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Cover

Illustration of a low-energy conformation of N-acetyllactosamine (lacNAc), a common terminal section of N-glycans. It can e.g. be bound by mammalian lectins (galectins), serving as docking point (kindly provided by Dr. C.-W. von der Lieth, Heidelberg, Germany).

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Review

Eukaryotic glycosylation: whim of nature or multipurpose tool?^{*}

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Abstract. Protein and lipid glycosylation is a ubiquitous phenomenon. The task of cataloguing the great structural variety of the glycan part has demanded considerable efforts over decades. This patient endeavor was imperative to discern the inherent rules of glycosylation which cannot affirm assumptions on a purely coincidental nature of this type of protein and lipid modification. These results together with theoretical considerations uncover a salient property of oligosaccharides. In comparison with amino acids and nucleotides, monosaccharides excel in their potential to serve as units of hardware for storing biological information. Thus, the view that glycan chains exclusively affect physicochemical properties of the conjugates is indubitably flawed. This original concept has been decisively jolted by the discovery of endogenous receptors (lectins) for distinct glycan epitopes which are as characteristic as a fingerprint or a

signature for a certain protein (class) or cell type. Recent evidence documents that these binding proteins are even endowed with the capacity to select distinct low-energy conformers of the often rather flexible oligosaccharides, granting entry to a new level of regulation of ligand affinity by shifting conformer equilibria. The assessment of the details of this recognition by X-ray crystallography, nuclear magnetic resonance spectroscopy, microcalorimetry and custom-made derivatives is supposed to justify a guarded optimism in satisfying the need for innovative drug design in antiadhesion therapy, for example against viral or bacterial infections and unwanted inflammation. This review presents a survey of the structural aspects of glycosylation and of evidence to poignantly endorse the notion that carrier-attached glycan chains can partake in biological information transfer at the level of cell compartments, cells and organs.

Key words. Cell adhesion; drug design; glycan metabolism; glycolipids; glycoproteins; glycosaminoglycans; lectins; recognition.

Introduction

Proteins are ubiquitous compounds in all living organisms with many well-established biological functions. They play important roles, for example as biocatalysts, transport and targeting devices, in establishing cell-type

specificity and in various aspects of cell sociology. Post-translational modification of proteins adds a further level of regulation to their activity and can thus enlarge their capacity to take part in regulatory events. The reversibility of the introduction of small inorganic groups into amino acid side chains such as phosphate allows proteins to be used as molecular switches. Evidently, the capacity of the peptide backbone is not restricted to serving as acceptor for a phosphate group. Another rather common type of posttranslational

^{*} Dedicated to Prof. Dr. F. Cramer on the occasion of his 75th birthday.

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modification is protein glycosylation. A potential indication of a biochemical role of the carrier-immobilized sugar units is furnished by the observation that a second abundant class of biomolecules shares the property of proteins to be decorated with oligosaccharides. Ceramide-based lipids as major constituents of biomembranes can carry attached glycan chains, and the phosphatide phosphatidylinositol with its lipid anchor part and hydrophilic inositol head group is likewise engaged as a means of glycan attachment. Glycoproteins and glycolipids are referred to as glycoconjugates and members of the group of complex carbohydrates. When anchored in cell membranes, their sugar parts are directed into the outer environment and thus constitute the glycocalyx. While the glycan chains of lipids rarely extend beyond a dozen monosaccharide units and therefore remain in close vicinity to the cell surface, those of glycoproteins can protrude further from the cell membrane due to either long glycan chains or linkage to an amino acid at a remote position within the peptide chain.

In the early years of research on these compounds, emphasis had to be laid on the development of analytical tools to allow structure determination as a prerequisite for any taking stock of functional aspects. Now a set of sophisticated and further continuously improved methods is available, consequently making it easier to put the initially puzzling complexity of saccharide sequences on record [1–6]. Only after having pursued structural analysis, which revealed a wide range of glycan structures, could attention be turned to the enzymatic routes of biosynthesis and to the delineation of a putative physiological meaning, these lines of reasoning reflected, for example, in reviews by Roseman [7] and Winterburn and Phelbs [8]. This historical development of the different phases of research on complex carbohydrates is nicely described by a series of authoritative reviews [9–18]. Currently, the enormous strides taken in the area of sugar-mediated recognition suggest that glycan constituents of proteins and lipids establish an alphabetical system, adding to the well-accepted coding importance of nucleic acids and peptide backbones [19, 20]. Citing from the article of Winterburn and Phelbs [8], 'carbohydrates are ideal for generating compact units with explicit informational properties since the permutations on linkages are larger than can be achieved by amino acids, and, uniquely in biological polymers, branching is possible'. Therefore, 'the significance of the glycosyl residues is to impart a discrete recognitional role on the protein' (or any other carrier backbone). This notion has meanwhile continuously received enough support to keep it in the eye of a readership from different areas of life sciences [1, 21–26]. In order to familiarize the reader with this concept, we will summarize the major features of glycan struc-

tures, analysis, conformation and biosynthesis, and outline the current concept of the biological role of these fascinating molecules.

The sugar code

The term 'complex carbohydrates' should not solely be thought to connote a technically demanding task of cataloguing structures. Principally, oligomers of any class of biomolecules can establish a coding system for information storage, resembling words in a language. In this sense, structural complexity entails the intriguing capacity to serve as versatile hardware for biological information transfer. The capacities for this duty are not equal for the biochemical coding systems. Amino acids and nucleotides are linked nearly exclusively in

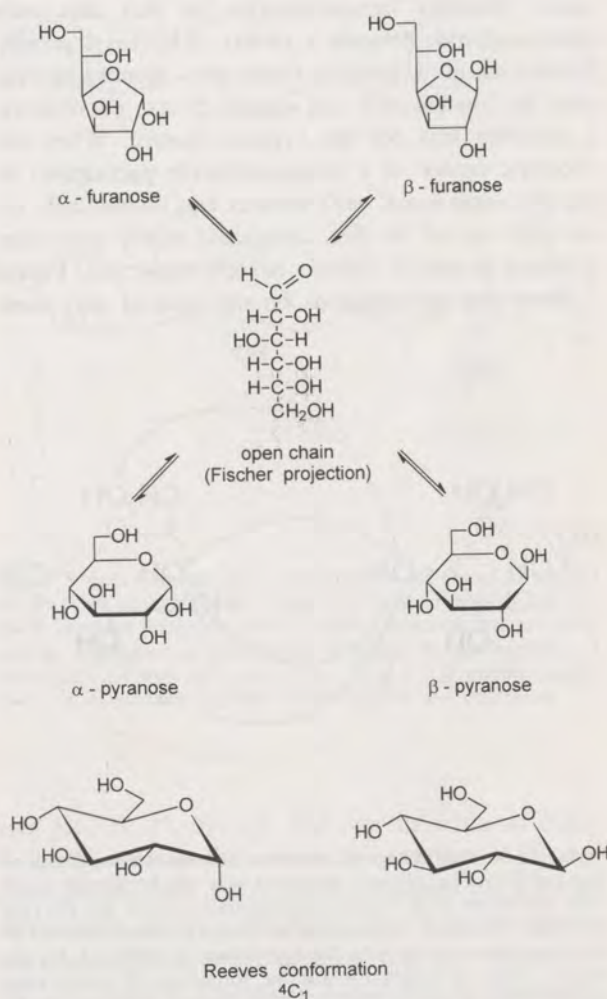


Figure 1. Mutarotation of D-glucose with the different representations of D-glucose as Fischer projection, Haworth projection and Reeves ${}^4\text{C}_1$ chair conformation.

linear chains via peptide or phosphodiester bonds. Only the primary sequence of the monomeric building units in the chains allows for variability in these two cases. This situation changes drastically when oligosaccharides are considered. It is pertinent to recall aspects of the basic biochemistry of sugars to appreciate these extraordinary properties of this type of oligomer, which can reach an unprecedented level of storage capacity.

Textbook biochemistry teaches mutarotation of monosaccharides such as D-glucose primarily from the perspective of a measurable physical parameter (optical rotation). Since linear forms of, for example, any hexose are normally present only in minute amounts, hemiacetals or -ketals are invariably found. The mutarotation of a hexose yields four different ring structures for each monomeric unit, as depicted in figure 1 for D-glucose (Glc). Viewed from the perspective of evaluation of hardware abilities, this result is of relevance. Similarly far-reaching is the fact that each monosaccharide presents a variety of hydroxyl groups. Formation of a bond between two monosaccharide units by condensation can engage diverse possibilities, a daunting task for the organic chemist. When the anomeric center of a monosaccharide participates in the glycosidic bond, each anomer can theoretically react with one of the five, chemically nearly equivalent hydroxyl groups of another hexopyranose unit. Figure 2 shows that permutations on the basis of only these

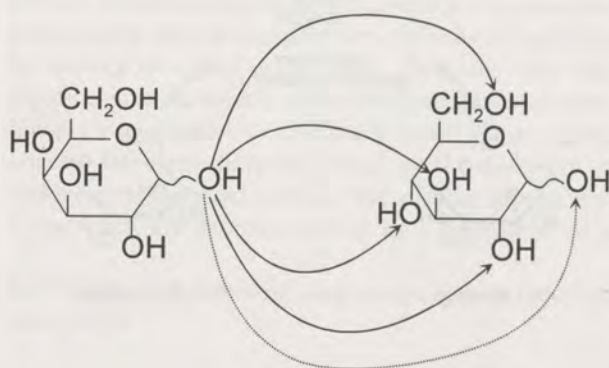


Figure 2. Possible linkage points for a disaccharide consisting of Gal and Glc. The different structures that can be formed result from variations in (i) anomeric configuration (α or β), (ii) ring size, that is furanose or pyranose, with only pyranose depicted in the figure (see fig. 1), (iii) linkage points as indicated by the arrows, with the dotted line for the formation of nonreducing disaccharides involving both anomeric centers, and (iv) the sequence of the two monomers. Excluding linkage via the two anomeric centers, each C1 hydroxyl group can be conjugated to any of the four available hydroxyl groups at C2, C3, C4 and C6 in α - or β -configuration, yielding 8 isomers for each of the two anomeric centers.

two factors (variations of linkage point and the two anomeric positions) will afford 16 theoretically possible hexopyranose structures with all dimers omitted, in which the two anomeric centers are linked to each other. Sequence permutation doubles this number. In this calculation, the potential for alteration of the ring size (pyranose/furanose, see fig. 1) has not even been incorporated. Using two amino acids or nucleotides, only two different structures can be generated.

If we allow the formation of any linear or branched trisaccharide from a pool of 20 different monosaccharides, we end up with 9×10^6 possibilities, whereas only 8000 tripeptides can be formed from the 20 amino acids [19]. For a hexamer this gap widens to more than seven orders of magnitude. This enormous coding capacity of oligosaccharides renders them highly suitable hardware for information storage. However, it is essential to add that the theoretical number of combinations has by far not been unearthed in nature, probably because the equally high number of corresponding glycosyltransferases is not available in the organism [5, 27–29]. A compensatory diversification is found instead by addition of 'small substituents' such as phosphate, acetate or sulfate [19, 26, 30–38]. As will be illustrated later in several places, single or multiple modifications with such substituents can even occur on one monosaccharide residue, affecting its character as code letter. For example, the 4-sulfation of N-acetyl-D-galactosamine (GalNAc) can be likened to the formation of an umlaut in German language. If now multiple saccharide chains, each containing a set of possible structural variations, are attached to one protein backbone as is the case for many glycoproteins, one can grasp the immediate relevance of these calculations and findings for the theoretical content of information stored in the glycoconjugate.

Posttranslational modification by glycosylation can even lead to the creation of discrete subsets of glycoproteins (glycoforms, according to Rademacher et al. [39]). They share an identical polypeptide sequence but differ in the glycan composition. As will exemplarily be outlined in the section on glycans and disease, monitoring the relative proportions of serum transferrin glycoforms has diagnostic value. It is indicative of clinically defined inherited syndromes or of impairment of liver function by distinct causes. In line with the given reasoning, it is tempting to speculate that this potential is not wasted as a costly whim of nature but rather establishes a multipurpose tool that is taken advantage of in diverse circumstances. Before the functional aspect can be discussed, it is essential to provide an insight into the structural organization of protein and lipid glycosylation.

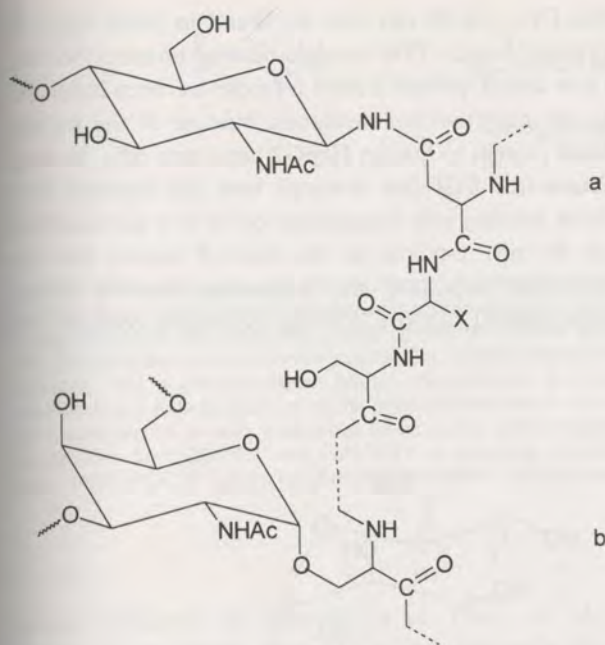


Figure 3. Linkage points for the covalent attachment of common N- and O-glycans to protein. The GlcNAc β 1-Asn-X-Ser sequon is characteristic for most N-glycans. The carbohydrate chain is extended at the C-4 of GlcNAc as indicated by the wavy line (a); for sites of core fucosylation, see figure 15. The GalNAc α 1-Ser structure of mucin-type O-linked glycoproteins can be extended by addition of further saccharides to C-3 and C-6, leading to the introduction of branches (b).

Glycan attachment to proteins

In glycoproteins, the glycan chains are nearly always covalently linked to suitable functional groups of amino acid side chains within a protein. The elucidation of the structures of the linkage region between carbohydrate and protein was initiated with the description of GlcNAc β 1-Asn for N-glycans and GalNAc α 1-Ser or Thr for O-glycans (fig. 3) [1, 10, 25, 26, 40]. Ensuing research considerably extended our knowledge in this

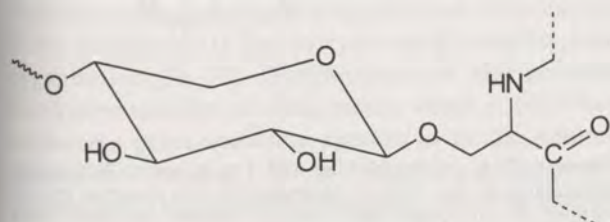


Figure 4. Structure of the -4Xyl β 1-Ser linkage of glycosaminoglycans.

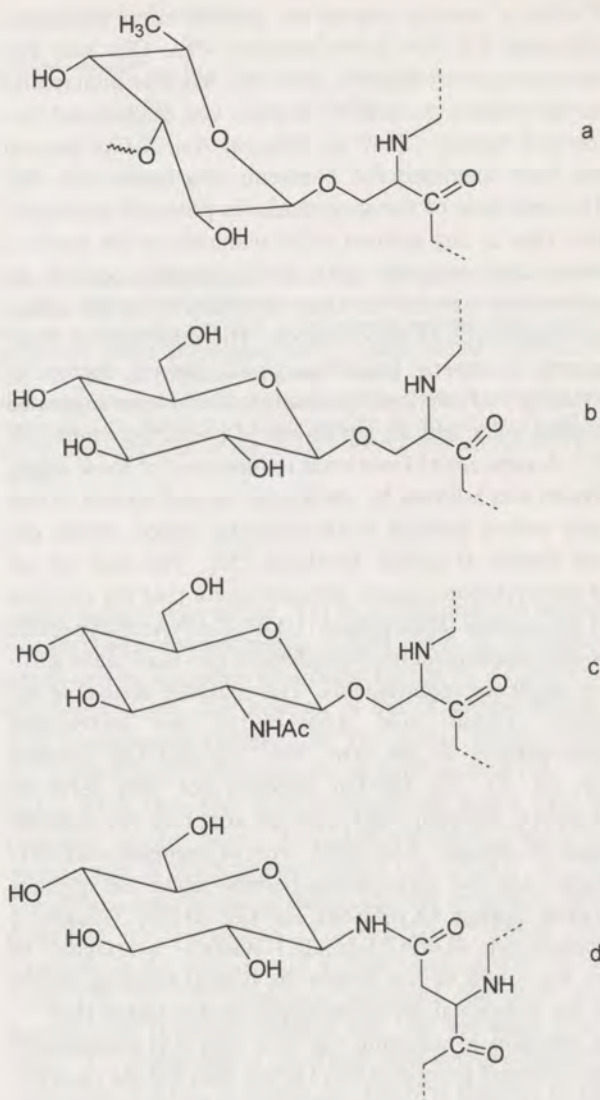


Figure 5. Less common Ser glycosylations are found in the (a) -3Fuc α 1-Ser linkage and (b) Glc β 1-Ser linkage in EGF-like domains. Another possible mode of O-glycosylation as well as one of glucose attachment to protein are presented by the linkages (c) GlcNAc β 1-Ser and (d) Glc β 1-Asn. Type (c) is predominantly found in intracellular proteins in the nucleus and cytoplasm.

area [15, 28, 29, 41, 42]. The current status of delineation of the rules that govern the selection of suitable glycosylation sites in the peptide sequence has enabled explanation of the nonrandom nature of this glycan attachment. It has been recognized that for N-glycosidic linkage a peptide sequence of Asn-X-Ser/Thr (a sequon) is required, in which the variable amino acid X seems to be important for the efficiency of recognition of the resulting sequon [43–45]. Additionally, the amino acid following a sequon (the Y-position in Asn-X-Ser/Thr-

Y) has a notable impact on glycosylation efficiency, especially for Asn-X-Ser sequons, Pro, Glu and Trp causing the most negative effect [46, 47]. For human and bovine plasma proteins C, human von Willebrand factor and human CD69 an unusual Asn-X-Cys sequon has been identified for N-glycan attachment [48, 49]. The definition of the sequon allows potential glycosylation sites in any protein to be deduced on the basis of amino acid sequence data. No equivalent peptide sequence requirement has been described so far for classical mucin-type O-glycosylation. The initiation of chain growth by several transferases with varying degrees of stringency of substrate recognition has at least suggested general rules and an algorithm of predictive value [50, 51]. A substantial functional redundancy of these transferases was inferred by mutagenic recombination of one gene with a deletion in the catalytic region, which did not impair O-glycan synthesis [52]. The lack of an O-glycosylation sequon does not mean that the reaction of the uridine diphosphate (UDP)-GalNAc:polypeptide N-acetylgalactosaminyltransferases can take place without sequence requirements. The spatially accessible acceptor amino acid appears to be surrounded preferentially by Ser, Thr, Pro, Ala and Gly residues [28, 29, 50, 53]. Ser/Thr residues not only serve as O-glycan acceptors but also as adaptors for another class of glycans. The third type of complex carbohydrates are the glycosaminoglycans using the glycan-protein linkage 4Xyl β 1-Ser/Thr (fig. 4) [54]. Usually, a trisaccharide 4GlcA β 1-3Gal β 1-3Gal β 1- is attached to the 4-position of Xyl before the typical building blocks of the individual glycosaminoglycan are added [55]. In addition to allowing GalNAc and Xyl attachment, the hydroxyl groups of Ser/Thr are sites for the incorporation of other glycan modifications. Despite their comparatively low frequency they should not be overlooked in this context. On the contrary, they illustrate the existence of cytoplasmic glycosylation pathways independent of these classical routes, each case an economic argument against a merely superfluous expression of the engaged glycosyltransferases. Epidermal growth factor (EGF)-like domains of several glycoproteins including blood coagulation factors VII, IX and XII, plasminogen activators and protein Z are the targets of a sequon-dependent O-fucosylation and O-glucosylation [56, 57]. The enzymatic introduction of α -L-fucose (α -L-Fuc); (fig. 5a) can initiate extension to Glc β 1-3Fuc α 1-Ser/Thr or to a sialylated N-acetylglucosamine sequence at the 3-position [58, 59]. The sequon dependence of fucosylation in the EGF-like domains of tissue-type plasminogen activator (t-PA) and urokinase was reinforced by the absence of this modification in the human chimeric plasminogen activator K₂tu-PA lacking this sequence element [56, 60]. The consensus sequence for the reaction of this fucosyltransferase is Cys-X-X-Gly-Gly-Ser/

Thr-Cys-, which can also be found in other types of proteins besides EGF module-bearing proteins [56, 59]. Once added, protein-bound L-fucose has been intimated to be important for mediating binding of the fucosylated protein to human HepG2 hepatoma cells, because fucose-free EGF-like domains lack this capacity [61]. Since binding and degradation of t-PA is not inhibited by 50 mM L-fucose in the case of human and rat hepatoma cells [62], this suggestion requires further experimental support. Another possibility concerns the

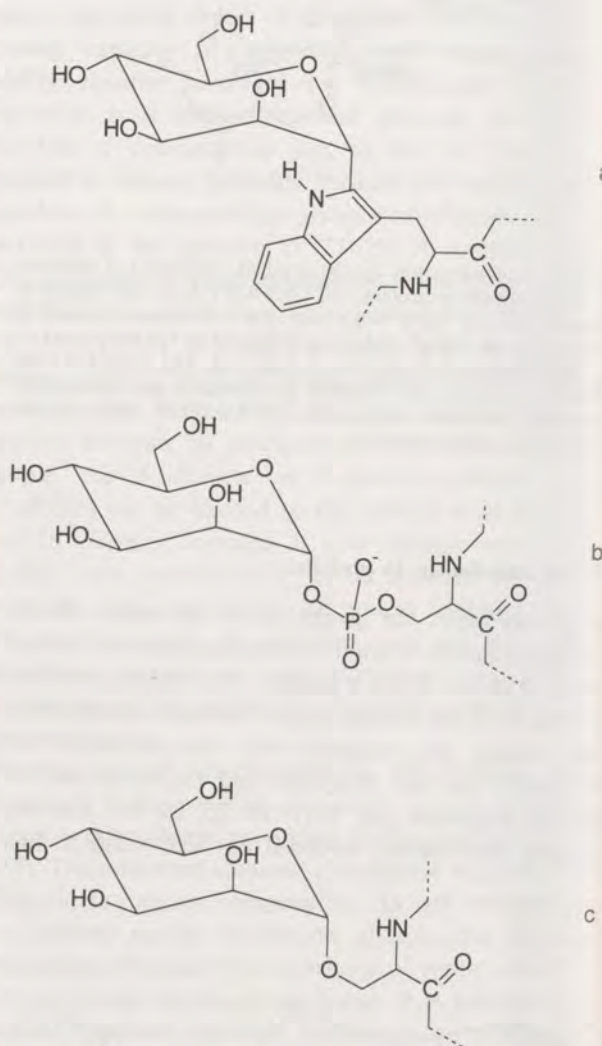
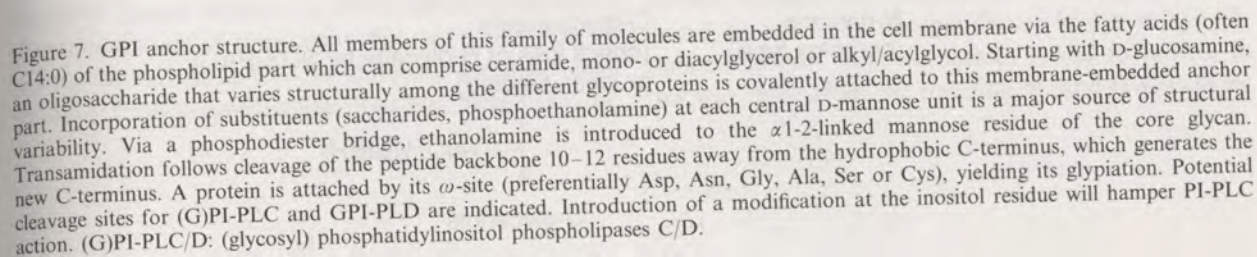
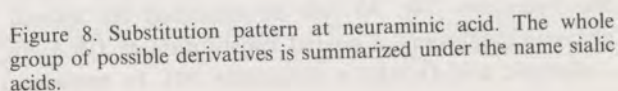


Figure 6. C-C linkage of Man and Trp in human RNase (ribonuclease) U_s (a). This is currently the only example known without N- or O-glycosidic linkage between saccharide and protein. Neither biological implications nor enzymatic ways for biosynthesis are known so far. Structure of the Man α 1-P-Ser linkage as an example of phosphoglycosylation (b). Instead of D-mannose, Fuc, GlcNAc and Xyl may also be found. Man α 1-Ser linkage as common glycosylation motif in yeast (c).



In contrast to the rather restricted appearance of these two modifications [56], a β -linked GlcNAc (fig. 5c) can be attached to an extended array of proteins [66–70]. So far, no elongation of this type of modification has been reported. It is confined to a single monosaccharide linked to the protein, and is present on a steadily growing list of intracellular proteins, for example cytoskeletal constituents such as erythrocyte band 4.1 protein, neurofilaments, cytokeratins 8, 13 and 18, transcription factor Sp1 or c-myc, estrogen receptor and p53 [70, 71]. A reciprocal relationship to phosphorylation is assumed for the reversible formation of GlcNAc β 1-Ser/Thr, as such glycosylation sites may also be targets of protein kinase(s) action. Functional implications also include a role in transcriptional initiation by RNA polymerase II and in cytoskeletal assembly and organization [70–72]. GlcNAcylation is also possible for hydroxyproline in slime molds, where a Gal/Fuc-containing pentasaccharide appears to be the nonimmunogenic product of a hitherto unrecognized cytoplasmic O-glycosylation pathway [73]. The lack of immunogenicity is reason to suspect occurrence of similar structures in mammals. Hydroxyl groups of amino acid side chains can further accommodate conjugation with α -Gal (Ser in plant glycoproteins such as the

In this context, an even more exceptional analogy to members of a different urkingdom can be drawn for a



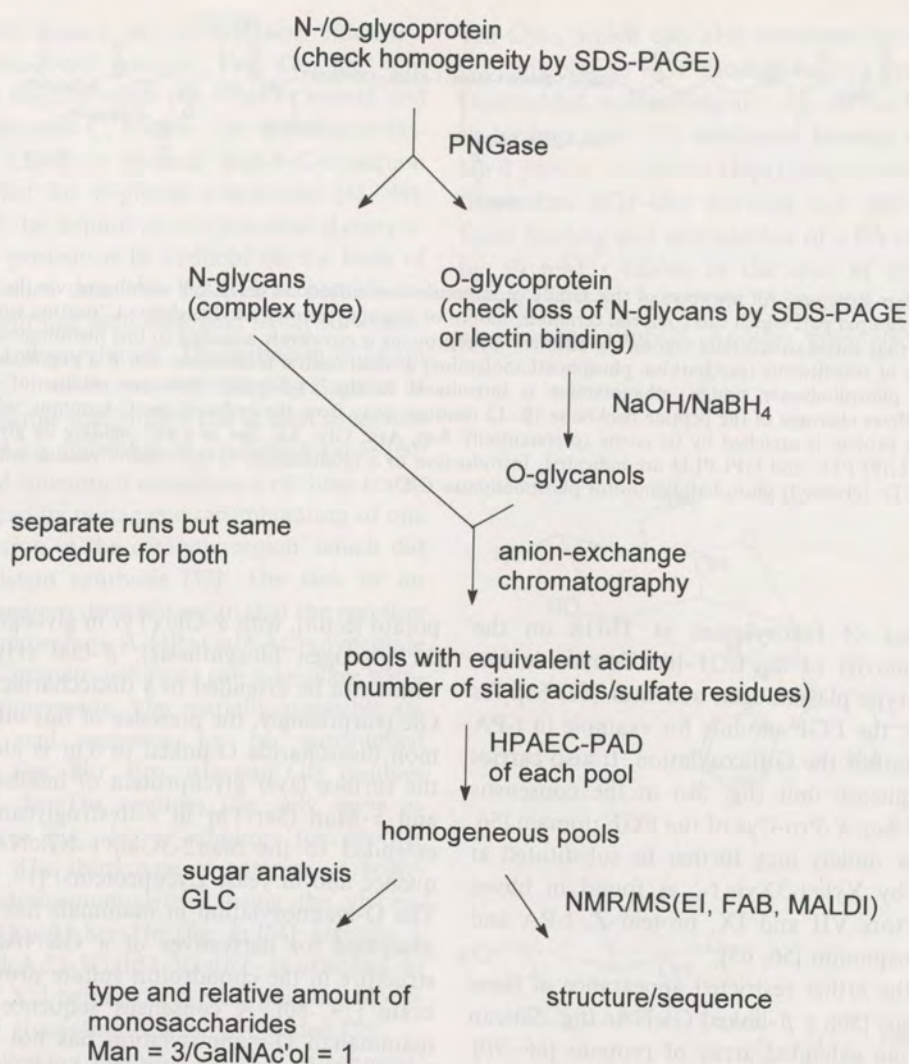


Figure 9. Flow diagram of a typical analytical strategy for the structural elucidation of N-/O-glycans. The given procedures represent commonly used steps. Depending on the type of glycans and the equipment available, other methods may also be used, as given in the text, for example separation of glycans with capillary zone electrophoresis as well as methylation analysis of the glycan followed by modified mass spectrometrical analyses.

second type of glucosylation besides that of EGF domains. Glc β 1-Asn (fig. 5d) is described with a stoichiometry of at least one modified site per molecule to occur in the B2 chain of mammalian laminin, a major basement membrane glycoprotein [82]. This linkage unit had been discovered before in surface glycoproteins of extreme halophilic archaebacteria [83]. In *Halobacterium halobium*, asparaginyglucose of an Asn-X-Thr(Ser) sequence is extended by a short chain of two or three β 1-4-linked D-glucuronic acid (GlcA) residues sulfated in the 3-position [74]. As will be described for animal glycosaminoglycans, a partial substitution of GlcA by L-iduronic acid (IdoA) is detectable [84]. In moderate

halophiles such as *Haloferax volcanii*, a chain of 9 or 10 β 1-4-linked Glc moieties replaces the highly charged glycosaminoglycan-like chain attached to asparaginyglucose [85]. A plant-specific modification of L-hydroxyproline-rich glycoproteins such as Ser-Hyp₄-containing extensins, repetitive proline-rich proteins, arabinogalactan-proteins or solanaceous lectins is arabinosylation, which appears to follow the rule of Hyp contiguity [86, 87]. In retrospect, all the multiple documented modes of O-glycosylation can confound nonspecialists. The distinction between the abundant mucin-type form starting with α -GalNAc and less frequent non-mucin-type structures has therefore taken root in the literature.

In the examples given the acceptor group on the protein is either an amide N atom or in a more promiscuous fashion a hydroxyl O atom in the side chains of Ser/Thr/Tyr/Hyl/Hyp. Although these two conjugation modes indubitably are the most abundant and prominent reactions in protein glycosylation, the enzymology of sugar incorporation has found at least two other means of glycan attachment. The recent description of C-mannosylation of tryptophan (fig. 6a) in the case of ribonuclease 2 from cells of different mammals [88] prompts further investigation into the mechanism of

this reaction. Its product is not found in recombinant ribonuclease 2 expressed in plant protoplasts, insect cells or *Escherichia coli*. In addition to the C-glycosylation, an indirect attachment of saccharide to protein is described with phosphoglycosylation [89]. Its occurrence explains why it would be incorrect to exclusively assume a direct connection between the glycan and the protein during attachment. For example, α -Man is linked to Ser via a phosphodiester bridge in this instance (fig. 6b). Oligosaccharides linked to protein in this way differ from the common N- and O-glycans by

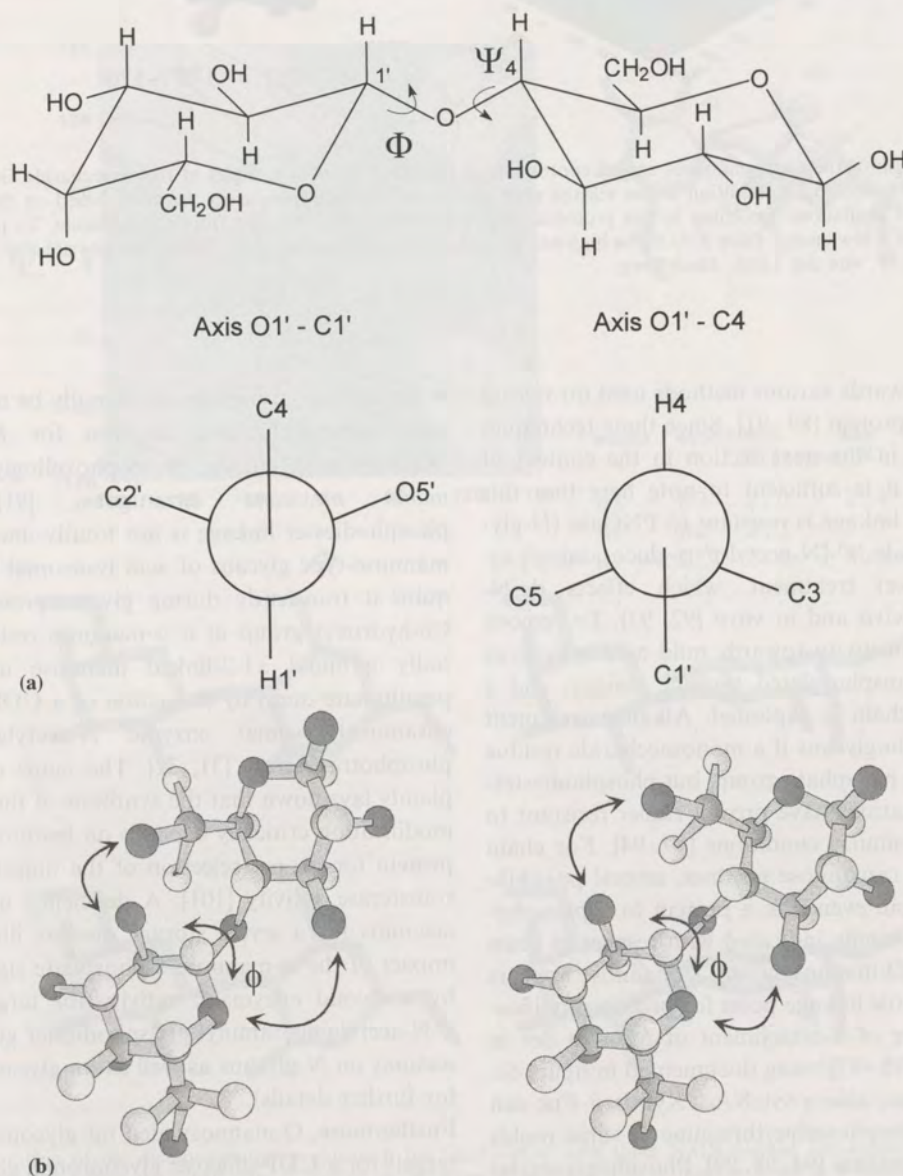


Figure 10. Torsion angles Φ and Ψ for the glycosidic linkage of lactose (see fig. 2: β 1-4-linkage) with the corresponding Newman projections (a). The impact of an alteration of only one torsional angle (Φ ; about 25°) on the relative positioning of two selected pairs of OH-groups in the two monosaccharide moieties is indicated by arrows (b).

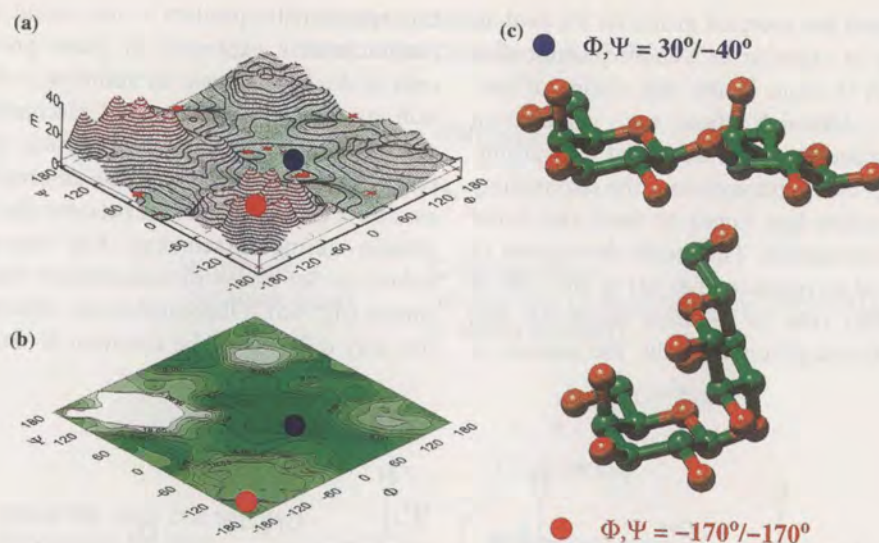


Figure 11. Illustration of the conformational energy contour maps for the Φ/Ψ -torsion angles of the disaccharide Gal β 1-3Gal in 3D- (a) and 2D-representation (b). In addition to the relaxed map the crosses indicate low-energy positions based on the conformational clustering analysis. Calculations according to this protocol remove the Φ/Ψ -grid constraint from the molecule. To provide an insight into the geometry of a low-energy (blue dot) and a high-energy (red dot) conformation, their representations are also given (c). Kindly provided by Dr. C.-W. von der Lieth, Heidelberg.

their behavior towards various methods used for release of glycans from protein [89–91]. Since these techniques will be outlined in the next section in the context of glycan analysis, it is sufficient to note here that this phosphoglycosyl linkage is resistant to PNGase (N-glycanase or peptide-N⁴-[N-acetyl- β -D-glucosaminyl]-asparagine amidase) treatment, which effects de-N-glycosylation in vivo and in vitro [92, 93]. To remove the glycan, its sensitivity towards mild acid hydrolysis resulting in a phosphorylated L-serine residue, and a released glycan chain is exploited. Alkaline treatment will cleave phosphoglycans if a monosaccharide residue is attached to the phosphate group, but phosphodiester-linked oligosaccharides have proven rather resistant to liberation under similar conditions [89, 94]. For chain extension on the D-mannose residues, several possibilities exist which can even link a protein to a phospholipid. As already briefly indicated with respect to yeast and mammalian O-mannosylation, D-mannose appears to be a very versatile linkage point for protein modification, the structure of α -attachment of Man to Ser in yeast [76, 77, 81, 95–97] being documented in figure 6c. Besides D-mannose, also α -GlcNAc, Xyl or β -Fuc can be linked to L-phosphoserine/threonine in slime molds and unicellular parasites [94, 98, 99]. Phosphoglycosylation with GlcNAc or Fuc has been identified so far in cysteine proteinases or spore coat proteins SP75 and SP96 from *Dictyostelium discoideum* [98–100] and with Xyl in phosphoglycoproteins of *Trypanosoma cruzi* [94].

A phosphate group can additionally be moved between two monosaccharides, as seen for R-Man α 1-PO₄-6Glc(or Gal)-R in the proteophosphoglycan of *Leishmania mexicana* amastigotes [91]. Such a phosphodiester linkage is not totally uncommon. High mannose-type glycans of acid lysosomal hydrolases acquire it transiently during glycan processing on the C6-hydroxyl group of a D-mannose residue (preferentially terminal α 1-2-linked mannose units but also penultimate ones) by the action of a UDP-N-acetylglucosamine:lysosomal enzyme N-acetylglucosamine-1-phosphotransferase [31, 32]. The name of the enzyme plainly lays down that the synthesis of the crucial sugar modification critically depends on features of the target protein for proper selection of the objects of glycosyltransferase activity [101]. A deficiency in this enzyme accounts for a severe storage disease, illuminating the impact of the D-mannose-6-phosphate signal generated by a second enzymatic activity for targeting, that is α -N-acetylglucosaminyl phosphodiester glycosidase (see sections on N-glycans as well as on glycans and disease for further details).

Furthermore, O-mannosylated rat glycoproteins are the targets for a UDP-glucose: glycoprotein glucose-1-phosphotransferase, which generates a Glc α 1-p-6-Man linkage cleavable by a α -glucose-1-phosphate phosphodiesterase [102, 103]. The functional role of this phosphodiester linkage is less unraveled than that of the

intermediate en route to the D-mannose-6-phosphate routing signal. This is also the case for another putative phosphodiester. Release of radioactive GalNAc-containing glycans by mild acid treatment from rat liver glycoproteins and acceptor properties of N-cadherin for the activity of a chicken neural retina cell surface N-acetylgalactosaminyl phosphotransferase argue in favor of the presence of phosphodiester-linked α GalNAc in further mammalian glycoconjugates [104, 105]. Turning back to the protozoan proteophosphoglycans, both the presently ill-defined functional aspect and the potential

to develop antiparasite drugs that interfere with the biosynthesis will spur further work. The provocative suggestion that this constellation of the various types of phosphoglycosylation might be a whim of nature can yet only be refuted by providing clear-cut evidence of biological relevance.

Functional reasoning for the phosphodiester is rather evident, when the phosphate group extends from a D-mannose residue to ethanolamine. A bridging role can be played by the glycan and its attached phosphatidylinositol, joining a protein's C-terminus to a

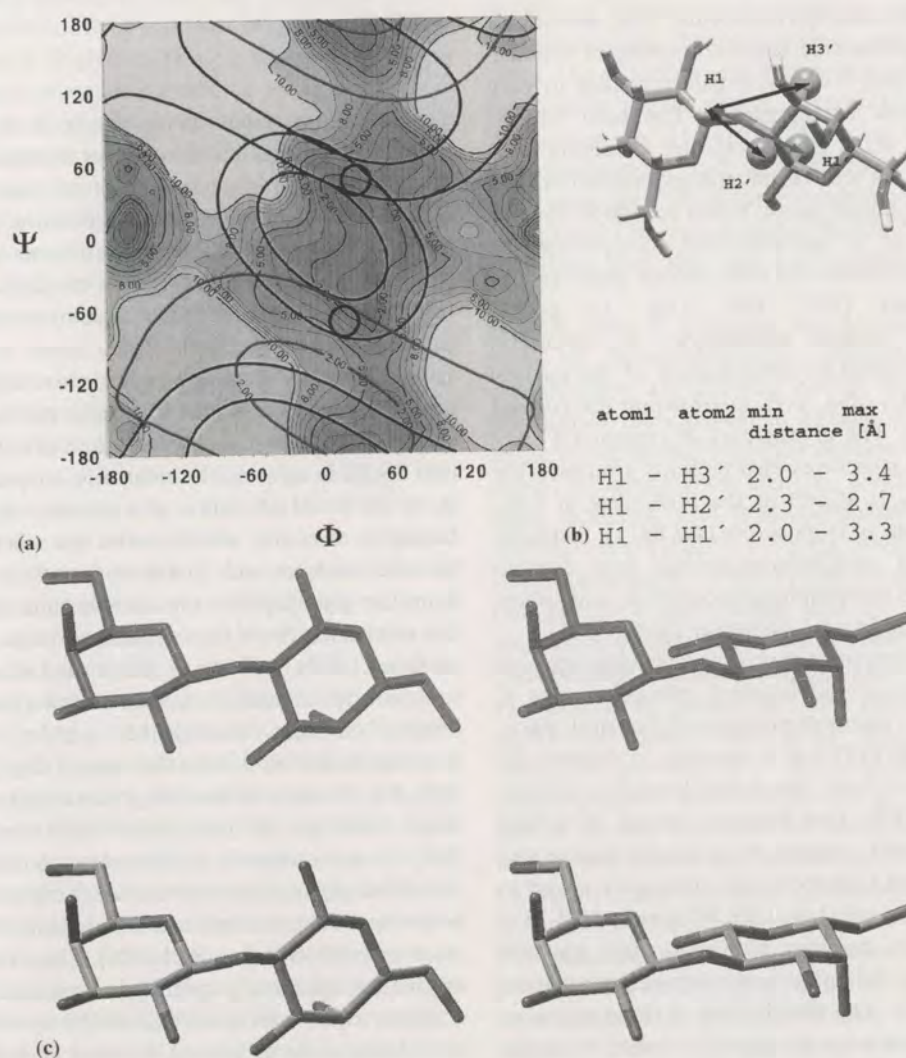


Figure 12. Illustration of the distance-mapping approach for Gal/β1-2Gal with three NMR-derived constraints. The three pairs of distance lines, calculated from the NMR data and given in (a), have no common area of overlap in the Φ,Ψ,E -plot. The characteristics of each pair are based on the signal intensities of an interresidual contact of the anomeric proton H1 with H1', H2' or H3', respectively, of the β1-2-linked sugar unit (b). Overlap between two sets of distance lines encircles sections of the two low-energy territories in the central valley of the Φ,Ψ,E -plot (a). This result of time- and ensemble-averaged NMR data argues in favor of two rapidly interconverting, energetically privileged conformations, presented to view in (c). Kindly provided by Dr. H.-C. Siebert, Munich, and Dr. C.-W. von der Lieth, Heidelberg.

membrane lipid, graphically demonstrated by the group of glycosyl phosphatidylinositol (GPI) anchors (fig. 7). They were first discovered in *Trypanosoma* [106]. In these structures the membrane anchor is formed by two fatty acids from a phospholipid often with defined chain lengths for the individual species, for example C14:0 for the variable surface glycoprotein (VSG) of *Trypanosoma brucei*. Diacylglycerol, 1-alkyl-2-acylglycerol and monoacylglycerol have been identified as hydrophobic membrane-entering domains [107, 108]. Moreover, diacylglycerol ceramide can serve as lipid part. Lipooligosaccharides containing ceramide do not belong to the group of GPI anchors and should be called glycosyl inositolphosphoceramides [107].

GPI anchors not only harbor differences in the lipid part. Via D-glucosamine α 1-6inositol one phosphate group carries a saccharide chain consisting of D-galactose, D-mannose and N-acetyl-D-galactosamine in variable species-specific arrangements. The basic unit is further extended via a phosphodiester involving a D-mannose residue, as seen in phosphoglycosylation, and ethanolamine. Its amino group forms an amide bond to the C-terminus of a (glyco)protein, completing the molecular bridge between the lipid anchor and a variety of (glyco)proteins [107, 109, 110]. In acetylcholinesterase of human erythrocytes the structural theme is further varied by esterification of the inositol residue with a fatty acid, even augmenting the contact to the membrane. The glycan part of certain GPI anchors and related structures also presents a noteworthy structural feature. It attests to the possibility of furanose ring formation of D-galactose (see fig. 1). Actually, the occurrence of galactofuranose has been demonstrated in glycosyl inositolphosphoceramide and -phospholipids of *T. cruzi* and *Leishmania* species [111].

Since the anchor structures expose specific cleavage sites for (glycosyl) phosphatidylinositol phospholipases C [(G)PI-PLC] and glycosyl phosphatidylinositol phospholipases D (GPI-PLD), it is possible to convert the (glyco)protein part from membrane-bound to soluble form [108, 112, 113]. This selective release of certain membrane-associated proteins by a phospholipase was substantial evidence to describe this linkage type and to guide structural analysis [114, 115]. Whereas (G)PI-PLC cleaves the linkage between phosphate and glycerol, GPI-PLD acts on the ester bond between phosphate and inositol (fig. 7). The metabolites of these reactions or other degradative steps produce a number of second messengers such as diacylglycerol, phosphatidic acid or inositolphosphate glycan [112, 116–119]. Besides the structural role that spatially associates a (glyco)protein to a membrane, GPI anchors are thus also reservoirs for signaling mediators upon stimulation or degradation. Especially the examples of rare modifications illustrate the enormous technical progress in detecting and delin-

eating new linker structures for glycans that occur often only in minor amounts. To think oneself capable of mastering the ensuing task of structure determination of the glycans is the result of an admirable increase in the power of the analytical tools available. The spectrum of natural ways of conjugating a glycan to a protein requires careful consideration of each step of the analytical procedures starting with the separation of the glycan from the carrier. Phosphoglycosylation, for example, may have escaped discovery for a long time because it is an acid-sensitive linkage and is thus hydrolyzed under conditions that are not typical for glycan–protein bond cleavage. Following their release from the protein part, the glycan chains are amenable to structural analysis.

Glycan analysis

The enormous potential for variation in glycan structures calls for analytical tools to unambiguously identify the nature and sequence of monosaccharides, anomeric configuration, linkage position as well as the nature and positioning of substituents on individual residues. Sophisticated strategies employing diverse approaches are required for complete structural elucidation [2–4, 6, 120].

Glycan analysis of glycoproteins may start with degradation of the protein part to a small peptide around the glycan linkage region. Hydrolytic cleavage of the peptide bonds is achieved by relatively unspecific proteases [3, 4]. To avoid formation of a mixture of small peptide fragments that may interfere with the subsequent carbohydrate analysis and therefore have to be separated from the glycopeptide, the already indicated release of the saccharide from the complete protein part is often preferred. This removal is performed chemically with sodium or potassium hydroxide/sodium borohydride or tritiated sodium borohydride yielding [^3H]-labeled oligosaccharide alditols in the case of O-glycans [1, 121, 122]. For the liberation of N-glycans, hydrazine is used, which destroys the polypeptide backbone completely [123]. In cases where it is desirable to retain partially or entirely deglycosylated protein for further analysis of its properties, trifluoromethanesulfonic acid (TFMSA) is a recommendable choice [124–126]. Since these methods are neither absolutely specific nor necessarily complete and may cause unwanted side reactions within the glycan chain such as loss of O- and N-acyl groups or peptide core degradation, the conditions have to be tested systematically to find a suitable way to remove all glycan chains with minimal harmful effects on the oligosaccharide chain. These problems are often addressed by enzymes requiring less drastic conditions for cleavage, although protein denaturation to ensure accessibility is still beneficial.

Endoglycosidases release complete or nearly complete glycan chains from the linkage area [127–129]. Several N-glycanases from different sources are available for action on N-glycans [93, 129, 130]. Similar to chemical deglycosylation, the enzymatic release of glycan chains may nonetheless be incomplete. Steric hindrance, which hampers accessibility of the N-glycosidic bond to the enzyme's reaction center, is usually overcome by protein denaturation. In the case of N-deglycosylation of native cell surfaces, the use of detergents has to be minimized or better avoided if the cells are to be taken for further studies. Commercially available enzymes exhibit fine specificities that allow a further discrimination between the various forms of N-glycans, for example high-mannose or complex types [93, 131, 132]. Enzymatic O-glycan removal is facilitated by at least two enzymes. Their activities towards O-glycans show a narrow specificity. Therefore, these enzymes cannot be expected to perform liberation of all O-linked saccharides [133–135]. An enzymatic means to demonstrate presence of O-glycans is the fragmentation of the glycoproteins by O-sialoglycoprotein endopeptidase [130].

Following release, the saccharide chains must be separated. Depending on the inherent complexity of the glycan mixture various techniques can be applied. Often, separation based on the number of acidic groups (sulfate or sialic acid) within one chain is performed as a first step. The availability of anion-exchange resins with high capacity and good resolution power also for high-performance liquid chromatography (HPLC) renders this step relatively easy. The choice of methods used for further separation of the fractions obtained depends on the characteristics of the components in the actual mixture. In many instances HPLC on a variety of resin types can be applied [136–139], for example anion-exchange, amino-phase, or reversed-phase columns as the case may be for individual saccharides or their derivatives to be separated. Relatively new and powerful methods are anion-exchange chromatography at high pH, mostly in combination with pulsed amperometric detection (HPAEC-PAD) [140–142], and capillary zone electrophoresis (CZE) which may be coupled, for example, to photometric or mass spectrometric detection systems [143–145]. Capillary electrophoresis with ultraviolet (UV) detection at 190 nm has been reported to be about 4000 times more sensitive than measurements by HPAEC-PAD [146]. At this stage, a characteristic fingerprint of neutral and mono- up to tetrasialylated N-glycans was obtained [147], pointing to the general applicability of this method.

To analyze the sugar composition of a glycan, methanolysis of the purified oligomers to monosaccharides is often performed by treatment with methanolic HCl yielding methyl glycosides of the monosaccharides or by aqueous hydrolysis with, for example, tri-

fluoroacetic acid followed by reduction of the monosaccharides formed with sodium borohydride to the corresponding alditols. A set of methods is available for the following determination of the nature of the individual sugars [3, 4, 6, 148], for example gas-liquid chromatography (GLC) analysis of either the methyl glycosides after trimethylsilylation to yield the per-O-trimethylsilyl ether derivatives or of the acetylated alditols [4, 149, 150]. HPAEC-PAD or HPLC after derivatization with, for example, 2-aminopyridine or 2-aminobenzamide are also exploited for monosaccharide analysis [4, 6, 138, 151–153]. When the hydrolysis of an oligosaccharide can deliberately be limited with site-specific tools, these techniques are the basis for establishing an approach to sequencing. By sequentially employing specific exoglycosidases such as sialidase, β -galactosidase, hexosaminidase and α -/ β -mannosidases, the sequence and anomeric configuration of the monomeric sugars within a typical N-glycan can be determined [2, 154–157]. Analyses of the liberated monosaccharides and the remaining oligosaccharides after each step of enzyme treatment are run in this procedure. This technique has further been refined up to the stage that kits are now commercially available [158].

At this point in chemical and enzymatic analysis the types of monosaccharides, sequence and anomeric configuration are clarified but in most cases not the position of each glycosidic linkage, a major source of sequence variability (fig. 2). Chemically, this problem is solved by methylation analysis, a rather old and ingenious technique [159]. It has been significantly improved over the years [160] by reducing possible contamination from the reagents, by modifying the procedure to ascertain complete methylation and by scaling down the amount of saccharide needed for modification. It is now even possible to analyze 100 ng of an oligosaccharide by this method in conjunction with gas-liquid chromatography/mass spectrometry (GLC-MS) analysis with selected ion monitoring of the permethylated, liberated and derivatized monosaccharides [6, 160]. A comparison of the old protocols for methylation of carbohydrates with up-to-date technology is probably one of the most telling examples for continuous improvement of already well-established methods.

For monosaccharide analysis there is still one major problem, the analysis of sialic acids. In contrast to other monosaccharides, this sugar is often already unwittingly removed from a glycan chain under relatively mild conditions. At the same time, it is easily degraded by acid or alkaline treatment. Thus, under conditions necessary to clip hexoses and (N-acetyl-)hexosamines from a glycan, sialic acids are almost completely destroyed. Moreover, the presence of a number of small substituents such as acetyl and lactyl groups (fig. 8) causes serious problems. These ester linkages are labile under

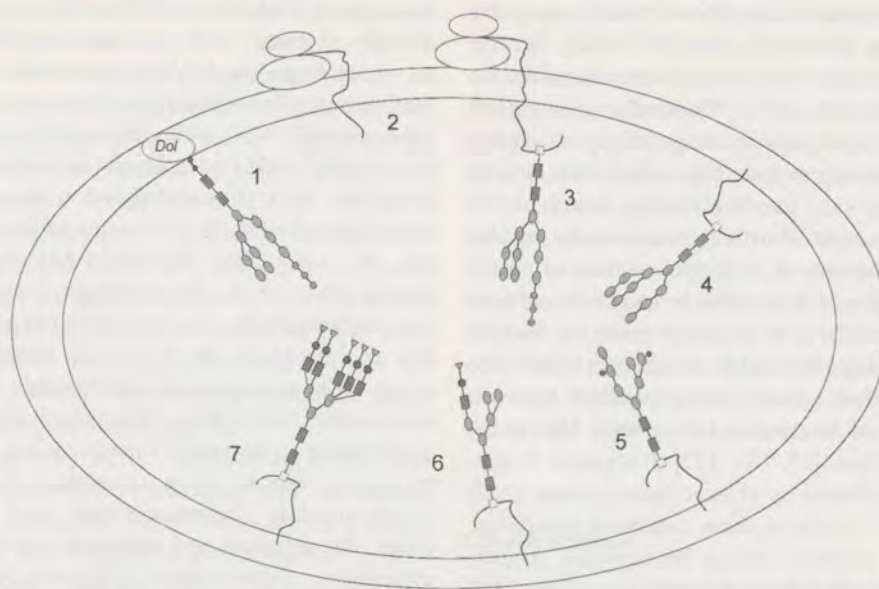


Figure 13. Illustration of the initial reactants and common products of processing of N-glycans in the endoplasmic reticulum. Starting with the oligosaccharyltransferase-mediated attachment (3) of the preassembled dolichol-linked precursor (1) to the nascent protein (2), several principal processing options for main types of products are possible, that is generation of high-mannose (4), hybrid (6) and bi- to pentaantennary (7) complex N-glycans. 6-Phosphorylation of D-mannose residues (5) decorates the protein for transport to the lysosomes by engaging P-type lectins (see table 1).

an array of conditions [30, 33, 34, 161–163]. The ester at the 4, 7, 8 or 9-hydroxyl positions can then be lost. In this context of sugar analysis the product of a peculiar monosaccharide modification reminiscent of Lys/Pro derivative formation deserves comment. In free sialic acids the amino function at C-5 is always acylated, in glycoconjugate-bound form in almost all cases. Acetylation to N-acetylneuraminic acid (Neu5Ac) does not occur at the stage of the amino-C9-sugar. It is already present on the biosynthetic precursor N-acetylmannosamine-6-phosphate, which is condensed with phosphoenolpyruvate to form 9-O-phosphorylated Neu5Ac [34, 164]. This phosphate group is finally displaced from Neu5Ac by a specific phosphatase. Unlike any N-acetyl group of other letters of the carbohydrate alphabet used in eukaryotes, this part of Neu5Ac is the target for hydroxylation to produce N-glycolylneuraminic acid (Neu5Gc). After activation to cytidine monophosphate (CMP)- β -Neu5Ac, monooxygenation can take place to supply CMP- β -Neu5Gc as the starting point for the synthesis of all Neu5Gc derivatives [165, 166]. The glycolyl OH function of Neu5Gc can even be acetylated, as found in rat thrombocytes [167], or can carry another sialic acid residue in glycoproteins from the starfish *Asterias rubens* [168]. Although Neu5Gc presence is widespread in mammals, normal human tissues do not contain this type of sialic acid [34, 164, 169]. The

reason for this lack in biosynthetic capacity is the loss of one exon coding for the N-terminal sequence from the gene of the human monooxygenase when compared with the genomic organization of the mouse homologue [170, 171]. This species-specific restriction notwithstanding, the sialic acid backbone is definitely the scaffold for the introduction of the largest number of modifications in the realm of carbohydrate biochemistry. Although the linkage establishment for the principal sugar unit is confined to α -2–3/6/8-bond formation [34, 172], manipulation at the functional groups graphically and positively impinges on the extent of potential coding units, as theoretically discussed in the section on the sugar code.

By derivatization of one or several of the OH functions of Neu5Ac or Neu5Gc at C-4, 7, 8 and 9, about 30 naturally occurring derivatives are formed carrying acetyl, lactyl, methyl, sulfate or phosphate as small substituents [30, 33, 34, 161–163, 168, 169, 173–176], with acetylation as the by far most abundant form of substitution. Another 10 modified forms are found in nature as derivatives of the 2,3-unsaturated sialic acid 2,3-dehydro-N-acetyl-D-neuraminic acid (Neu5Ac2en) [34, 169, 177]. Interestingly, Neu5Ac2en and its N-glycolyl derivative are very effective inhibitors of sialidases [178, 179]. This sugar has been used as lead structure for computer-aided drug design of a potent inhibitor of

influenza virus sialidases to fight the flu [180, 181]. It has also proven pertinent in inferring the biological role in growth and differentiation control of regulatable cell surface ganglioside sialidase activity of cultured human neuroblastoma cells [182–185]. It goes without saying that the presented structural complexity surely calls for an adequate panel of analytical techniques to accurately map the presence of any sialic acid. The methods used for sialic acid identification are colorimetry, thin-layer chromatography (TLC), HPLC, GLC-MS, different MS techniques and NMR. The enormous problems in sialic acid analysis, including loss and migration of acetyl groups even under physiological conditions and strategies that should solve them to avoid artifacts, have been detailed in several reviews [33, 34, 162, 163, 186]. Besides improving the handling of these delicate substances, solid insights into their enzymatic production have been gained. After many years of research the proteins essential for 9-O-acetylation of sialic acids appear to have been identified [187, 188]. If they conferred no functionally relevant properties, then the constitutive presence of a viral esterase, an enzyme that destroys sialic acid 9-O-acetyl groups [34, 189], to initiate a futile cycle would be without consequences. Obviously, the indispensable nature of 9-O-acetyl groups is underscored by a report on their destruction *in situ* in transgenic mice, which leads to arrest of development at the two-cell stage [190]. The addition of this 'small substituent' is therefore not a random event but most probably a site-specific modification regulating the ligand and properties of the sugar unit [34, 38, 191, 192]. Together with site-specific routing signals (e.g. Man-6-p or SO₄-4GalNAc, to be further discussed in the section on N-glycans) and docking points (e.g. 3-O-sulfation of the central modified GlcN unit in the serpin-binding site of heparin to be detailed in the section on proteoglycans), this result makes clear how important 'small substituents' are even on the level of the organism. Instead of processing by monosaccharide analysis, whole purified oligosaccharide chains can often directly enter structural investigation. The various mass spectrometric techniques are excellently suited to precisely determine molecular weights, branching or substitution patterns of glycans, when a battery of standards is available. Employing electrospray ionization or matrix-assisted laser desorption with, for example, 2,5-dihydroxybenzoic acid as matrix, even pseudomolecular ions have become readily accessible for analysis without prior derivatization. The major drawback of this technique is that structural information is obtained in terms of hexose, N-acetylhexosamine and so on; discrimination between epimers such as D-glucose and D-galactose is not possible. With MS-MS techniques, analysis of rather complex mixtures is also feasible. In the course of such measurements, a mass spectrometric peak resulting

from the ionized 'parent molecule' is further fragmented, and the resulting 'sequence ions' originating from this individual peak are recorded in the spectrum (daughter-ion scan). In addition, it is possible to screen for the original fragment from which a certain peak is formed (parent-ion scan). In this way, interpretation of a mass spectrum does not only depend on the occurrence of a given fragment that may also be derived from a contaminant but also on a set of interdependent ions, which increases the reliability. Mass spectrometry is also very often performed after initial runs with separation methods such as GLC, HPLC or CZE [193–201]. Homogeneous glycans can then be subjected to the most powerful analytical tool for glycan analysis. ¹H-NMR analysis invariably demands highly purified substances. With this method it is not only possible to identify individual monosaccharides, in almost all cases including type and position of substituents on these residues, but also to deduce anomeric configurations, linkage points and sequence of the residues [202]. This analytical step concludes the processing, as summarized in a scheme for a strategy of glycan analysis (fig. 9). This figure illustrates that a complete run obviously asks for access to expensive equipment and precious methodological know-how. If the experimental question does not require a complete analysis, versatile and less demanding one-step methods would be ideal to detect the presence and type of glycosylation and of defined sequences. A rapid and efficient means of documenting the presence of glycans with certain structural features in a mixture is the monitoring with epitope-specific probes, that is antibodies or lectins. They are conveniently purified by the versatile technique of affinity chromatography on resins with immobilized carbohydrates or glycoconjugates [203–208]. Either solid-phase assays or (serial) affinity chromatography with well-characterized marker proteins (lectins or monoclonal

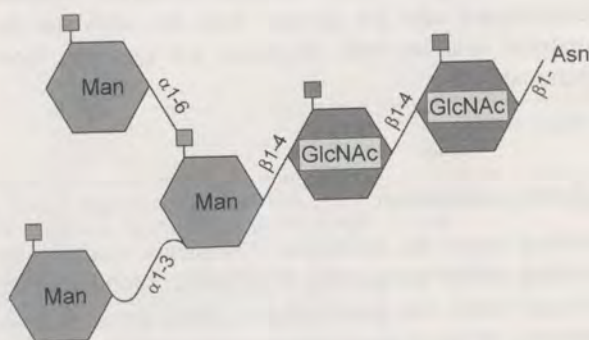


Figure 14. Core structure of N-glycans, that is Man₃GlcNAc₂-Asn (see fig. 13; common part of structures 4–7).

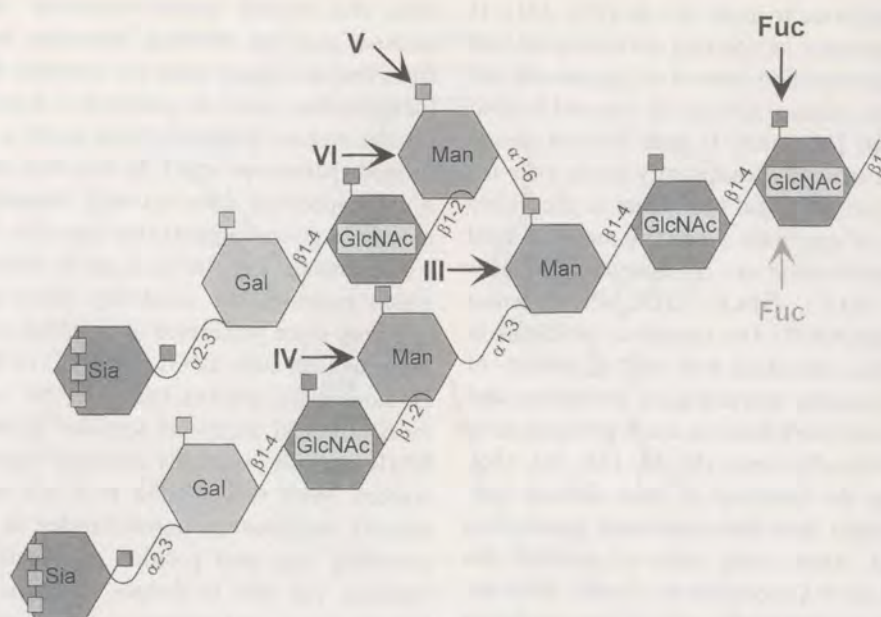


Figure 15. Mature biantennary N-glycan. In the process of maturation this N-glycan can accommodate several substituents, which initiate the formation of other complex N-glycans, for example a pentaantennary product given as structure 7 in figure 13. Further GlcNAc residues may be attached to the core at the positions indicated by the arrows to enable addition of antennae (see structure 7 in fig. 13 for scheme). The roman numbers refer to the temporal sequence, in which the corresponding GlcNAc transferases have to act to assemble tri- to pentaantennary structures. An exception is the transferase III inserting a so-called bisecting GlcNAc, which cannot serve as acceptor for further chain elongation. Fucosylation at the peptide linkage GlcNAc (see fig. 3) occurs at the 3- and 6-position antennae may not be complete or may contain varying lengths of N-acetylglucosamine repeats and distinct modifications such as LacdiNAc-SO₄. The LacdiNAc terminus can alternatively be modified by α 2-6 sialylation or α 1-3 fucosylation.

antibodies) may be pertinent to prove the presence of a certain glycan type or sugar epitope in one step [208–215]. The tools will not only act on isolated compounds but can also localize carbohydrate epitopes in cell preparations and tissue sections [208, 216–220]. Beyond the solution of a technical problem, this application also points to a role of this recognition process in the cell biological context. It also argues in favor of the validity of the assumption of an already mentioned ‘discrete recognitional role’ for glycans, when the tools and the analyzed samples both originate, for example, from mammals.

Glycan conformation

Having solved the problems of sequence analysis, including correct assignment of anomeric configurations, linkage points and positioning of substituents, the description of the structural characteristics of oligosaccharides can move to the next level, that is the three-dimensional (3D) depiction of the conformation. To address this issue properly, a combined approach

has proven valuable. Already at this stage it should be mentioned that the thorough and reliable description of glycan (ligand) conformation in solution will have a far-reaching impact for drug design [221, 222]. By letting the thumbs of each hand touch, the reader can easily form an instructive model of the flexibility of a disaccharide. By then starting to rotate each hand separately, the major source of inherent flexibility about the dihedral angles is readily visualized (fig. 10a). Naturally, not all possible conformations defined by the two angles Φ and Ψ will be equivalent in terms of their energy levels. As illustrated for a change in only one torsion angle for lactose (fig. 10b), the distances of pendant groups are affected markedly by this alteration. To calculate the correlation between the geometric and energetic factors, a force field has to be established. Considering each energetic contribution such as bond deformations, valence angle distortions or nonbonded interactions, topographic maps can be drawn linking energy levels to sets of conformations [223–231]. They can be presented in 3D- or 2D-formats, as shown in figure 11a,b. To familiarize the reader with the view of the molecule’s shape at different energy levels, the con-

formations of two positions from high- and low-energy levels are shown in figure 11c. It is remarkable that not only one low-energy position in the map can result from these calculations. As seen in figure 11 for Gal β 1-3Gal and in figure 12 for Gal β 1-2Gal, several area sections are energetically preferred regions [20, 232]. To underscore that this result is not exceptional, it is worth pointing out that conformers of β -lactoside derivatives in the low-energy region occupy approximately 5% of the total potential energy surface [233]. Evidently, it is possible that different area sections are on similar energy levels, for example in two parts of the central valley (figs 11, 12). The histoblood group A tetrasaccharide, too, appears to lack rigidity and may switch conformational parameters between two energetically rather equal positions [234]. In contrast, Le^x and Le^y epitopes are essentially rigid molecules, highlighting the conclusion that no general a priori statements are justified.

In order to change the conformation and move to a new position in the Φ, Ψ, E -maps, energy barriers must be crossed with the amount of motional energy available to the atoms. By running molecular-dynamics simulations, the question can be answered whether the different energetically preferred regions in the entire Φ, Ψ -space will actually be populated. These calculations gain access to 'snapshots' of the molecule, which will not complete stretch vibrations of a C-H-bond between two computational recordings [229]. Consequently, the time interval between two computational recordings will be in the order of 1 fs. These calculations for Gal β 1-2Gal, whose topographical Φ, Ψ -map is shown in figure 12a, reveal a rapid oscillation in the central low-energy valley with only a rare crossing of energy barriers to one side minimum [20, 235]. The computer-assisted calculations thus allow us to discern an important aspect of oligosaccharide behavior, that is the inherent mobility. This factor will certainly not be without impact on the chances for success of crystalliza-

tion. Indeed, it can be the cause of frustration for crystallographers. Despite the logical development of the algorithms and these truly unpleasant indications of their validity, these calculations inevitably are in need of further experimental confirmation.

The experimental data basis originates from NMR spectroscopy using the nuclear Overhauser effect (NOE, a through-space magnetization transfer with r^{-6} -dependence) as ruler for atomic distances up to a limit of 3.5 Å for oligosaccharides [224, 236–238]. One crucial difference between the calculations and the NMR measurements concerns the different time scales [237, 239–241]. Within a period of milliseconds during NMR monitoring, the molecules can frequently change conformations, yielding time and ensemble averaging [237, 239–241]. At any rate, each NOE contact provides a distance constraint for the molecule. In the topographical maps this constraint is incorporated as pair of lines which delimit the allowed Φ, Ψ -section [242]. For Gal β 1-2Gal, three contacts are measurable (fig. 12a,b) [20, 232]. Since no conformation can be seen upon examination of the Φ, Ψ, E -map which can satisfy the three pairs of experimental distance constraints (fig. 12a), such a conformation is referred to as 'virtual' [237, 243]. However, two areas of overlap for two pairs of contour lines exist (fig. 12a), and their positioning makes it reasonable to assume that two conformations in the two low-energy sections of the central global valley (fig. 12c) fulfill the NMR-derived criteria [20, 232, 235]. This interpretation reconciles theoretical and experimental data. In cases where signal overlap causes severe problems, additional spectral dispersion is offered by heteronuclear experiments with isotope (¹³C)-enriched oligosaccharides [244, 245]. To extend such measurements to the level of glycan chains, for example for glycan intermediates during intracellular processing and cell surface-presented oligosaccharides [192, 246, 247], it is essential to have sequence information of cellular glycoconjugates available.

Table 1. Main families of animal lectins.

Family	Structural motif	Carbohydrate ligand	Modular arrangement
C-type	conserved CRD	variable (mannose, galactose, fucose, heparin tetrasaccharide)	yes
I-type	immunoglobulin-like CRD	variable (Man ₆ GlcNAc ₂ , HNK-1 epitope, hyaluronic acid, α 2,3/ α 2,6-sialyllactose)	yes
Galectins (formerly S-type)	conserved CRD	Gal β 1,3(4)GlcNAc core structures with species- and galectin type-dependent differences in affinity for extensions to blood group A, B or H epitopes; internal stretches of poly(N-acetyllactosamine) chains	variable
Pentraxins	pentameric subunit arrangement	4,6-cyclic acetal of β -galactose, galactose, sulfated and phosphorylated monosaccharides	yes
P-type	conserved CRD	mannose-6-phosphate-containing glycoproteins	yes

CRD, carbohydrate recognition domain; from [298] with modification.

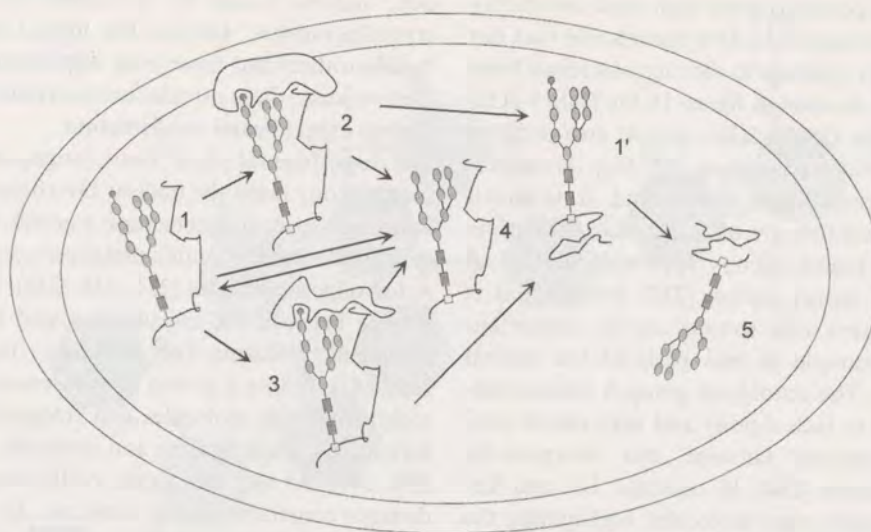


Figure 16. Illustration of a role of the monoglucosylated glycan intermediate(s) in quality control. The molecular chaperones calnexin (type I membrane protein) and its soluble homologue calreticulin bind this sugar structure (2, 3) and can affect the folding process positively, leading to a mature product (1'). Cleavage by an endo- α -mannosidase at this stage (5) or by sequential action of α -glucosidase II and the ER α -mannosidase removing the α 1-2-linked mannose moiety not from the α 1-3 arm but from the α 1-3 branch of the α 1-6 arm yielding the classical isomeric $\text{Man}_8\text{GlcNAc}_2$ structure precludes further retention of the glycoprotein in this compartment and re-entry into quality control. This is otherwise possible via the concerted actions of α -glucosidase II and UDP-glucose:glycoprotein glucosyltransferase (1,4).

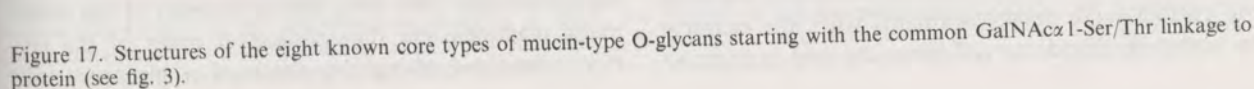
N-Glycans

Glycoproteins of this type usually contain the $\text{GlcNAc}\beta$ 1-Asn linkage (fig. 3) discussed above. The sugar chains mainly contain GlcNAc, Man, Gal, Fuc and sialic acids. An essential feature of these glycans is their preassembly on a lipid-based anchor in the endoplasmic reticulum. This glycan chain composed of nine Man, two GlcNAc and three Glc residues and attached to dolichodiphosphate is transferred en bloc onto the amide function of an appropriate Asn residue in a sequon within a nascent protein chain by an oligosaccharyltransferase (fig. 13) [28, 29, 248–253]. Glycosylation and folding of a nascent protein chain can compete in vivo, which explains the lack of utilization of sequons which are rapidly rendered inaccessible [254, 255]. Following the transfer of the preassembled glycan to the protein, this chain is processed immediately. Nonetheless, the cotranslational addition of a glycan can instigate shifts in the folding pathway of the nascent protein chain [251, 256]. In a defined order individual sugars are released from this glycan. Remarkably, computer-assisted calculations of the mono-, di- and triglucosylated $\text{Man}_9\text{GlcNAc}_2$ -chains reveal full accessibility of the glucose moieties to any putative receptor protein [246]. This process is the prerequisite for enzymatic remodeling of the sugar chain, producing one of the three common subtypes of N-glycans, that is high-mannose,

hybrid and complex-type chains [1, 25, 75, 257–259]. The topological arrangement of glycosyltransferases and the levels of substrates are decisive and fine-tunable factors which control the glycosylation potential of the Golgi apparatus [260–262]. These factors introducing natural variability are accounted for by rigorous batch controls of the products of recombinant gene expression in mammalian cells by techniques, which have been outlined in the section on glycan analysis, to ensure uniform pharmaceutical quality [263–265]. As depicted in figure 13, the main types of N-glycans can be generated by enzymatic extension of the processed precursor. They have the core structure $\text{Man}_3\text{GlcNAc}_2$ in common (fig. 14). In this structure only the five monosaccharides closest to the $\text{GlcNAc}\beta$ 1-Asn linkage region of the original high-mannose type ($\text{Man}_9\text{GlcNAc}_2$) are still present. Especially in the case of the complex type, the outer part of the antennae can present unique termini, giving distinct glycoproteins a characteristic signature. The crucial step in complex-type glycan biosynthesis is the attachment of GlcNAc at the α 1-3-linked D-mannose residue even before the α 1-6 arm has been completely processed. Further introduction of GlcNAc molecules paves the way to multiantennary structures. In figure 15 an example of a resulting biantennary N-glycan is depicted together with the positions, where elongation of additional antennae is initiated. The Glc-

A control point committing glycan synthesis to hybrid or complex N-glycans is provided by the action of GlcNAc transferase III. Whereas GlcNAc transferase I acts as a GO signal, the modification of the inner β -linked mannose moiety prior to α -mannosidase II

processing (incorporation of bisecting GlcNAc) prevents the actions of this $\alpha 3/6$ -trimming enzyme, GlcNAc transferases II, IV and V and core $\alpha 1$ -6-fucosyltransferase [28]. The product of GlcNAc transferase III at this stage can thus be considered as a STOP signal. On the other hand, foregoing processing by α -mannosidase II, which trims the $\alpha 1$ -6-linked antenna, guides the glycan in the direction of complex-type chains, often terminating with a sialic acid in branches. If sialic acid is added, it will be in $\alpha 2$ -3 or $\alpha 2$ -6 linkage, as already indicated in the preceding chapter. A peculiar sialylation can enzymatically follow at an $\alpha 2,3$ -linked sialic acid moiety of an N-glycan of a neural cell adhesion molecule (N-CAM). Surprisingly, this $\alpha 2$ -8-linked polysialic acid with a chain length of 8 to above 100 is structurally identical to a component of the surface coat of Gram-negative bacteria [266–268].



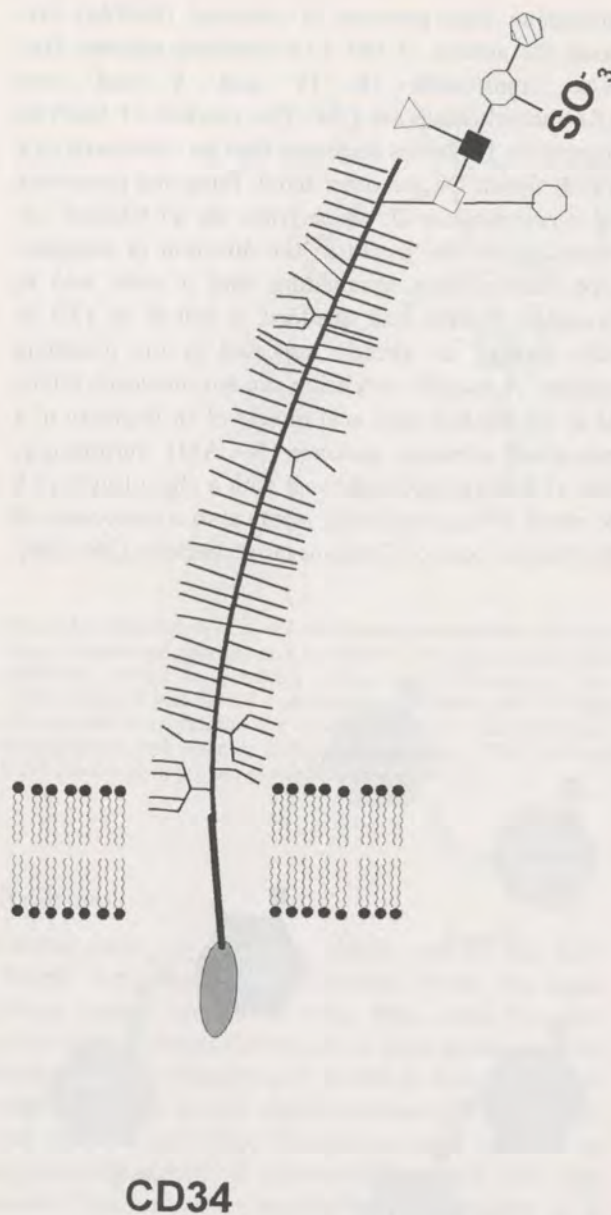


Figure 18. Illustration of the appearance of a mucin-type glycoprotein, that is CD34 expressed on hematopoietic precursor cells and vascular endothelium. The terminal part of the glycoform expressed by high endothelial venules which is accessible for interactions most readily contains a structure serving as docking point for L-selectin (see fig. 31), that is a sulfated Le^x-epitope. Kindly provided by Priv.-Doz. Dr. H. Kaltner, Munich.

The two types of ubiquitous enzymatic sialylation may even occur on different antennae in one glycan. Remarkably, chemoenzymatic synthesis has mastered the problem of delivering these glycan chains and introducing custom-made neoglycoproteins with, for example, an undecasaccharide to cell biological and histopatho-

logical testing [269–272]. Coupling of purified glycans from natural sources is thus no longer the only approach to recruit complex oligosaccharides as tools for receptor search [273–276], whose concepts and successes will be presented in the section on glycan recognition. When no further processing will reduce the size of the Man₅GlcNAc₂ chain, it is committed to the generation of high-mannose-type glycans, which are common products of yeasts but are also present in animal glycoproteins such as ovalbumin. Interestingly, monitoring their expression has unraveled dynamic remodeling even after reaching the cell membrane. For high-mannose-type N-glycans on the plasma membrane of rat hepatocytes and hepatoma cell lines, remodeling of the glycan chains at the cell surface or during endocytosis was reported. Postbiosynthetic modification includes removal of up to three mannose residues by stepwise demannosylation [277]. Trimming already processed glycoproteins by reglycosylation has also been suggested for complex-type N-glycans on the basis of the significantly shorter turnover rates of terminal sugars (sialic acid, D-galactose, L-fucose) when compared with the protein [278]. Thus, reglycosylation may serve as a repair mechanism of these complex compounds which had encountered glycosidases [279, 280]. This process is different from the appearance of free oligomannosides in the cytoplasm after their formation by hydrolysis as a bypass of the dolichol cycle [281].

Besides elaboration of the outer sections of the antennae, the immediate core residue is also the target of a modification. L-Fucose can be added to the GlcNAc residue linked to Asn. In mammals it is bound to the 6-position, in plants at the 3-position [40, 75, 87, 282]. In insect cells difucosylation is observed [283]. This modification requires two different enzymes and follows a strict order in adding first the α 1-6-linked fucose and then the α 1-3-linked residue [284] (fig. 15). The ensuing immunogenicity of the α 1-3-modified structure is one reason for concern to employ insect or plant cells for the production of therapeutic glycoproteins [285–287]. This aspect will be further discussed at the end of this chapter.

Along the route that N-glycans move within the cell the corresponding enzymes needed to complete the synthesis of these complex molecules are located. In the endoplasmic reticulum, the en bloc transfer from the dolichol precursor to the protein takes place followed by the first steps of modification, before any trimmed N-glycan moves to the cis-Golgi. Within the Golgi network further modification to the complex-type N-glycans takes place, and sialylation as the final modification occurs by the corresponding sialyltransferases in the trans-Golgi [288–292]. The complexity of the enzymatic reactions and the topological order of the enzymes involved along the route of a glycoprotein's

biosynthesis determine the display of glycans on the cell surface and the sorting of N-glycosylated proteins. One example is provided by the preferential labeling of lysosomal enzymes with mannose-6-phosphate in a two-step reaction [31, 32, 293, 294]. The transient presence of a phosphodiester linkage after the first step has already been noted in the chapter on glycan attachment to proteins when talking about phosphoglycosylation. As pointed out in figure 13 and its legend, this signal is the equivalent of a postal code taking advantage of specific receptors (P-type lectins; see table 1 and the section on animal lectins for further information) for prompt delivery to the final destination. Without it, misrouting of the hydrolases destined to end up in lysosomes will occur, and a severe lysosomal storage disease with dense inclusion cells (I cells) develops, as further explained in the section on glycans and disease. The analogy between defined (uncommon or unique) sugar compounds and postal codes is tempting. Indeed, analysis of intracellular transport of glycoproteins contradicts the view of the cargo system as a singular fortuitous event. In the routing of N-glycoproteins between the endoplasmic reticulum and the cis-Golgi and further post-Golgi trafficking, several lectins (calnexin, calreticulin, ERGIC53, VIP36, comitin) are involved [295–299]. Evidence in the case of ERGIC53 has accu-

mulated that this lectin plays a role in efficient intracellular transport of a certain subset of glycoproteins [300, 301]. Having already emphasized the spatial accessibility of the only transiently present glucose units on each processed N-glycan, it is intriguing that the putative receptors have been defined, that is calnexin and calreticulin [296, 302, 303]. The presence of one glucose residue is needed for recognition by the molecular chaperones calnexin and calreticulin, which are engaged in aiding correct folding of various proteins as an early quality control during biosynthesis (fig. 16). If the recognition signal constituted by the monoglucosylated glycan (GlcMan₉GlcNAc₂) is prematurely clipped off by a glucosidase II, incompletely folded glycoproteins can still be recognized by UDP-Glc: glycoprotein glucosyltransferase and relabeled for re-entention in the endoplasmic reticulum [304, 305]. As with UDP-N-acetylglucosamine:lysosomal enzyme N-acetylglucosamine-1-phosphotransferase and UDP-N-acetylgalactosamine:glycoprotein hormone N-acetylgalactosaminyltransferase, a recognition site on the target glycoprotein is indispensable for this glycosyltransferase to regenerate monoglucosylated N-glycans [101]. Although the monoglucosylated form serves as an active but not essential tag in this control system, with several backup mechanisms to compensate any loss in

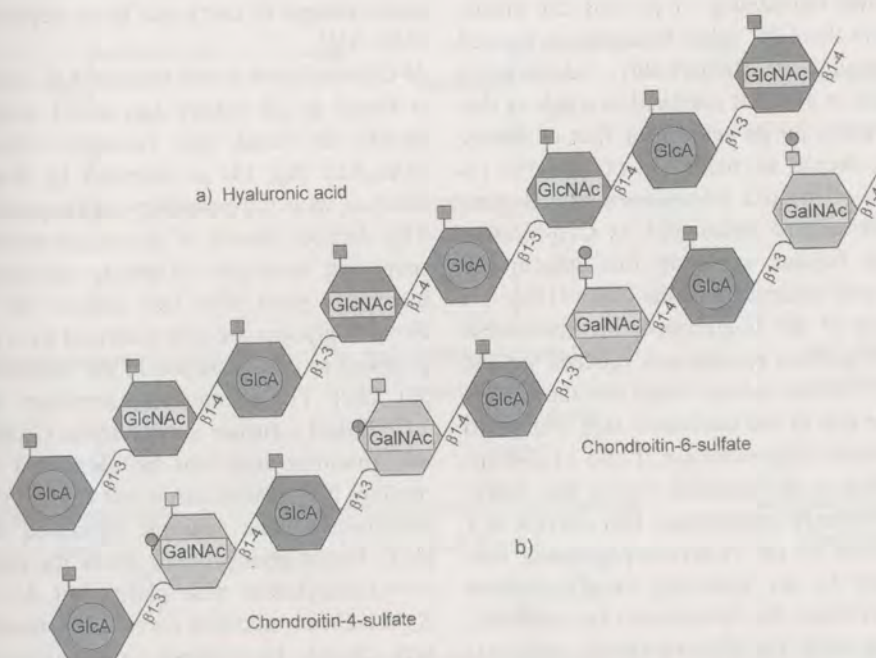


Figure 19. Structures of hyaluronic acid built of the repeating disaccharide unit $-4\text{GlcA}\beta 1-3\text{GlcNAc}\beta 1-$ (a) and the typical units of chondroitin-4-sulfate (b, left) and chondroitin-6-sulfate (b, right). The structure of this glycosaminoglycan differs from hyaluronic acid by substitution of GlcNAc by GalNAc, with further modification of this amino sugar via sulfation in the 4- or 6-position. Usually, blocks with both types of sulfation occur within one chain.

[287, 338]. In contrast to the biosynthesis of the N-glycans with its initiation by a preassembled precursor, other types of glycosylation can proceed entirely in a stepwise manner, as shown at first for mucin-type O-glycans in the next section.

Mucin-type O-glycans

As already indicated, O-glycans differ from N-glycans in their attachment point to the protein (fig. 3). In comparison to the multiantennary structures decorating the N-type glycoprotein parts, O-glycans are rather compact structures. Already at the GalNAc residue attached to the protein branching can occur [28, 29, 42, 57, 339–341]. Eight different core structures are presently known for extension beyond the GalNAc α 1-Ser/Thr linkages, as shown in figure 17. Introduction of, for example, sialylation and other modifications further increases the diversity of O-glycans. Therefore, the total number of known O-glycan structures is considered to be much higher than that of N-glycans. To give a compelling example, human lung mucin alone contains more than 100 different O-glycans [342, 343]. It is speculated that the high diversity in O-glycan structures of, for example, bronchial mucins is responsible for trapping inhaled microorganisms and thus protects the

underlying epithelia [343, 344]. The structural variations are not made feasible by an extension of the pool of monosaccharides. A common feature of all of these structures is the presence of the limited number of different monosaccharide residues, namely GalNAc, Gal, GlcNAc, Fuc and sialic acids. Sulfation is an additional, frequently detected modification of mucin-type O-glycans. From comparison with the constituents of N-linked saccharides, it is clear that Man is a typical monosaccharide for N-glycans. Owing to the discovery of the presence of GalNAc in N-glycans from invertebrates and vertebrates [345–349] already spoken of with respect to the pivotal 4'-sulfated derivative at the termini of glycan antennae of pituitary glycoprotein hormones [37], this sugar should no longer be considered as typical for O-glycans.

Biosynthesis of O-glycosidically linked saccharide chains [29, 42, 339, 340] is completely different from that of N-glycans. Instead of transfer of a preassembled carbohydrate precursor individual nucleotide-activated monosaccharides are added by site-specific glycosyltransferases in a stepwise manner. This process is highly organized in such a way that at certain branching points of biosynthesis the addition of the next sugar determines the route for further elongation, as detailed in informative reviews on this aspect [28, 42, 339, 341]. Many of these transferases have meanwhile been char-

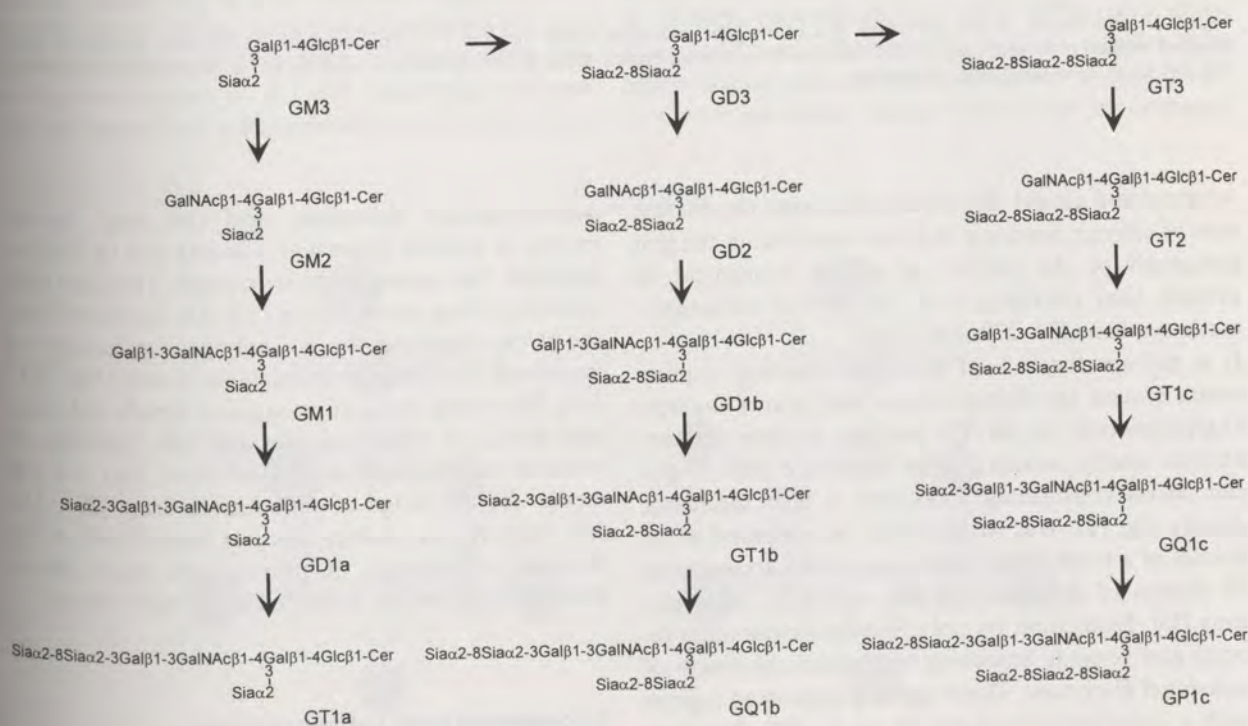


Figure 21. Biosynthetic pathway for gangliosides of the ganglio series a, b and c.

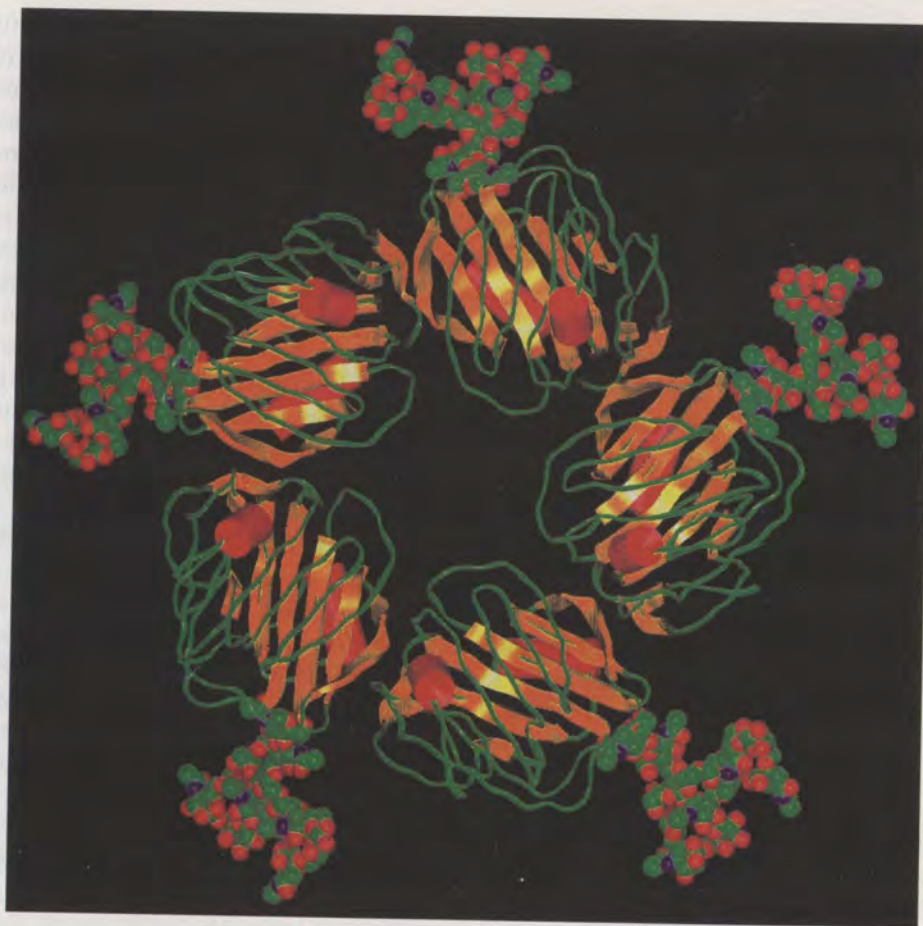


Figure 22. Illustration of the secondary structure of the pentraxin serum amyloid P component with its N-linked glycan on Asn32 attached to each monomer (entry 1SAC into the Brookhaven Protein Data Bank). Kindly provided by Dr. E. Tajkhorshid, Heidelberg, and Dr. C.-W. von der Lieth, Heidelberg.

acterized and cloned, as already mentioned for the first type of enzyme initiating O-glycan synthesis in the first paragraph of the section on glycan attachment to protein, thus providing tools for refined chemoenzymatic synthesis [29, 270, 350, 351].

It is perhaps the lack of stringent structural requirements around the linkage region that allows multiple O-glycosylation on Ser/Thr residues in close vicinity. Mucins usually contain a large number of short O-glycan chains constituting areas with a high saccharide density (fig. 18). This structure can be compared to the bristles of a brush [352], reminiscent of the arrangement of clusters of glycosaminoglycan chains in proteoglycans [55]. Elongation by poly(N-acetyllactosamine) repeats and versatile branching can extend the length of individual O-glycans. These highly glycosylated regions with a carbohydrate content of up to 80% furnish a dense local clustering of negative charge by the constituent sialic acids and sulfate. Similar to the gly-

cosaminoglycan hyaluronic acid (see next section) mucins in solution cause high viscosity and on surfaces a barrier that cannot easily be crossed. They also offer protection from proteolytic attack. On the other hand, the glycan structures are not only passive barriers but implicated in molecular interactions as well [340, 353–356]. Since they serve as recognition signals and adhesion points for other molecules and cells, initiating cell contacts in inflammation and infection, they are role models for the design of antiadhesion drugs [309, 343, 357–362]. As an analogy has just been drawn to the structural appearance of proteoglycan sugar chains, their synthesis will be presented in the next section.

Glycosaminoglycans and proteoglycans

Proteoglycans are conjugates of at least one covalently bound glycosaminoglycan chain with perplexing poten-

tial for inter- and intrachain heterogeneity attached to a protein core. In addition to this special type of glycan incorporation via the linkage unit shown in figure 4, the protein part, itself belonging to different families as discussed in the second part of this section, can often carry N- and O-glycans. Although issues of nomenclature are rarely inspiring, it is not too difficult to condense the essentials of glycosaminoglycan structure in order to provide the reader a conceptual grasp of this section of glycosciences.

The glycosaminoglycans form a distinct group of complex carbohydrates. Generally, they are defined as unbranched (linear) acidic polysaccharides formed from repeating disaccharide units. They are composed of a uronic acid (D-glucuronic acid or L-iduronic acid) or D-galactose and an amino sugar (D-glucosamine or D-galactosamine) [55, 363–366]. Monosaccharides in the chain can richly be modified by 'small substituents', enhancing the potential for variability enormously. The amino sugars are in most cases N-acetylated, sometimes N-sulfated, yielding an intrachain heterogeneity most notable in the case of heparin and heparan sulfate [367–372]. The term 'heparin' is conscripted to refer to the presence of a high proportion (generally > 80%) of N-sulfation of the GlcN residues. As a clue for the organization of the enzymatic assembly line, this factor entails abundant O-sulfation and a high IdoA:GlcA ratio. Since current methods do not attain a definitive sequence assignment of intact microheterogeneous glycosaminoglycan chains, manageable fragments are produced by nitrous acid or heparin lyase treatment. The polydisperse mixture of di- and oligosaccharides can then be fractionated with sophisticated techniques such

as HPLC or CZE, as already outlined in the section on glycan analysis. Unambiguous structure determination mainly relies upon NMR and mass spectrometrical analysis [373]. Besides the presence of N-sulfation, sulfate substituents can also decorate OH groups of the amino sugars and/or uronic acids.

Characterization and cloning of the sulfotransferases responsible for the individual reactions on proteoglycans but also on N- and O-glycans of glycoproteins and on glycolipids has advanced considerably [36]. Sulfotransferase-dependent substrate modification has already been shown in the last section to endow the pituitary glycoprotein hormones with a routing signal. It is therefore not surprising that a similar interpretation with the positioning of these groups as sensors for adaptive processes is advocated, especially for heparan sulfates [374].

The lowest degree of modification among the group of glycosaminoglycan chains is recorded for hyaluronic acid (fig. 19a), with D-glucuronic acid and N-acetyl-D-glucosamine as building blocks of the repeating disaccharide unit [375–377]. It is the only member of this group which is not covalently linked to protein and which is free of sulfate groups. All other types are integral parts of proteoglycans with the sulfate positioning deserving special attention. In chondroitin sulfates, D-glucuronic acid is found together with N-acetyl-D-galactosamine, which is further substituted by sulfate at positions 4 or 6, yielding chondroitin-4-sulfate or chondroitin-6-sulfate (fig. 19b). Within one chain there can be sequence sections with 4- together with others displaying 6-sulfation, designated as copolymer. A similar structural feature also occurs in dermatan

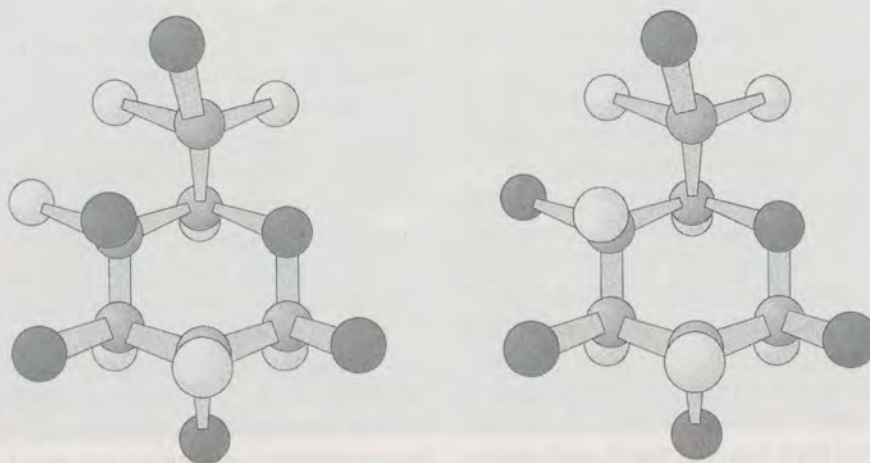


Figure 23. Stick and ball conformation of D-galactose (left) and D-glucose (right).

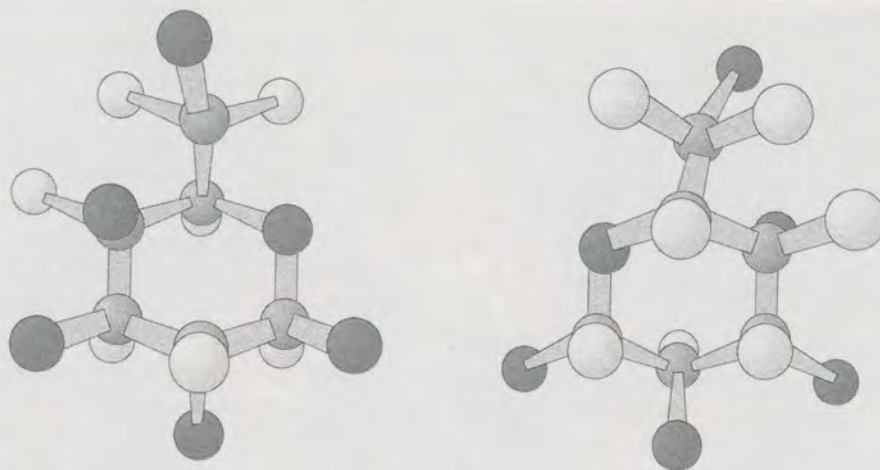


Figure 24. Polar A and unpolar B sides of D-galactose as stick and ball model.

sulfate, in which a part of the uronic acid residues of chondroitin sulfate is enzymatically epimerized to L-iduronic acid that additionally may carry O-sulfate groups.

Owing to its clinical application as anticoagulant heparin is the most popular proteoglycan. Its sugar chains contain either D-glucuronic acid or L-iduronic acid together with D-glucosamine as the basic disaccharide [363, 367]. An insight into its structure is given in figure 20. It illustrates intrachain heterogeneity of monosaccharides due to differential modification by sulfate and the extent of C5-epimer formation of the uronic acid. The glycosidic linkage points of the uronic acids to the amino sugars remain unaltered; the change from α to β is a consequence of the stereochemical alteration (from D to L). Due to the interconversion of IdoA conformers (1C_4 , 2S_0) essentially through rotation around the C2–C3 bond, the shape of the polysaccharide is amenable to energetically inexpensive modulation, establishing an extra degree of intrachain freedom of mobility [378]. Sequence heterogeneity in heparin and heparan sulfates extends to at least 10 different -GlcN- HexA and 17 different -HexA- GlcN disaccharide units with definitely nonrandom occurrence despite mechanistic constraints during synthesis [369]. Theoretically, 48 different disaccharides, including the presence of rarely found GlcN, are possible [379]. Similar to N- and O-glycans, not all devisable structures are actually biological. Nonetheless, the actually measured variability is still amazing. Again, the exquisitely well-ordered structure of the product depends on a high degree of topological organization of the biosynthetic machinery in the Golgi apparatus and the *trans*-Golgi network. Fundamentally, two catalytic activities are sufficient to fulfill the job of

chain elongation. Prior to this process the preparation of the core must be accomplished. It starts with the transfer of Xyl onto Ser within the peptide chain (fig. 4). The subsequent addition of two Gal residues is catalyzed by two different β -galactosyltransferases because the substrates are Xyl β 1-Ser and Gal β 1-4Xyl, respectively. With the attachment of GlcA to Gal in β 1-3-linkage, the common core structure for further elongation is completed [380]. This -4GlcA β 1-3Gal β 1-3Gal β 1-4Xyl β 1-Ser linkage is identical for chondroitin sulfate, dermatan sulfate, heparin and heparan sulfate. A deficiency of galactosyltransferase I in a 4-year-old Danish male was associated to an Ehlers-Danlos-type of connective tissue disorder with impaired decorin (a secreted proteoglycan with normally one dermatan sulfate chain) [381]. The clinical relevance of this biochemical defect in glycan modification merits devoting a separate section to the general aspect, merging clinical medicine and glycoscience.

That settled, we can continue to dig into the workings of glycosaminoglycan construction. The chain elongation makes progress by stepwise operation of only two enzyme activities. UDP-N-acetyl-D-glucosamine or -D-galactosamine and UDP-D-glucuronic acid (UDP-D-galactose) are the activated forms that enable alternate transfer of the monosaccharides onto the growing chain. A GlcNAc or GalNAc transferase center, depending on the type of N-acetylhexosamine to be incorporated, and glucuronosyl(galactosyl)transferase activity accomplish this task. Elegant work on heparin/heparan sulfate biosynthesis spearheading this aspect of work on glycosaminoglycans has revealed that two activities reside in a single 70-kDa protein [382]. All modifications which are characteristic for the individual

glycosaminoglycan are thereafter introduced in a defined sequence during ongoing chain elongation [370, 383, 384]. In the case of heparin, a series of five enzymatic activities is coordinated: N-deacetylase followed by N-sulfotransferase and then O-sulfotransferase and GlcA C5-epimerase [370, 385]. The first two activities are harbored in one protein for which mouse tissues express two different genes [386]. For the two latter enzymes N-sulfation is required for substrate recognition. Finally, a specific 3-O-sulfotransferase introduces an infrequent modification. This feature is crucial to turn the sequence stretch into an antithrombin-binding unit. In addition to this special sulfation three additional sulfate groups at monosaccharide unit I (O-sulfation), III and V (N-sulfations) complete the changes of the framework for high-affinity binding [369, 387–389]. Actually, these modifications must already be present to impart substrate properties for the 3-O-sulfotransferase to the pentasaccharide. The product of the shaping of the high-affinity serpin ligand is shown in figure 20. Circulating heparan sulfate can mimic the potent anticoagulant heparin and thus contribute to a fatal clinical outcome by serious bleeding in individual

patients [390]. Neutralization of the anticoagulant activity by protamine sulfate or platelet factor 4 is mandatory in such a case. The exception already noted owing to the presence of an uronic acid is seen for keratan sulfate chains. In this case Gal alternates with GlcNAc. Sulfate incorporation at C-6 of Gal and GlcNAc maintains the acidic appearance of this glycosaminoglycan, a chick corneal form being linked to its core protein via Asn [391]. Moreover, O-linked attachment is possible for this chain-type in aggrecan [392].

Not only the sequences of the glycosaminoglycan chains and the intra- and interchain heterogeneity furnish sites for regulation. Reactivity with binding partners in their vicinity and/or attachment to protein cores with glycoform formation are the material for a series of captivating stories on the versatility of glycosaminoglycan functions [55, 366, 371, 384, 392–395]. In this respect, the discussion is simplified for hyaluronan, because the long (up to 10 μm) chains are not attached to a protein core. Their physicochemical properties, including swelling with high viscosity of solutions in water and inherent elasticity, are ideal for a lubricant in joints and on surfaces sliding along each other. In addition, this



Figure 25. Illustration of the peptide backbone of one monomer of bovine galectin-1 (see table 1; entry 1SLT into the Brookhaven Protein Data Bank) with emphasis on the close spatial contact between Trp68 and the ligand. The solvent-accessible surface is highlighted. Kindly provided by Dr. C.-W. von der Lieth, Heidelberg.

polymer can be likened to a highway for cell migration and the scaffolding for a molecular network in the extracellular space. It is thus not surprising that a variety of hyaluronan receptors have been put on record, among them CD44, a receptor for hyaluronic acid-mediated motility (RHAMM), cartilage link protein as well as aggrecan and the other members of the modular proteoglycan subfamily, versican, brevican and neurocan [376, 395–399]. Already very early in development, the entry of migrating mesenchymal cells into the blastocoel exploits glycosaminoglycan to set the stage for the multilayered body plan, as underscored by the harm done by hyaluronidase injections. Similarly, cell movements from the dorsal neural tube appear to make headway on hyaluronic acid.

Concerning elasticity, proteoglycans with the sulfated types of glycosaminoglycan chains can contribute to regulating this physicochemical property. A variety of subfamilies of protein cores without a unifying sequence motif can be enlisted as carriers of the chains. From the point of view of molecular networking, the extracellular hyaluronic acid-binding proteoglycans are evidently endowed with bridging properties for various partners due to their imaginative modular design [366, 395, 400]. The irreplaceable character of aggrecan is convincingly proven by the detrimental effects of a mutation in an animal model [401]. Together with the correlation between decorin deficiency and development of a progeroid syndrome, these model studies help to embrace the concept of the vital relevance of the proteoglycans. The structural trait shared by aggrecan, versican, neurocan and brevican is a tridomain structure. The central region harboring the glycosaminoglycan chains is flanked by N-terminal hyaluronan-binding sequence stretches and a C-terminal section with EFG-like domains, a C-type lectin domain and a complement-binding consensus repeat. Clustering of these three modules is known from the mosaic-like structure of selectins and *Limulus* factor C [298], which will be discussed in the chapter on animal lectins (see also fig. 31 for an illustration of the modular architecture of selectins). Chimeric structural design is also characteristic of the other subgroup of extracellular proteoglycans. They lack capacity for binding to hyaluronic acid, but have acquired other networking abilities. Perlecan, agrin and testican present an array of modules which can include laminin domain G-, III- or IV-like, low-density lipoprotein (LDL) receptor-like or secreted protein acidic and rich in cysteine (SPARC)-like sequences or a Cys-Trp-Cys-Val (CWCV) domain [395]. A prototype molecule of this group is perlecan with its matrix protein (for example laminin and collagen type IV)- and cell-binding properties [366, 402]. The staggering growth of our knowledge on proteoglycan cores is further attested by the fact that secreted pericellular

proteoglycans even comprise another distinct category. Their members, decorin among them, display a central domain composed of leucine-rich repeats flanked by two cysteine-rich regions with a total molecular weight of 36–42 kDa [366, 403]. These SLRPs (small leucine-rich proteoglycans) also participate in matrix assembly and confer cell adhesive properties extending to the regulation of cell proliferation [366, 395, 404].

The assumed impact on matrix assembly is readily apparent for certain transmembrane proteoglycans, whose name is derived from the Greek verb *syndein* (to bind together). Syndecans and other transmembrane proteoglycans such as CD44 or thrombomodulin can connect the extracellular environment to the actin filaments of the cytoskeletal organization [366, 392, 405–410]. Anchoring the core does not exclusively depend on a transmembrane domain. As with other proteins a core protein can be associated to a membrane by the GPI anchor, introduced in figure 7. Tissue- and stage-specific expression and work on the *dally* (division abnormally delayed) locus in *Drosophila melanogaster* implicate the glypicans (currently five vertebrate forms are known; mutations in the glypican-3 gene are connected with the Simpson-Golabi-Behmel syndrome characterized by visceral and skeletal abnormalities and pre- and postnatal overgrowth) in regulation of growth control and patterning during development [366, 411].

Besides the modules in the diverse protein cores the glycosaminoglycan chains will contribute noticeably to the interactive potential complementing their rather passive physicochemical properties. Monitoring of specific binding of probes constituted by labeled glycosaminoglycans to cells in tissue sections is one approach to detect this aspect of proteoglycan function [412]. This receptor-directed activity does not only encompass the famous textbook example of serpin binding by the heparin pentasaccharide shown in figure 20. Also, a variety of proteins including laminin, thrombospondin, fibronectin, cell adhesion molecules such as N-CAM, lectins and virulence factors are proven binding partners for the sugar part of proteoglycans [298, 371, 372, 413–421]. This property is likewise relevant for cytokines and growth factors. By attachment to a glycosaminoglycan chain they are apparently either safely stored for a period and released upon stimulation of protease or heparitinase activities or acquire a functionally effective state in a ternary complex with their protein receptor [371, 422–431]. In striking analogy to oligosaccharides released from plant cell walls in a defense reaction, glycosaminoglycan components can also serve as signaling molecules after enzymatic generation from the chain [432]. From a merely passive role on the basis of their charge density, our judgment of glycosaminoglycans has steadily made a remarkable transition towards the appreciation of their 'recogni-

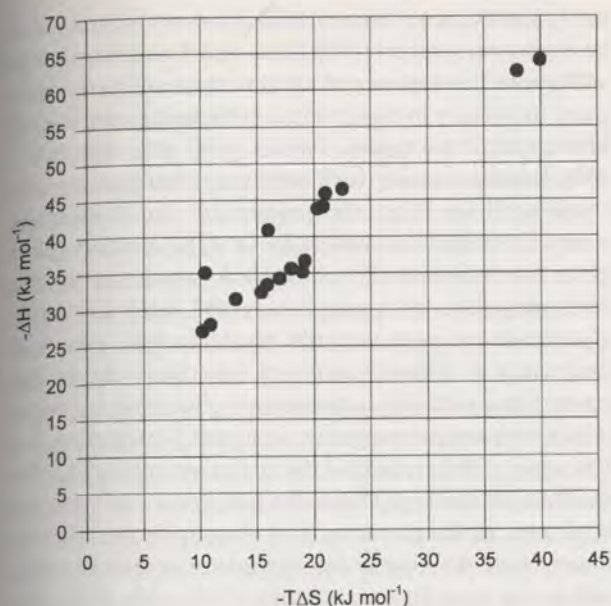


Figure 26. Enthalpy-entropy compensation plot for the thermodynamic data of binding of mono- and disaccharides to the galactoside-specific mistletoe lectin.

tional' capacity with impact on biosignaling. The pattern of maturation of scientific reception is similar in the case of glycolipids.

Glycosphingolipids

In contrast to glycoproteins and proteoglycans, glycolipids are not high molecular weight compounds on the basis of their structure, with only about 1150 Da for, for example, sialyllactosylceramide (GM3; fig. 21), a widely occurring glycolipid. They contain as noncarbohydrate part a lipophilic adapter by which they become a part of the outer leaflet of the lipid bilayer. While the ceramide portion can display a certain degree of heterogeneity due to varying chain length, unsaturation, hydroxylation and branching, classification of a glycolipid calls for homogeneity in its carbohydrate part. Sialic acid-containing glycosphingolipids are called gangliosides, and their biosynthetic relations are shown in figure 21. The membrane anchoring of the glycolipids and the comparatively short chain length preclude extensive reaching of glycolipids into the extracellular space. Hence, the saccharides are rather in close vicinity to the membrane surface. Owing to their lipid part most glycolipids are poorly soluble in water. In an aqueous phase they usually form micelles, with the polar sugars facing outward. Isolation of glycolipids from biological materials therefore requires organic solvents or detergents.

As seen for the mucin-type O-glycan chains, there are numerous structural variations within this group, although they are likewise built with only few different monosaccharides, that is Glc, Gal, GalNAc, GlcNAc, Fuc and Sia [433, 434]. Man has been reported to be a constituent of invertebrate glycolipids, leading to the arthro- and mollu-oligosaccharide series [435]. As reflected in figure 21 for gangliosides, the biosynthetic pathway of glycosphingolipids follows the strategy used for O-glycans or glycosaminoglycans. Individual monosaccharides are added in a stepwise manner from nucleotide-activated precursors by specific glycosyltransferases [436–440]. As accentuated in figure 21, the order of the individual biosynthetic steps is well elucidated. Final stages of ganglioside synthesis take place in the *trans*-Golgi network. Already in the *cis*-Golgi synthetic routes are committed to certain product lines. The acceptor sugar of major importance is the Gal moiety of GM3. As soon as a GalNAc moiety is transferred to the 4'-position of this D-galactose unit, no further α 2-8-sialylation of the GM3 sialic acid can occur but only chain elongation via GalNAc. A similar behavior evocative of the guiding role of the introduction of a bisecting GlcNAc residue in N-glycan synthesis or of N-sulfation in heparin/heparan sulfate synthesis is found in the case of GD3, channeling sialylation. Thus, the number of sialic acid residues attached to the D-galactose unit of GM3 is the basis for distinguishing a, b and c series gangliosides. The continuous elaboration of purification protocols and glycan analysis techniques, the latter already outlined in this review, fueled a stunning increase in the number of gangliosides. From 1978 to 1989 the number of characterized species jumped from 28 to 79 [441]. The same is true for the different classes of neutral glycosphingolipids (i.e. ganglio- and isoganglio-, globo- and isoglobo-, lacto- and neolacto-, muco- and gala series), sulfatoglycosphingolipids and fucoglycosphingolipids [438, 442]. By 1989 267 identified glycosphingolipids had been registered [441]. Especially cells with aberrant glycosylation after malignant transformation are a rich source for newly detected variants [439, 443, 444]. Once these glycoconjugates have reached their final destination primarily on the cell surface, the sugar chain can be subject to enzymatic remodeling similar to the N-glycans of glycoproteins. We have already referred to the presence of a cell surface ganglioside sialidase in the section on glycan analysis. This enzyme activity elevates the levels of presentation of the ganglioside GM1 and of lactosylceramide with consequences on cell proliferation [182, 184, 185, 445]. Since this regrouping in glycolipid subpopulations is associated with growth control and differentiation in cultured human neuroblastoma cells, as, for example, shown with the already mentioned sialidase inhibitor Neu5Ac2en, the biological rel-

evance of the display alterations offers an insight to functional traits. The common degradation of glycosphingolipids proceeds after endocytosis in lysosomes by exoglycosidases without accessory proteins. However, once the sugar chain has been shortened below a length of five units, the remaining distance to the intracellular membrane surface is no longer sufficient to guarantee that the degrading enzymes can find a solid grip on their substrate. For hexosaminidases acting on GM2 (for structure, see fig. 21) it has actually been shown that for steric reasons cleavage of glycosidic linkages cannot take place [446, 447]. Therefore, an additional protein, a so-called activator protein, is essential, which works as a 'molecular handle' or 'kind of detergent' in interacting with an appropriate glycolipid and lifting it measurably out of the membrane, thus rendering the sugar chain accessible to the hydrolase [447]. If the action of this protein is impaired, degradation is abrogated [446, 448]. Mutations in activator proteins such as the GM2 activator are the cause of clinical symptoms of lysosomal storage diseases, in this case nearly indistinguishable from the classical Tay-Sachs disease, where the mutation resides in the α -chain of the β -hexosaminidase A gene [448]. This aspect will be discussed further at the end of this section. Accumulation of glycolipids due to defective degradation can be alleviated by therapeutically routing enzymes to the badly afflicted cell types such as macrophages, an approach termed enzyme replacement therapy, with the enzyme's trimmed complex-type N-glycans as a routing signal to the C-type lectins of the target cells [298, 449]. Instead of exoglycosidase treatment of the naturally occurring β -glucocerebrosidase or expression in GlcNAc-transferase I-deficient mammalian cells, insect cells may also be a source of mannose-presenting enzymes, as explained in the section on N-glycans [450, 451]. This example again emphasizes the role of glycans in cell-targeted delivery. If the routing signal itself is not correctly synthesized, for example a mannose-6-phosphate group in lysosomal acid hydrolases, clinical symptoms can be directly assigned to a lack of functionality in the glycan chains, as reported in the context of phosphoglycosylation and N-glycan processing and discussed more in detail in the next section.

Owing to their strategic positioning in the outer leaflet of the mammalian cell membrane, the head group of glycolipids can be accessible for protein/carbohydrate-carbohydrate interactions, which will be explained in the section on glycan recognition. The most famous example is the highly specific binding of cholera toxin to ganglioside GM1, an irresistible argument against a merely arbitrary role of sugars as headgroups in glycosphingolipids [442]. Growth control by sialidase-dependent ganglioside modification in neuroblastoma cells has already been cited as a further example, most prob-

ably involving protein-carbohydrate interactions with an endogenous lectin [185]. This report underscores the well-appreciated potential of this class of glycoconjugates to partake in biosignaling extending to the level of kinases or transcription factors [437–439, 446, 452–455]. Besides reacting with their sugar headgroups, glycosphingolipids can be converted to bioreactive compounds such as ceramide or sphingosine-1-phosphate or N,N-dimethylsphingosine, which are signals for protein kinases among others [453, 456]. Evidently, glycolipids are very versatile modulators of a host of reactivities at different stages of their life cycle. In this context the putative formation of glycolipid microdomains with unique properties in ligand presentation is a hot topic of debate [457, 458]. A further analogy to, for example, mucin-type O-glycans (see figs. 17 and 18) can be drawn, as the glycan part of glycolipids can likewise impersonate docking points for microbes, an attractive target for antiadhesion therapy [358, 442, 459]. The underlying structural similarity of parts of glycans from both classes of complex carbohydrates can mean that aspects of their functions can be shared by lipid- and protein-linked determinants [460]. Similarly intriguing, the appearance of glycosphingolipid-based stage-specific embryonic antigens suggests possible explanations of molecular events which are programmed by this signal [461, 462]. Since the prefix *sphingo* derives from the Greek myth of the sphinx, the magnitude of the functional riddle posed by the molecules is adequately signified.

A recently emerging approach to discovering the functions of glycolipids is to generate knockout mice with deficiencies in a distinct glycosyltransferase, as reviewed recently by Hathaway and Shur [463]. While only subtle defects in neuronal functions (for example neuronal conduction velocity) were measurable in mice lacking complex gangliosides due to disruption of GM2/GD2 synthase, galactocerebroside and its sulfatides have been shown to maintain the insulator function of the membrane bilayer of oligodendrocytes and Schwann cells and to target axons by oligodendrocyte processes [464–467]. The same strategy, that is to disrupt genes for selected enzymes at the stem cell level, has been employed to study glycolipid degradation. Of remarkable clinical interest is the production of animal models for heritable neurodegenerative disorders caused by incomplete hydrolysis of the glycan part with excessive accumulation of gangliosides [448, 468]. However, the study of the variants of GM2 gangliosidosis reveals that an identical genetic defect in mice and humans will not necessarily cause the same symptoms. Despite deficiency in the GM2-degrading β -hexosaminidase A, the 'Tay-Sachs' mice are phenotypically normal. The explanation of this finding is attributable to a mouse sialidase. It is able to convert GM2 to a substrate of the

remaining intact mouse β -hexosaminidase, whose action produces lactosylceramide [469]. In this case, species differences in enzyme specificity preclude obtaining the desired animal model.

The crucial role of an enzyme defect for the apparent consequences on the cellular level can be unequivocally demonstrated by gene import into cultured fibroblasts. The deliberate deficit correction ensues significant clearance of accumulated substrates, as demonstrated with transgenic 'Fabry' mice expressing a human α -galactosidase [470, 471]. Clearance of accumulated material will also be beneficial for alleviating defects in lysosomal degradation of other glycoconjugates. Given the strict order of enzymatic steps in such a pathway, glycan analysis of the abundant material clarifies the nature of the deficiency on the analytically accessible level, which concerns glycosphingolipids [439, 472–474], glycogen [475–477], glycosaminoglycans [478, 479] and glycoproteins [480, 481]. Notably, the compilation of the predominant products of diverse variants with inherited defects in N-glycan processing makes it possible to schematically represent the chain of events in degradation, comparable to the example given for GM2 gangliosidosis [481–483]. Data from rigorous testing with site-specific glycosylation and processing inhibitors can easily be reconciled with these results, proving the chain of events independently [484–489]. The further analysis of the responsible enzymes from the patients will define the site of mutation and its actual impact on the protein's features [490–494]. This research can also provide clues to a more complicated regulation than originally thought when one degradation step is impaired with disparate consequences. While, for example, in mucopolidosis I sialylated N- and O-glycans are excessively found due to a lack of sialidase activity, only corresponding O-glycans are detected in Kanzaki disease, although the same enzyme is faulty [495, 496], necessitating clarification of the precise nature of the biochemical defects.

In our context, supportive evidence for a clear-cut answer to the provocative question in the title would be inarguable if it were possible to present clinical examples for an indubitable correlation of aberrant glycosylation and clinical symptoms. Beyond the importance for basic science, the diagnosis can pave the way for an effective rational therapy at least on the level of the symptoms. Such a case has already been discussed for autosomal recessive Morbus Gaucher by the targeted enzyme (i.e. β -glucocerebrosidase) replacement [449, 451, 497].

Glycans and disease

A disease is considered as an aberration from the normal status. If the molecular cause of this experiment of nature can be defined, and no further additional defects compli-

cate the interpretation, the reduced efficiency of the organism in one or several aspects highlights the necessity for strict regulatory events and a functionality of the respective target of the deviation. The fact that glycans should not be randomly added to any protein is graphically proven by a mechanism for pathogenesis of severe hemophilia A. Abnormal N-glycosylation, introduced as a consequence of mutations in, for example, positions 572 or 1772 of the factor VIII-coding DNA sequence, which create new sequons for N-glycosylation, blocks the procoagulant activity of factor VIII [498]. Decreased functionality by this mechanism has also been observed for antithrombin Rouen-III. A mutation from Ile to Asn at position 7 established a new sequon within the heparin-binding domain [499]. Once added, all glycan chains are subject to the events of processing. They appear to be derailed in certain aspects in various diseases [500–503]. Acquired dysfibrinogenemia seen in patients with hepatitis or primary hepatoma involves a delayed rate and extent of fibrin polymerization. A correction of this defect and a normalization of the thrombin time was brought about in vitro by an enzymatic decrease in the hypersialylation of the four biantennary N-glycans [500]. Various degrees of liver dysfunction are associated with the elevated retention of α_1 -trypsin inhibitor in the hepatocyte as a consequence of glycosylation abnormalities linked to mutations in the peptide backbone, for example Glu342Lys [500]. The substantial heterogeneity of glycan structures of other acute-phase glycoproteins in response to pathophysiological conditions intimates that glycan presentation is at least of diagnostic relevance [501]. Monitoring of this aspect for α_1 -acid glycoprotein has even encouraged the notion that distinct glycoforms harboring the sialyl Lewis^x-epitope may have antiinflammatory potency to restrict an excessive selectin-mediated leukocyte extravasation into the inflamed tissue section [504, 505]. The importance of the fucosylated sugar determinant will be discussed further in this chapter with respect to an inherited deficiency in producing GDP-Fuc de novo, the essential precursor of fucosylation.

In addition to affecting remodeling, impairment of liver functions can translate into the production of carbohydrate-deficient glycoproteins up to the level of a lack of glycosylation. Such a deficiency for the serum glycoprotein transferrin has debatable value as a diagnostic indicator for example for chronic alcohol abuse [506–508]. In this case the patient himself can be blamed for the self-inflicted symptoms on the level of the serum marker. Hypoglycosylation with a characteristic pattern of distribution of transferrin glycoforms is also indicative of a family of presently four subtypes of inherited disorders whose initial description dates from 1980 [359, 503, 509–514]. From the panel of analytical tools isoelectric focusing or anion exchange chromatography are instrumental in delineating the relative proportions

of isoforms, for example tetra- and disialotransferrin [515], in the serum of patients with the multisystemic problems. Electrospray mass spectrometry has verified the assumed loss of one or two N-glycans [359, 511]. Currently, the molecular defects causing a characteristic pattern of hypoglycosylation in the carbohydrate-deficient glycoprotein syndromes (CDGSs) are defined for type Ia, Ib and II. Clinical abnormalities in type Ia include severe neurologic defects (for example brainstem atrophy, cerebellar hypoplasia and alternating esotropia), dysmorphic features, developmental delay, hypogonadism, lipodystrophy and blood coagulation defects. The severity of the symptoms can vary. The responsible genetic abnormality often lies in the gene for phosphomannomutase, the enzyme which converts D-mannose-6-phosphate to D-mannose-1-phosphate [513, 516, 517]. This deficiency causes a disturbance of GDP-D-mannose production, representing an example of impairment in the supply of the activated form of this hexose for N-glycan precursor assembly as $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\text{-P-P-Dol}$ (fig. 13) and also for biosynthesis of GPI anchors and GDP-L-fucose. In assays with skin fibroblasts from CDGS type I patients, D-glucose deprivation or D-mannose supplementation exerted a corrective influence on the size of the lipid- or protein-linked glycans [518, 519]. A variant with a rather mild symptomatology is based on impaired glucosylation of this lipid-linked precursor, which constitutes a suboptimal substrate for transfer to the nascent protein [520]. Phosphomannomutase activity is normal, leaving open the answer to the question on the primary defect.

Besides this most common form and the newly defined variant, the subtype Ib has recently been categorized as a primarily gastrointestinal disorder with protein-losing enteropathy and no indication for psychomotor or mental retardation [521, 522]. While diagnostically indistinguishable from common type Ia on the basis of transferrin analysis, enzyme assays revealed a single-site defect for phosphomannose isomerase. This enzyme converts D-fructose-6-phosphate into D-mannose-6-phosphate. Thus, the precursor for GDP-D-mannose can still be synthesized by a hexokinase, provided the mannose supply is sufficient. Dietary sources and salvage pathways indeed deliver the material for direct D-mannose use, which can make a significant contribution to N-glycan biosynthesis [523]. This reasoning has prompted the design of a rational therapeutic approach with diet supplementation. It worked well in the treated patient [522]. The success of this approach also provides hints to explain dissimilar clinical presentations. Since mannose uptake and/or reuse can be different among cell types, the molecular defect in this enzyme can well be compensated to varying extents by D-mannose-6-phosphate

generation, bypassing the shortage in isomerase functionality.

Abnormalities in serum transferrin analysis upon isoelectric focusing were also conducive to pinpoint the type II CDGS, with considerably elevated disialo levels and dramatically reduced tetrasialo values. Peripheral neuropathy could not be diagnosed, in contrast to delayed myelination and global cortical atrophy. The defect in this rare disease was traced to N-acetylglucosaminyltransferase II [513, 517, 524, 525]. As can be deduced from figure 15, the α 1-6-linked Man arm of the N-glycan core will not be extended to complex-type chains upon losing this enzyme's activity. Differences in the clinical presentation relative to type I patients are also seen for type III and type IV patients with characteristic changes in the serum transferrin pattern following isoelectric focusing [513, 526]. Although the biochemical nature of the defects in these two inherited diseases has not yet been defined, the fact that abnormal glycosylation can be linked to a variety of clinical phenotypes is further legitimate incentive to teach the basics of glycan structure to medical students to keep them abreast of this development.

It is essential not to disregard that a certain symptom may even depend on two entirely different defects in glycan synthesis. For example, hypergonadotropic hypogonadism is not only associated with CDGS type I but also with galactose-1-phosphate uridylyltransferase deficiency [513, 527]. A deficiency in N-acetylglucosaminyltransferase II will not only be responsible for the development of CDGS type II. Expression of a mutant enzyme can lead to mild anemia, hepatomegaly, cirrhosis and hemosiderosis of the liver, classified as congenital dyserythropoietic anemia type II or hereditary erythroblastic multinuclearity with positive acidified-serum test (HEMPAS) [359, 513, 528]. The conversion of the common glycan precursor (see fig. 13) to complex-type N-glycans is also reduced with impaired α -(3/6)-mannosidase II activity. This processing enzyme acts after N-acetylglucosaminyltransferase I on the two residual extensions of the central α 1-6-linked mannose prior to N-acetylglucosaminyltransferase II (fig. 15). Again, two different biochemical defects are responsible for the symptoms of one clinically defined syndrome [503, 528]. A clue to understanding the development of the dyserythropoietic anemia in the course of the disease caused by the α -mannosidase II aberration was obtained by knockout mice mutants [529]. In the null mice, nonerythroid cell types avert severe problems via an alternate pathway. The theoretical concept of invoking the presence of a separate α 3/6-processing enzyme, termed α -mannosidase III, for removal of the two mannose residues from the α 1-6 arm despite a lack

of prior GlcNAc-extension by N-acetylglucosaminyltransferase I was experimentally proven. Both pathways end up in the same substrate for N-acetylglucosaminyltransferase II. The detection of this compensatory (possibly exemplary) backup mechanism can be interpreted as a sign of the vital importance of maintaining correct processing of N-glycans. This view is corroborated by independent experience with (i) glycosylation inhibitors such as swainsonine from plants of the genus *Swainsona*, which cause a disease known as locoism [530], or tunicamycin whose deadly effects due to blocking N-glycosylation at the first step of the dolichol cycle, that is the formation of Dol-P-P-GlcNAc, are counterbalanced by gene amplification [531] as well as (ii) with deliberately engineered loss-of-function mutants after altering a glycosyltransferase gene in the mouse genome [463, 532, 533]. Knockout mice with gene disruption in N-acetylglucosaminyltransferase I, which effectively reduces glycosylation to a 'yeast-or insect-like' status, are invariably subject to embryonic lethality, although mutant cells in vitro suffer no overt consequences [463, 532, 533]. Growth retardation and semilethality were apparent in mice with engineered defects in a β 1-4-galactosyltransferase gene [534, 535]. In contrast, absence of the bisecting GlcNAc residue (fig. 15) caused no behavioral or neuromuscular alterations, accessory processes such as cell homing requiring further attention [536].

Genetic defects with clinical impact are not only linked to enzymes of N-glycan processing. A variety of other diseases, including variable hemolytic anemia by increased GalNAc α 1-Ser presentation due to reduced O-glycan chain extension, progeroid syndrome with connective tissue abnormalities due to reduced core unit assembly of glycosaminoglycan chains, osteochondrodysplasias and macular corneal dystrophy due to (assumed) undersulfation of certain glycosaminoglycan chains (defect in sulfate transport, 3'-phosphoadenosine-5'-phosphosulfate (PAPS) synthesis or sulfotransferases) and paroxysmal nocturnal hemoglobinuria due to defective assembly of the GPI anchor precursor and ensuing failure of GPI anchoring, prove that the other types of glycans are also relevant for upholding a normal phenotype [26, 503, 513, 537]. As a further resource for valuable insights, the conditional lethality of mutant yeast cells (*gpi1*) with a defect in the synthesis of N-acetylglucosaminyl phosphatidylinositol, the first intermediate in GPI synthesis (see fig. 7 for GPI anchor structure), underlines the importance of GPI anchoring and its potential exploitation by devising antifungal drugs that interfere with GPI synthesis [538], as similarly suggested with respect to parasitic phosphoglycosylation in the section on glycan attachment to proteins. But these are not the only lessons we can learn from the

analysis of the biomedical consequences of natural changes in glycosylation. Tracing the pathway between mutation and phenotype has delineated ligand properties of carbohydrate structures in two clinically relevant cases. As already noted in the context of processing of high-mannose-type N-glycans (fig. 13), a D-mannose-6-phosphate signal is attached to acid hydrolases via a two-step stamping process, outlined in the context of presentation of phosphoglycosylation. As a direct result of the elucidation of the molecular basis of the I (inclusion)-cell disease (mucopolipidosis II) and its milder variant (Pseudo-Hurler polydystrophy), the P-type lectin-mediated targeting of marker-bearing glycoproteins to the lysosomes was discovered [31, 32]. The reduced activity of the UDP-N-acetylglucosamine: lysosomal enzyme N-acetylglucosaminyl-1-phosphotransferase abrogates proper enzyme targeting. As a consequence misrouting of D-mannose-6-phosphate-lacking hydrolases into the secretory pathway mimics a catabolic enzyme defect of a severe lysosomal storage disease. The expedience of this knowledge is also documented by experimental attempts of enzyme replacement therapy in murine mucopolysaccharidosis VII with D-mannose-6-phosphate-bearing β -glucuronidase obtained in vitro from receptor-deficient L-cells which secrete most of the desired signal-containing enzyme [539, 540].

Severe consequences with mental and growth retardation and recurrent infections are also apparent in another inherited disease, autosomal recessive leukocyte adhesion deficiency (LAD) type II syndrome [541]. This disease has already been referred to in the first part of this section with respect to the potentially antiinflammatory properties of glycoforms of α ₁-acid glycoprotein. Its molecular cause is attributed to glycan synthesis in contrast to the type I syndrome. The type I syndrome has been traced back in biochemical terms to mutations in the β ₂ integrin (CD18) gene. Either no product, a defective form unable to associate with the partner α chains to build the three heterodimeric integrins LFA-1, CR3 and p150,95 (CD11a,b,c CD18) or dysfunctional β ₂ integrins are the hallmarks of the genetic deviation [542, 543]. Under all conditions, recurrent and/or chronic infections despite persistent leukocytosis in the circulation point to a conspicuous inability of leukocytes to migrate into the inflamed tissue part. An inherited defect in production of the activated fucose donor, GDP-Fuc, accounts for the underlying defect in the type II syndrome [541]. Explicitly, the de novo pathway at the stage of regulation of GDP-D-mannose-4,6-dehydratase activity by a yet unidentified regulatory protein is likely to be affected [544, 545]. The requirement for fucosylated ligands on

leukocytes was elegantly underscored by knockout mice for the α 1-3-fucosyltransferase VII. Their immunophenotype resembled that of LAD type II syndrome patients [546]. The dependence on fucose incorporation into cell surface glycans for proper activity as addressins is different for leukocytes and nonlymphoid endothelial cells, only the former relying on correct fucosylation for their trafficking behavior [544]. The ongoing debate about the actual nature of the ligand structures in this process notwithstanding [547–549], it is safe to conclude that fucosylated and sialylated/sulfated carbohydrate epitopes are implicated in the initiation of leukocyte-endothelium contacts during inflammation at least on the level of leukocyte glycans. The findings in I-cell disease and LAD type II syndrome collectively and strongly argue in favor of a special case of operative glycan recognition, that is the role as a ligand for an endogenous lectin. In this sense, our discussion on glycan-related diseases has guided us to appreciate that natural tampering with enzymes of the glycosylation machinery engenders a variety of syndromes. Moreover, the documented medical relevance prompts us to look more deeply into the aspects of glycan recognition and glycan receptors (lectins).

Glycan recognition

As attested by a causal relation between a defect in glycosylation and symptoms of a clinically manifest disease, the glycan part of glycoconjugates, already coined as bioinformation hardware, is clearly able to modulate biomolecular features of carrier biomolecules. For proteins, physicochemical factors include solubility and charge variations, passive steric hindrance guiding folding, oligomer assembly, proteolysis and immunogenicity, and active intramolecular interactions stabilizing protein conformations and affecting enzyme and receptor activities [25, 26, 75, 249, 512, 550–552]. Especially the core constituents such as the N,N'-diacetylchitobiosyl or attached fucose moieties can be tightly associated to the protein backbone as if they are an integral part of the carrier [553–555]. This intimate contact is able to modulate and/or restrict the mobility of glycan chains, for example the α 1-6-branch of the Asn78-linked biantennary complex-type undecasaccharide of the α -subunit of human chorionic gonadotropin (for illustration of the structure, see fig. 15) [556]. Although the terminal parts of a glycan chain appear to be extended in solution, as shown for the Asn32-attached glycan of human serum amyloid P component in figure 22, the nature of the immediate surroundings constituted by the peptide backbone and side chains can impart differential presentation to the environment and accessibility of the oligosaccharides. The interdepen-

dence of conformational preferences between individual torsion angles of glycosidic linkages and between protein (or other groups in the immediate vicinity) and glycan parts will be crucial for the actual selection of conformational minima, which necessitates looking more deeply into the concept of environment-dependent shaping of the potentially bioactive glycan [192, 247, 557–559]. Assessment of the relaxation behavior in NMR analysis or avidity of lectin binding is conducive to delineating such effects [271, 556, 560–562]. Recalling the description of the remarkable flexibility of free oligosaccharides in the section on glycan conformation, the restriction of the inherent mobility of the sugar part of glycoconjugates up to freezing conformations into bioactive or bioinert constellations will have implications for the ligand properties of the corresponding glycans.

The term 'ligand properties' can inadvertently imply binding exclusively to a protein. However, intermolecular contacts can be made to a suitably positioned glycan (homotypic and heterotypic carbohydrate-carbohydrate interaction) as well, warranting a comment. Clustering of individual low-affinity sites in carbohydrate-carbohydrate recognition adds up to yield stable cell aggregates involving aggregation factors (polyanionic glycans) in sponges and neutral or sialylated glycolipids in higher eukaryotes [453, 455, 563, 564]. Intermolecular protein-carbohydrate interactions have already been substantiated at several places in this article, for example concerning intracellular N-glycan-presenting glycoprotein routing. This molecular interaction is governed by common principles of recognition involving cooperative or bidentate hydrogen bonds, dispersion forces and

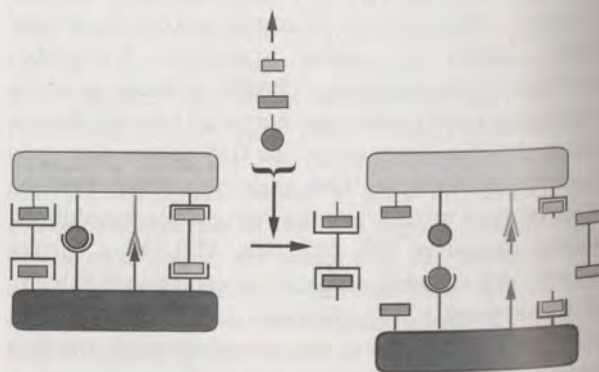


Figure 27. Illustration of the concept for lectin-based antiadhesion therapy. Therapeutic inhibitors (glycostructures or glycomimetics) block the intercellular interaction established by lectin-ligand contact including cellular association by bridging of epitopes. Kindly provided by Dr. H.-C. Siebert, Munich, and Dr. C.-W. von der Lieth, Heidelberg.

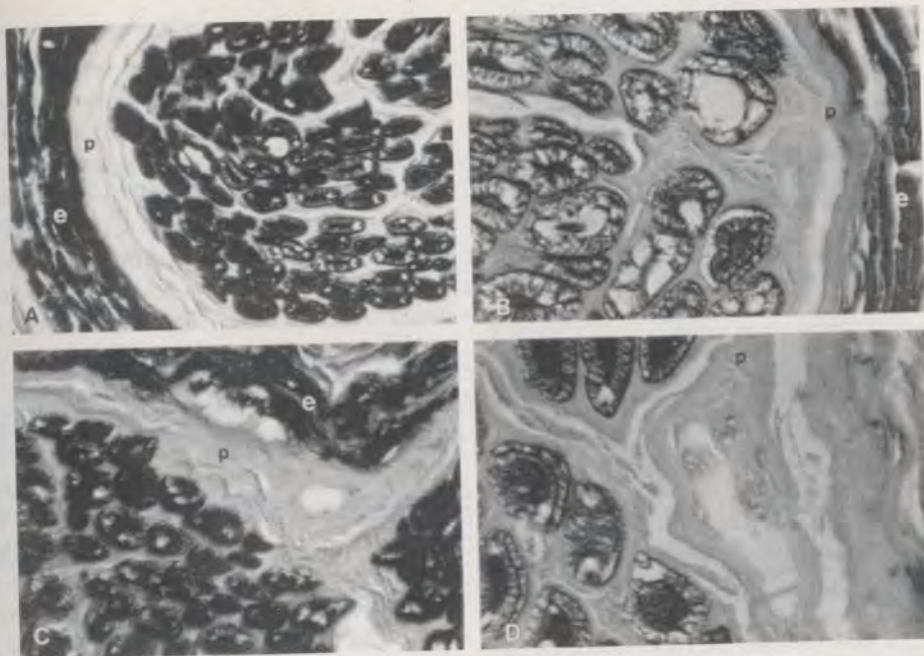


Figure 28. Localization of specific binding sites for β -D-xyloside (A), α -D-mannoside (B), β -D-galactoside (C) and N-acetyl- β -D-glucosaminide (D) in the epineurium (e) of fixed sections of human accessory nerve. Perineurium (p) and endoneurium failed to present staining, obtained by the common DAB reaction and metal ion intensification; magnification $\times 422$ (A), $\times 845$ (B), $\times 528$ (C) and $\times 1056$ (D).

hydrophobic stacking [222, 298, 565–572]. While the abundance of hydroxyl groups naturally intimates the relevance of donor/acceptor hydrogen bonds, the actual involvement of stacking and dispersion forces requires an explanation which necessarily falls back on the inspection of the monosaccharide structure given in the section on the sugar code.

The three basic hexoses for N- and O-glycans are assumed to be selected for incorporation owing to their relative levels of thermodynamic stability [573]. Unfavorable 1,3-diaxial interactions of bulky groups are minimized in glucose and its 2- and 4-epimers. For the consideration of interactions not only the relative positioning of the hydroxyl groups is important. As compellingly illustrated in figure 23, an epimerization also affects polarity distribution. Moreover, the upper and lower sides of a hexose unit can be disparate with respect to this property. Explicitly, the two surfaces of D-galactose present undeniably different characters in terms of polarity (fig. 24). The B-face with its aliphatic protons is thus likely to stack against aromatic side chains, as seen in the crystal structure of bovine galectin-1 (fig. 25). Since this aromatic amino acid is thereby shielded from solvent exposure by the carbohydrate ligand, some of its protons will no longer be accessible for nuclear polarization by a laser photoacti-

vated dye. Monitoring spin polarization of the aromatic side chains by NMR spectroscopy has thus been instrumental in verifying this structural role of the only tryptophan residue in galectin-1 in solution [241]. Also, plant lectins such as hevein domain-containing proteins with affinity for N-acetyl-D-glucosamine harbor aromatic residues in their combining sites for a similar purpose, as shown in solution by NMR spectroscopy and microcalorimetry [574–577].

This example teaches that a variety of enthalpic forces add up to result in the measurable enthalpy of the entire binding process. The enthalpic gain is inevitably linked to changes in ligand flexibility. When a disaccharide such as Gal β 1-2Gal with its rapid transition between two low-energy conformers is accommodated into a binding site, the necessity of an ensuing entropic penalty is readily apparent. To document the validity of the concept of an assumed conformer selection in solution, NMR techniques are the method of choice, especially the monitoring of interresidual ligand signals based on a through-space magnetization transfer, termed transferred nuclear Overhauser effects [20, 222, 228, 240, 557, 578–580]. This technique was instrumental in proving differential conformer selection by plant and animal galactoside-specific lectins [232, 235]. While the free ligand population is distributed between the two

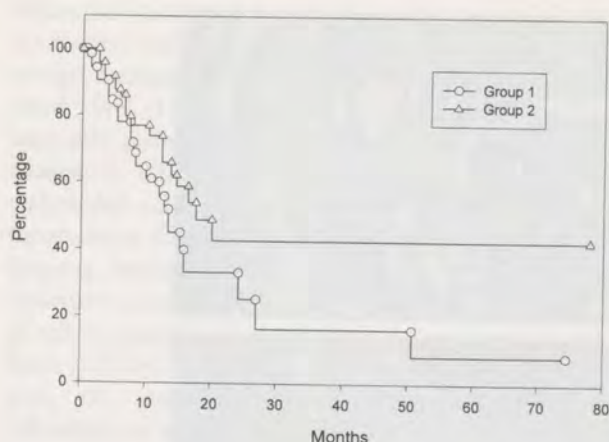


Figure 29. Illustration of the survival of lung cancer patients (in months) after surgery, given as percentage of the total number of patients, at each time point. Category formation is based on the capacity of tumor cells to specifically bind the histoblood group H (type 1) trisaccharide (group 1: no binding, group 2: binding), presented as part of a histochemically inert and labeled carrier, that is substituted poly(2-hydroxyethyl acrylamide).

low-energy positions in the central valley of the ϕ , ψ -plot, documented in figure 12a, its association to a plant or animal lectin accounts for shifting the dynamic equilibrium to one of the two conformers. A single saccharide sequence can thus be viewed as a 'bunch of keys' [239], which can open more than one lock. E. Fischer's famous 'lock and key' analogy for enzyme activities [581] is thus extended, placing emphasis on recognition as a shape, not a sequence problem [557]. Another documented case for this entropically unfavorable process even reveals its operation in homologous

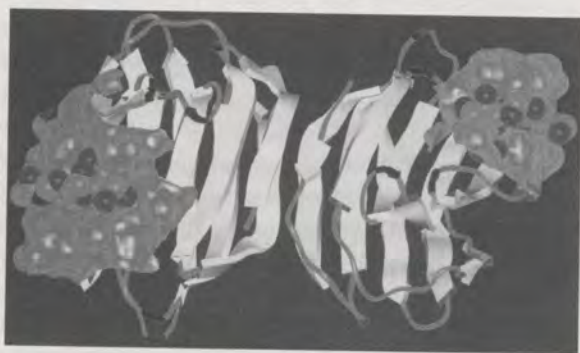


Figure 30. Illustration of the apparently strategic positioning of the two ligand-binding sites on homodimeric bovine galectin-1 (entry 1SLT into Brookhaven Protein Data Bank) to form a versatile cross-linking reagent (see also fig. 27). Kindly provided by Dr. C.-W. von der Lieth, Heidelberg.

locks (proteins), that is the selectins [582] (for further information on selectins, see next section). Evidently, the strategic design of sterically hindered derivatives (glycans or their mimetics) could be a safe section of the tour of the minefield towards reaching the envisaged destination in rational drug design, that is the perfect inhibitor. However, the complexity of the entire binding process warns against ignoring other, potentially decisive factors. In addition to the actual interactions between receptor and ligand, which extend beyond hydrogen bonding, the systematic testing of monodeoxy ligand derivatives, which are valuable for chemical mapping of the binding site [583], has clearly underscored the importance of other terms such as solvent and protein effects in the case of trimannoside binding to the model lectin concanavalin A [584]. Another argument in favor of this line of reasoning is the observation that extended glycan structures appear to be bound with different energetic mechanisms despite conservation of binding sites by *Diocleinae* lectins [585, 586].

A fundamental principle of the binding process is enthalpy-entropy compensation, balancing out for an enthalpic stabilization of approximately 5 kcal/mol [222, 228, 587–589]. Solvent reorganization and freezing of degrees of motional freedom by strong enthalpic interactions will certainly play a salient role in explaining this general property of weak intermolecular interactions, illustrated for mono- and disaccharide binding to a plant lectin as model in figure 26. However, it is essentially premature to assign a molecular cause to the properties determined by microcalorimetry. An example from recent research illustrates this prickly issue. Despite similar extents of restriction of ligand mobility of β 1-2(3)-linked digalactosides, the enthalpic gain and the entropic penalty can vary significantly, although still obeying the mutual compensation [235, 590]. Again, the meticulous analysis of protein and solvent features is understood to be imperative to delineating crucial parameters for entirely reconciling thermodynamic and structural studies. It is not preposterous to predict that this approach garnered from several areas will have merit for the development of high-affinity agents with testable perspective in lectin-mediated drug delivery or antiadhesion therapy in, for example, inflammation [222, 324, 357, 591–597]. A scheme for successful performance of a carbohydrate inhibitor or a mimetic thereof is presented in figure 27.

Coupling of these high-affinity binding partners to a biologically inert matrix without impairing their ligand properties is also a major step towards generating a versatile class of lectin-detecting tools, that is neoglycoconjugates [317, 320, 327, 598–604]. Their application in solid-phase assays, in cell biological test systems and in tissue sections fosters the detection of sugar-binding molecules which retain activity under experimental con-

Table 2. Functions of animal lectins.

Activity	Example of lectin
Ligand-selective molecular chaperones in endoplasmic reticulum	calnexin, calreticulin
Intracellular routing of glycoproteins and vesicles	ERGIC-53, VIP-36, P-type lectins, comitin
Intracellular transport and extracellular assembly	nonintegrin 67-kDa elastin/laminin-binding protein
Cell type-specific endocytosis	hepatic asialoglycoprotein receptor, macrophage C-type lectins, hepatic endothelial cell receptor for GalNAc-4-SO ₄ -bearing glycoproteins
Recognition of foreign glycans (β 1,3-glucans, LPS)	CR3 (CD11b/CD18), <i>Limulus</i> coagulation factors C and G
Recognition of foreign or aberrant glycosignatures on cells (incl. endocytosis or initiation of opsonization or complement activation)	collectins, pentraxins (CRP, limulin), C-type macrophage receptors, L-ficolin
Targeting of enzymatic activity in multimodular proteins	acrosin
Bridging of molecules	homodimeric and tandem-repeat galectins, cytokines (for example IL-2:IL-2R and CD3 of TCR), cerebellar soluble lectin
Effector release (H ₂ O ₂ , cytokines etc.)	galectins, selectins, CD23
Cell growth control and apoptosis	galectins, C-type lectins, amphoterin-like protein, cerebellar soluble lectin
Cell routing	selectins, I-type lectins, galectins
Cell-cell interactions	selectins and other C-type lectins, galectins, I-type lectins
Cell-matrix interactions	galectins, heparin- and hyaluronic acid-binding lectins
Matrix network assembly	proteoglycan core proteins (C-type CRD), galectins, nonintegrin 67-kDa elastin/laminin-binding protein

See [298, 669–671].

ditions [272, 320, 602, 603, 605–607]. Besides commercial offers for labeling kits, the natural activity of enzymes can be exploited by covalent conjugation to visualize a binding event of the sugar part in simplified protocols [606, 608–610]. Concerning ligand synthesis, the already mentioned combination of chemical and enzymatic techniques (see section on N-glycans) has matured considerably and is competitive to the purification of oligosaccharides from natural sources. This progress extends the range of testable ligands and their complexity considerably [29], as recently shown for bi-antennary N-glycans [271]. An especially charming aspect of this work aside from its scientific merit is the inherent genuine beauty of histochemical slides. As exemplarily documented in figure 28 for carbohydrate-reactive sites in the epineurium of a human accessory nerve [611, 612], specific glycoligand-dependent binding can readily be assessed. Thus, the monitoring of accessible binding sites on cells and in tissues by glycohistochemistry [218, 219, 606, 613, 614] can guide further efforts to purify the observed activity. In histopathology, this class of markers leads to results equivalent to

the application of antilectin antibodies [220, 615–622]. Glycohistochemistry with neoglycoconjugates is, for example, helpful in muscle fiber typing in fixed sections and tumor diagnosis in different classes such as brain, breast, colon, lung or prostate malignancies [326, 616, 617, 623–631]. In addition to diagnostic questions, the binding of distinct sugar epitopes such as A- and H-toblood group determinants, as shown in figure 29 for the H-epitope, is of predictive importance for prognostic evaluations in lung cancer [632, 633]. The pursuit of this analysis can also contribute to clarifying the riddle of the relevance of blood group presence in tumor pathology [634–637]. Since endogenous lectins have been implicated in growth control and induction of apoptosis [298], this glycohistochemical information is wedded at least phenomenologically to the concept of glycan function.

Indeed, the structural analysis of glycan-lectin interactions and their functional implications are a hallmark of glycan relevance. Research on animal lectins, whose path through the decades has been traced elsewhere [638–640], has matured to the stage of classification of

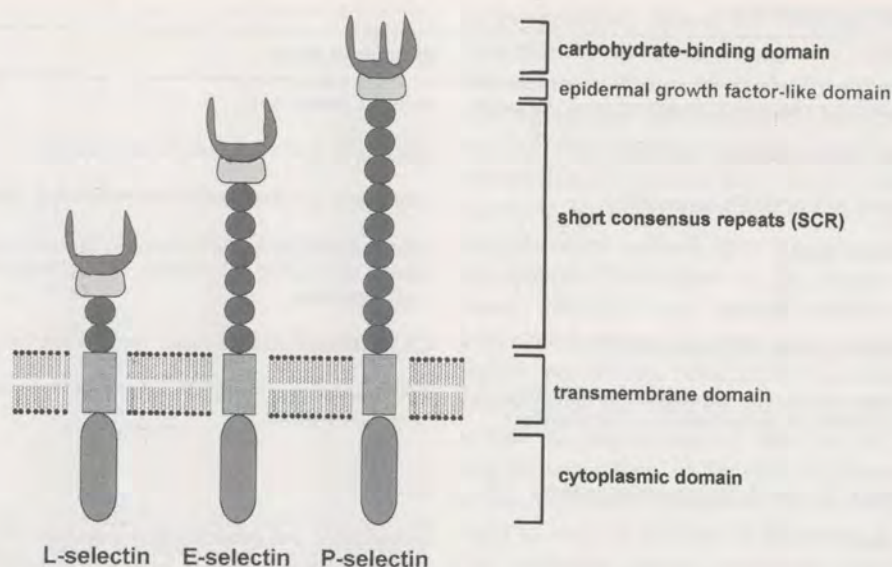


Figure 31. Illustration of the modular architecture of the members of the group of selectins, a subfamily of C-type animal lectins (see table 1). Kindly provided by Priv.-Doz. Dr. H. Kaltner, Munich.

main families and elucidation of various functions to be reviewed in the next section.

Animal lectins: current classification and function

Structural characterization of invariant sequence motifs and distinct folding patterns has paved the way for a well-accepted classification scheme (table 1). The question of the design of carbohydrate-binding sites is presently answered, for example, by noting the compactness of β -strand organization, seen in the jelly-roll topology with its antiparallel β -sandwich arrangement (fig. 30), the stacked β -sheets of the Ig-superfamily members or the flattened β -barrel arrangement of a P-type lectin with similarity to avidin [298, 568, 569, 571, 641–645]. Compilations of the frequency of lectin occurrence with respect to their target sugars have unveiled a preponderance for receptors specific for D-galactose and its derivatives such as sialyllactose [573, 646]. This phenomenological evidence points to a correlation between the spatial accessibility of these determinants at the outer part of sugar antennae and their operativity as lectin ligands. A similar conclusion had been drawn on the basis of the transient presentation of a glucose unit during N-glycan processing and the presence of calnexin and calreticulin (fig. 16). These two lectins lead off the compilation of functions, given in table 2.

The suggestion of a noncoincidental positioning is reinforced when the modular constitution of several lectins

implicated in cell adhesion is scrutinized [298, 361, 647–650]. The C-type subgroup of the selectins which has been mentioned in preceding sections as targets for anti-inflammatory drug design expose their lectin domain well beyond the glycocalyx by spacer modules (fig. 31). Thus, leukocytes and platelets in rapid circulation can sense the presence of docking sites on inflamed endothelium leading to initial contact formation [298, 361, 362, 651–653]. As seen for hyaluronic acid-binding extracellular proteoglycans (see section on glycosamino-

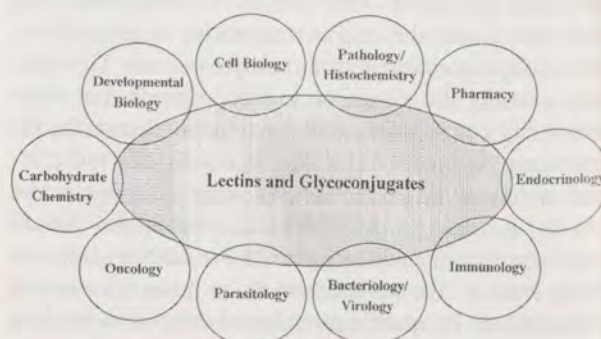


Figure 32. Illustration of the relevance of lectins and carbohydrates for various disciplines in basic and applied natural and clinical sciences.

glycans and proteoglycans), we again meet EGF-like and complement-binding consensus-repeat-like domains in the vicinity of the C-type lectin fold. A similar principle holds true for the sialoadhesin group of I-type lectins employing C2-set immunoglobulin-like tandem repeats, and most galectins can readily bridge two accessible glycans by the strategic occurrence of their binding pockets at opposing sides of homodimeric and tandem-repeat proteins, as shown in figure 30 [298, 654]. This binding, effectively forming cross-linked complexes [655–658], may not only establish or strengthen cell clusters. It is most probably a trigger for the role of galectins in cell growth control and apoptosis [119, 185, 298, 654, 659], the available evidence justifying an entry into table 2.

Evidently, positioning of the binding sites is essential for the consequences which follow molecular association. This correlation is not only encountered in the case of cell surface contacts on 'self' cells. Since bacteria and yeast cells differ markedly in the presentation of glycans, for example harboring a high density of mannans on infectious yeasts like *Candida albicans* (see section on N-glycans), it is not surprising when accepting the concept of a role of sugars as recognition units that components of the innate immune system take advantage of this glycosignature to identify invaders [298, 660–663]. Either α -helical coiled-coil-mediated noncovalent association of lectin-containing subunits ('bundle-of-tulips' arrangement) or tandem-repeat presentation of receptor sites in one contiguous polypeptide chain enable the construction of sensors to track down a 'nonself' pattern (table 2). Subsequent complement activation or opsonization lead from initial contact to destruction of the invading microorganism. Interestingly, the generation of the coagulin gel which will engulf Gram-negative bacteria in the hemolymph of the horseshoe crab to render them targetable by antimicrobial substances (anti-bacterial lipopolysaccharide (LPS) factor or tachyplesins) is initiated by two β -glucan sensors [664, 665]. The spatial vicinity of this unit to an activatable proteolytic center is a prerequisite to transform the signal on the presence of a foreign glycosignature into the eventually protective reaction cascade (table 2). Similar to the already mentioned relevance of endocytosis receptors for routing determinants such as GalNAc-4-SO₄ on the terminal part of N-glycan antennae of a limited set of glycoproteins [37], the occurrence of pattern-recognizing lectins in host defense attests how meaningful messages of the glycode can be deciphered to the benefit of the organism. As likewise emphasized, therapeutic routing has also been beneficially exploited in enzyme replacement therapy involving the C-type tandem-repeat mannose receptor of macrophages [298, 335, 449, 451]. These examples and further information given in

table 2 make easily understandable why current research activities on lectins and glycoconjugates touch on various disciplines from the realms of basic and applied science (fig. 32).

Concluding remarks

Having started this review with a provocative question, we have furnished the reader with a wealth of information to rebuff the notion that glycosylation is a superfluous, nonfunctional event in protein and lipid processing. Experience with recombinant expression of glycoproteins has helped teach instructive lessons in not underestimating the role of glycans in protein stability and therapeutic performance [263–265, 666, 667]. Albeit still laborious, glycan analysis continues to nourish the concept of lasting value that the sugar code has an unsurpassed and actually operative capacity to store biological information. It is reassuring that the envisioned 'discrete recognitional role' [8] has turned out to be prophetic. It is a great asset for this view that endogenous receptors (lectins) for distinct glycoepitopes have been purified. Their classification based on folding patterns and the architecture of carbohydrate-binding sites and the delineation of functions by molecular rendezvous between lectins and their sugar partners are making rapid progress, encouraging scanning of the horizon for potentially remunerative clinical applications which do not reek of science fiction. It is thus presently fair to conclude that it is not clear how to contradict the positive answer to the initial question in principle, although we still face the challenge of clarifying further, yet undiscovered aspects of glycan structure and functionality. We are reminded of an apt allegory, given by J. Montreuil in 1975 [668] quoting A. Gottschalk, who compared the realm of glycoconjugate research to a great unexplored continent. Montreuil concluded in 1995 that 'we have crossed the shores of the great unexplored continent announced by A. Gottschalk and, filled with wonder, we are discovering its fascinating secrets. We have entered the golden age of glycoconjugates' [16]. Yes, indeed!

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