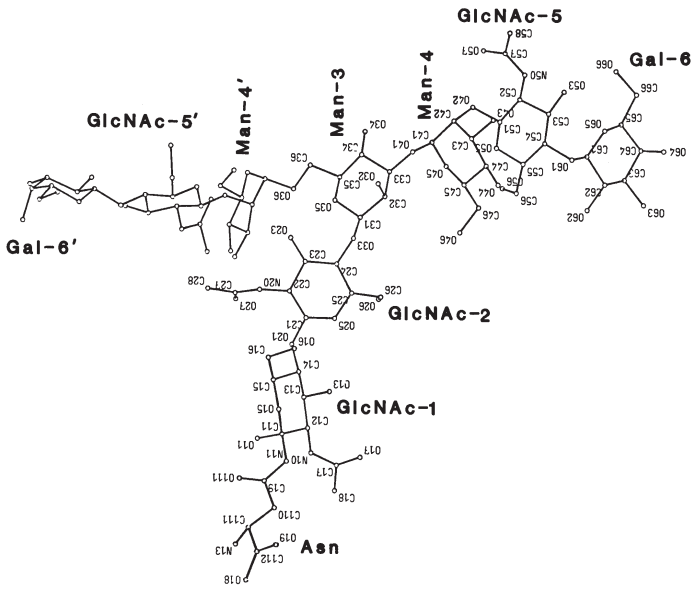

Fig. 13. Computerized model of the preferred conformation of human serum transferrin asialoglycan (desialylated structure 2 of Fig.10) (Ref. 63).

3°) Crystallographic structural studies of Fc fragment from human IgG (Ref. 19, 20-23, 54 & 55) are in favour of an extended conformation of glycan residues.
4°) More recently, Bock *et al.* (Ref. 51) explored the most favoured conformations in aqueous solution of synthetic oligosaccharides related to the N-acetyllactosaminic type using high-resolution NMR (both ^{1}H and ^{13}C) combined with hard-sphere *exo*-anomeric (HSEA method) calculations. The results they obtained indicate that the N-acetyllactosamine units are arranged in such a way that they are widely separated in space.

Broken-wing conformation. On the basis of NMR experiments (Ref. 11, 53 & 64) or by using the HSEA-calculations (Ref. 65), authors proposed the model described in Fig. 11D, I suggested to call *broken-wing conformation.* In this utmost conformation which takes advantage of the large angle of rotation of the α-1,6 linkages (see below), the α-1,6 antenna folds back towards the protein backbone and sets along the inner-core. According to Carver *et al.* (Ref. 64) this conformation is favoured by a bisecting N-acetylglucosamine residue.

Conformation of glycans of the oligomannosidic type. Spatial conformation of glycans of the oligomannosidic type has been explored by high-resolution NMR by Vliegenthart *et al.* (Ref. 13 & 66). On the basis of the NMR-spectral features, the authors proposed that the favored conformation of the glycan with 9 mannose residues (Structure 1 ; Fig. 5) is as depicted in Fig. 14. In this model, the orientation of the entirely rigid $\underline{\underline{4}}$-$\underline{\underline{C}}$-$\underline{D}_1$ trisaccharide antenna

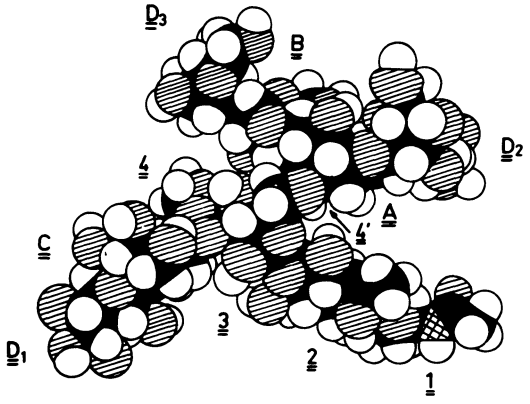

Fig. 14. Molecular model proposed for the oligomannosidic type glycan that contains 9 mannose residues. Numbers and letters correspond to the coding used in structure 1 of Fig. 5 (Ref. 13 & 66).

with respect to the Man(β1-4)GlcNAc(β1-4)GlcNAc(β1-N)Asn core is similar to that of the $\underline{\underline{4}}$-$\underline{\underline{5}}$ unit in N-acetyllactosaminic type structures. Regarding the positional arrangement around the 1,6-linkage of mannose-$\underline{\underline{4}}$' residue, the two disaccharide antennae $\underline{D}_2$-$\underline{\underline{A}}$ Man(α1-2)Man(α1-3) and $\underline{D}_3$-$\underline{\underline{B}}$ Man(α1-2)Man(α1-6) are in an extended-conformation and the $\underline{\underline{4}}'$-$\underline{\underline{A}}$-$\underline{D}_2$ part of the molecule is in close proximity of the di-N-acetylchitobiose unit and folds back towards the peptide chain.

2. Are extended conformations compatible with the properties and biological behaviour of glycans and glycoproteins ?

Interaction with lectins. As I mentioned above, the extended conformations of glycans seem to be far from the concept of the terminal monosaccharides as recognition signals. But, we know now that lectins could recognize and bind "laterally", and not "terminally", mono- or oligosaccharides in an *internal* position. For example, as demonstrated by Kornfeld and Ferris (Ref. 67), the most active part towards ConA of an N-glycosidically linked glycan is not constituted of an α-mannose residue in a terminal position, but of the disaccharide GlcNAc(β1-2)Man(α1-3 or 6). Thus, the glycans in an extended conformation provide good targets for recognition by other proteins in general, by lectins in particular.

Accessibility to enzymes. The extended conformations are the most satisfactory ones from the point of view of the glycan biosynthesis because all substitutable hydroxyl groups are readily accessible to glycosyltransferases, as shown by the following examples :

1°) Substitution of C-4 of the β-mannose residue by a bisecting N-acetylglucosamine, like in human IgG (Fig.10 ; Structure 3). This residue interacts with the two α-mannose residues $\underline{\underline{4}}$ and $\underline{\underline{4}}$' and covers in particular a wide area of the surface of GlcNAc(β1-2)Man(α1-3)Man(β1-) unit (Ref. 57 & 64), thus explaining *i*) the shifts of the H-1 of the three mannose residues observed in NMR (Ref. 11 & 18) ; *ii*) the non-reactivity with Con A of this kind of glycans because of the steric hindrance induced by the bisecting N-acetylglucosamine residues ; *iii*) the inhibition of certain glycosyltransferases (Ref. 35 & 68).

2°) The substitution by fucose residues of C-6 of N-acetylglucosamine-1 and of C-3 of N-acetylglucosamine-5', like in human lactotransferrin (Fig.10 ; structure 4). As shown in Fig. 15, the glycan structure becomes compact and 4 methyl groups (2 from the fucose

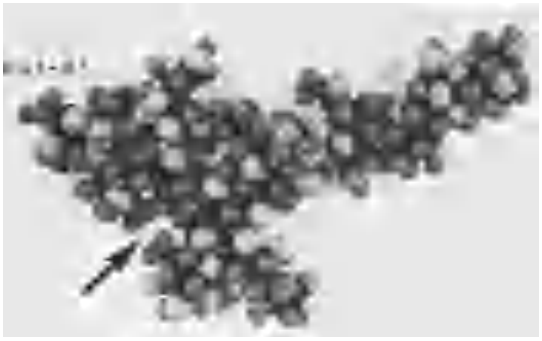

Fig. 15. Molecular model of a difucosylated biantennary glycan (Structure 4 of Fig.10). Arrow indicates the confluence of four methyl groups.

residues and 2 from the acetamido groups of N-acetylglucosamine residues) become confluent and can interact and link by hydrophobic area (arrow marked), so explaining the inactivity towards this kind of structure of certain α-fucosidases and of the endo-N-acetyl-β-D-glucosaminidase from *Basidiomyces* which splits the di-N-acetylchitobiose residue of glycans of the N-acetyllactosamine type (Ref. 69). Moreover, the glycan molecule becomes rigid as a whole because of the invariance of the α-1,3 antennae. So could be explained that the desialylated glycan of human lactotransferrin has the highest inhibitory effect on a galactose-lectin released from guinea pig colonic epithelial cells, the particularly rigid spatial conformation of the glycan rendering the terminal galactose of the α-1,3 antenna more accessible to the lectin (Ref. 70).

3°) Substitution of C-4 of mannose-4 and C-6 of mannose-4' by two additional antennae.

The generalization of the concept of the extended conformation to the additional antennae leads to the molecular model of the human α_1-acid glycoprotein tetraantennary glycans (Fig. 10 ; structure 7) an aerial view of which gives Fig. 16, I proposed to call the *umbrella-conformation* (Ref. 8, 14 & 47).

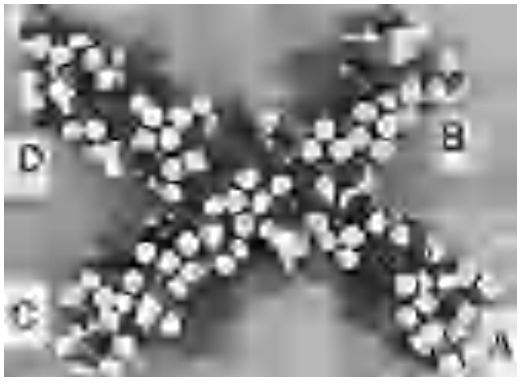

Fig. 16. Aerial view of a tetra-antennary glycan in the umbrella-conformation. Letters refer to the four antennae of the structure 7 of Fig.10.

This speculative view has been confirmed by Bock *et al.* (Ref. 51) using high-resolution NMR combined with HSEA-calculations and by Li *et al.* (Ref. 71) on the basis of small-angle neutron scattering experiments on human α_1-acid glycoprotein. Li *et al.* proposed the conformation depicted in Fig. 17 and concluded that most of the glycans were of the types (b) and (c), model (a) been disfavoured for reason of the negative charges of sialic acid residues.

Relationships between glycans and protein

a - Protective effect of glycans towards the protein moiety. Considering these pictures, a question arises : what are the dimensions and the surfaces of such glycans in comparison with those of the protein moiety ?

In the case of a sialylated biantennary glycan like that of human serum transferrin (Fig.10 ; structure 2), the dimensions determined by measuring the molecular model itself, by

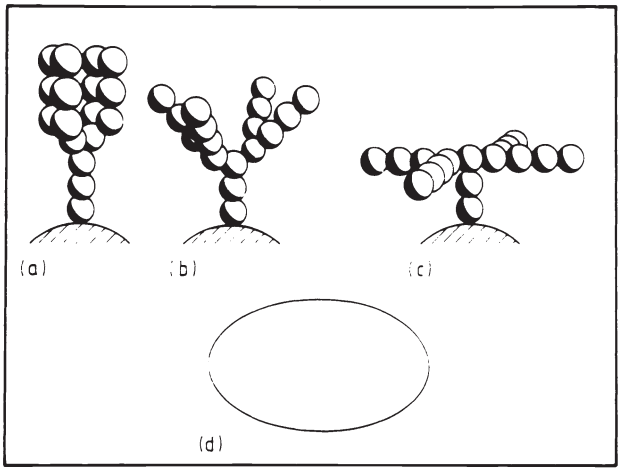

Fig. 17. Spatial conformations of a tetra-antennary glycan (Structure 7 of Fig. 10) determined by small-angle neutron scattering of human α_1-acid glycoprotein (Ref. 71). The ellipse of dimensions 5.3 x 3.1 nm to correspond to the protein core is drawn to scale in (d).

questioning computer and by using the data of Douy *et al.* (Ref. 59) are as follows : length 5.5 nm, height 2.4 nm; thickness 0.5 nm; area 2.75 nm^2. As shown in Fig. 18, these

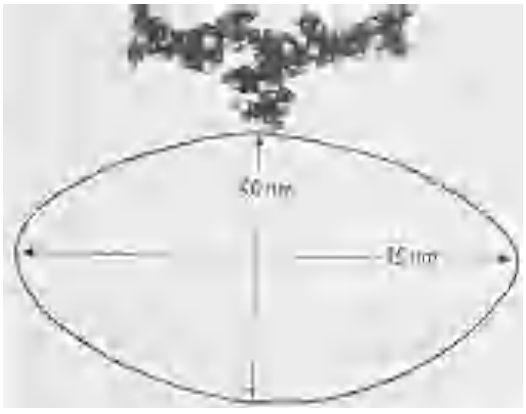

Fig. 18. Relative size of biantennary glycan and protein in human serum transferrin (Ref. 8, 14, 47 & 58).

dimensions are important compared with those of a globular protein like the human serum transferrin which is an oblate spheroid with semi-axes of length 4.75 nm, 3.00 nm and 2.50 nm (Ref. 72).

In the same way, a tetraantennary glycan, in adopting an umbrella-conformation, covers an area of about 20-25 nm^2. In the case of glycoproteins, like the human α_1-acid glycoprotein (NW : 37,000) which contains 5 glycan moieties which are essentially of the tri- and tetra-antennary type, the protein could be almost completely enveloped by the glycans (Fig. 19).

Fig. 19. Area covered by two tetra-antennary glycans associated with a protein having the same size as that of human serum transferrin (Ref. 8,14,47 & 58).

So could be explained the resistance of certain glycoproteins and their weak antigenicity, glycans acting as protective shields. Moreover, the umbrella conformation of glycans is solidly maintained by ionic bonds between the electronegative charges of sialic acid residues and electropositive charges of basic amino acids, as demonstrated by the small-angle neutron scattering study of Li *et al.* (Ref. 71). Removal of sialic acid residues, by making the antennae free and consequently mobile, abolishes the protective effect of glycans, so rendering the protein more antigenic and accessible to proteases.

If such structures are present in viral envelopes, one can appreciate at what level the viruses have brought the art of the camouflage and understand how the glycans may play a role in masking or altering the nature of the protein's antigenic sites and, hence, play a role in the process by which the viruses could escape recognition by the immune system. The possibility that glycans modulate recognition of viral proteins by the immune system is discussed in a paper of Wiley *et al.* (Ref. 73).

The protective effect that play the glycans towards the protein moieties could also explain the peculiar behaviour and resistance of metastatic cancerous cells since we know now that the membrane glycoproteins of cancerous cells are significantly enriched in tri- and tetra-antennary glycans as demonstrated by several authors (Ref. 58, 74-80). Table 2 clearly illustrates this fact. Moreover, the new glycan structures carried by the metastatic cells could explain the tissue specific localization of the latter by specific lectin recognition.

TABLE 2. Increase of the proportions(*) of tri- and tetraantennary glycans of glycoproteins in the case of virus-transformation of BHK 21/C_{13} fibroblasts

Branching type of glycans	Biantennary	Tri + tetraantennary
"Normal" BHK 21/C_{13}	69	31
HSV-transformed BHK 21/C_{13}	32	68

(*) Proportions are expressed in per cent

In the same way, Delannoy *et al.* (Ref. 81) recently observed that fibronectin from cancerous cells in culture is enriched in tri- and tétraantennary glycans leading to a complete inversion of the ratio biantennary/tri + tetraantennary structures (Table 3). This transformation is accompanied by an increase of the sialylation. The profound modifications we observed could be related *i)* to the loss of reactivity of the protein moiety of the fibronectin by masking the lectin-sites, *ii)* to the disappearance of fibronectin from cancerous tissues and *iii)* to the mechanism of metastasis itself.

TABLE 3. Increase of the proportions(*) of tri- and tetraantennary fibronectin glycans and of the degree of sialylation associated with the viral malignant transformation of BHK 21/C_{13} fibroblasts.

Branching type of glycans	*Biantennary*		*Tri + tetraantennary*		
"Normal" BHK 21/C_{13}	60		40		
HSV-transformed BHK 21/C_{13}	28		72		
Sialic acid residues per glycan	*0*	*1*	*2*	*3*	*4*
"Normal" BHK 21/C_{13}	36	45	14	4	1
HSV-transformed BHK 21/C_{13}	12	25	33	23	7

(*) Proportions are expressed in per cent

b - Glycan and protein conformation. The concept of the extended conformation of glycans allows a better understanding of the role the carbohydrate moiety plays in the conformation of proteins through specific protein-carbohydrate and carbohydrate-carbohydrate interactions.

A very fine demonstration was recently brought by Sutton and Philips (Ref. 54) by using X-ray diffraction. It concerns the Fc fragment of rabbit IgG molecule which derives from the classic association of two half-molecules, each carrying a glycan residue of the biantennary N-acetyllactosaminic type. The latter is in an internal position in the C_H2 domain and its integrity is indispensable for the maintenance of all properties of immunoglobulins. The

above mentioned authors have demonstrated that (Fig. 20)

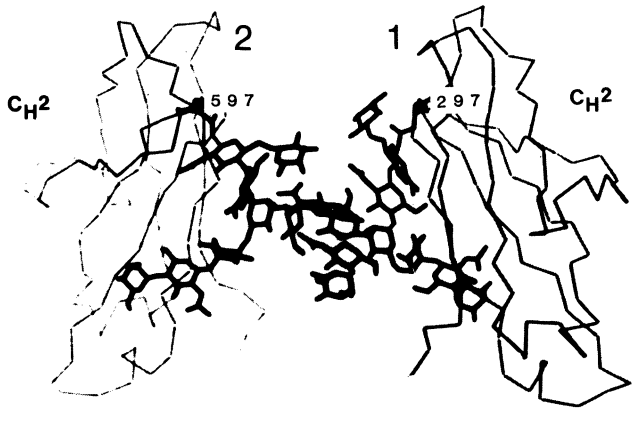

Fig. 20. Glycan structure at the Asn-297 residues (indicated 297 and 597) with the backbone of polypeptide chains 1 and 2, in the C_H2 domains of rabbit IgG (Ref. 54).

i) the pentasaccharidic innercore lies close to the surface of C_H2 domain and interacts with Phe-241, Val-264 and Asp-265 residues ;
ii) the two antennae of each glycan are in an extended conformation, the orientation of the α-1,3 antenna being T-shaped and invariant ;
iii) the α-1,6 antennae of each glycan make several contacts, mainly hydrophobic, with amino acid residues on the surface of the protein, principally with Phe-243, Pro-246 and Thr-260 ;
iv) on the contrary, the α-1,3 antennae have no contact with the surface of the C_H2 domain but do interact with each other between the GlcNAc-5 residue of the one chain and the GlcNAc-2 and Man-3 residues of the other chain, and it therefore appears that they are responsible for maintaining the disposition of the two C_H2 domains.

Concerning the interactions between glycans and hydrophobic amino acids, it is of interest to note that the terminal trisaccharide of the inner-core is relatively hydrophobic because of the presence of the two N-acetylglucosamine residues carrying acetamido groups and of the two- and sometimes three-fold substituted β-mannose residue. The hydrophobicity of the trisaccharide is still more enhanced when the N-acetylglucosamine-1 residue is fucosylated. Thus, it is not unreasonable to presume that this hydrophobic part of glycan could interact with hydrophobic regions of the protein moiety. Its flat structure (see Fig. 12) could be related to this property.

3. Conformational changes of glycans. Mobility of the antennae.
Glycan structures must not be considered as rigid conformations, because when they have a certain degree of freedom, for example, when they present no carbohydrate-carbohydrate or carbohydrate-protein interactions as it is the case for glycans or glyco-oligopeptides in solution. In fact, glycans present points of flexibility leading to a relative mobility of the antennae due to the rotation of the rigid pyranose rings around the two bonds attached to the glycosidic oxygen. The rotation is limited in the case of 1 → 2, 1 → 3 and 1 → 4 linkages, the 1 → 3-linkage being considered as the most rigid one. On the contrary, the 1 → 6 linkages are more flexible because of the additional rotational freedom.

In the case of biantennary structures of the N-acetyllactosaminic type (Fig. 10 ; structure 2), the most important point of flexibility is located at the α-1,6 bond linking the mannose-4' residue to the mannose-3 residue explaining that the Y-, T-, bird- and broken wing-conformers are interconvertible as demonstrated by the following series of experiments :

Low angle X-ray scattering and freeze fracture of artificial glycoconjugates. Gallot *et al.* (Ref. 59) showed that the glycan moiety of synthetic glycopeptides in aqueous solution adopts a bird- or a Y-shaped conformation depending on the molecular weight of the polypeptide block and on the concentration of the glycopeptide, molecular weight of the polypeptide between 2,000 and 3,500 and high concentration favouring the Y-conformation.

In the same way, Gallot *et al.* (Ref. 82 & 83) synthesized amphipatic liposaccharides by coupling glycoamino acids with fatty acids containing from 8 to 24 carbon atoms. Mixtures of these glycolipids and of phospholipids lead to the formation of long and parallel lamellar structures, glycans and water filling the space between the layers. As long as the

glycolipids are in small quantity in the ternary system glycolipid/phospholipid/water, the glycan takes up a bird-conformation. When the quantity is high, it becomes Y-shaped.

Electron paramagnetic resonance of spin-labelled glycans. The internal degree of freedom of rotation in space of the antennae has been pointed out by an electron paramagnetic resonance study by using spin labelled bi- and triantennary glycans (Ref. 84). Spin-spin interactions originating from collision effects between probes have been demonstrated. The interactions were higher in the case of triantennary structures. The addition of ConA reduced the mobility of the probe bound to the biantennary glycans which are able to bind ConA. It does not affect the spin-labelled triantennary glycans which are well known to be unable to associate with ConA.

Small-angle neutron scattering study of human α1-acid glycoprotein has shown that also in the case of tetraantennary structures all antennae are mobile and able to adopt an "umbrella-conformation" (conformer (c) of Fig. 17) or a "reversed umbrella-conformation" (conformer (b) of Fig. 17) (Ref. 71).

4. Concluding remarks

The above data bring out the fundamental role that plays the mannotriose core in the conformation of glycans of N-glycosylproteins. In this connection, it is amazing to note that all "strategic" branching points systematically associate a rigid 1,3-linkage with a mobile 1,6-linkage as shown in Fig. 21. What is the reason for the relatively invariant orien-

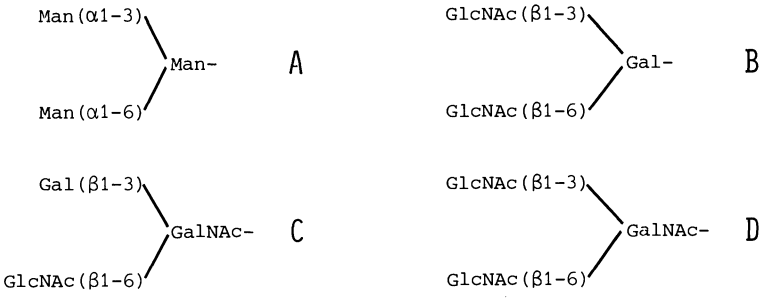

Fig. 21. "Strategic branching" associating 1,3- and 1,6-linkages. A : inner-core of N-glycosidically linked glycans ; B and C : branchings currently found in O-glycosylprotein glycans of the mucin-type ; D : inner-core of Ii active glycans from sheep gastric mucin (Ref. 85).

tation of a T-shaped 1,3-bond with a highly flexible and mobile 1,6-bond ? Probably in order to favour, when necessary, an extended conformation of the antennae which favours itself *i*) the occupation of their "vital space" by the glycans ; *ii*) the interaction of the glycans with their own protein ; *iii*) the glycan-glycan interactions ; *iv*) the action of enzymes ; *v*) and/or the recognition of glycans by receptors.

CONCLUSIONS

Ten years ago, I wrote as a conclusion of my report entitled "Recent data on the structure of the carbohydrate moiety of glycoproteins. Metabolic and biological implications" : "At the end of this brief review, we can conclude that the problem of determination of the primary struture of glycans is virtually solved. However, it remains to miniaturize the procedures in order to extend their application to very small quantities of biological substrates.Thus, we can look forward and claim that the future belongs to Biophysics the role of which will be to determine precisely the spatial arrangement of glycans and the conformation of glycoproteins in order to understand the mechanism of their action. The most exciting and marvellous age of the history of glycoproteins starts right now".

Ten years later, we can claim that the problem of determination of the primary structure of glycans is actually solved. The procedures have been miniaturized and applied to the study of cell membrane glycoproteins. In this field, the introduction of high-resolution NMR has been a decisive event. In fact, this procedure is rapid, non-destructive and very sensitive since it requires amounts of glycans varying from 25 to 100 micrograms only.

In the same way, the recent intervention of the Biophysicists lead to the definition of the spatial morphology of glycans and to the concept of their steric conformational changes fitting to the role they have to play. In fact, experimental results obtained in the past two years by exploring the spatial conformation of glycans through physical methods : X-ray diffraction, nuclear magnetic resonance, electron paramagnetic resonance and small-angle neutron scattering, led to the fundamental finding that the antennae, which support the biologically active part of the glycans, are not rigid formations at all. On the contrary, they must be considered as flexible and mobile parts of glycan molecules

solidly planted on the rigid arm constituted of the terminal trisaccharide linked to the peptide chain. In fact, the trisaccharide arm probably fixes in part the conformation of the glycan, preventing the twist of the whole molecule, due to the relative rigidity of the GlcNAc-Asn linkage (Ref. 86). So could be explained why glycoasparagines are often more potent inhibitors of haemagglutination by certain lectins : for example, the relative amounts of glycan required to inhibit red cell agglutination by *Vicia faba* agglutinin is 8-fold higher than for the corresponding glycoasparagine (Ref. 58).

The concept of the antennae changing their conformation according to the environment fits perfectly with the concept of glycans acting as recognition signals. Moreover, the double character of rigidity and flexibility of glycans may be central to the biological role of the carbohydrate moiety of glycoproteins.

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