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Complex Carbohydrates in Drug Development

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I. Introduction

Complex carbohydrates and complementary carbohydrate-binding proteins mediate a wide variety of physiologically and pathologically important interactions. Recent advances in carbohydrate chemistry and biochemistry afford the opportunity to develop bioactive complex carbohydrates, per se, as drugs or as lead compounds in drug development.

Complex carbohydrates are unique among biopolymers in their inherent potential to generate diverse molecular structures. While proteins vary only in the linear sequence of their monomer constitutents, individual monosaccharides can combine at any of several sites on *each* carbohydrate ring, in linear or branched arrays, and with varied stereochemistry at each linkage bond (e.g., α and β anomeric forms). Theoretically, 4 different monosaccarides can be linked together to generate >35,000 distinct tetrasaccharide structures, while 4 different amino acids can form only 24

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distinct tetrapeptides (Sharon and Lis, 1989). Further oligosaccharide structural variation commonly occurs via sulfation, acetylation, or phosphorylation of specific carbohydrate hydroxyl groups. With recent improvements in carbohydrate analytical chemistry, it has become apparent that nature utilizes the versatility of carbohydrates to generate a wide variety of molecular structures.

Unlike proteins, which typically fold back on themselves to form highly compact structures, oligosaccharide chains spread out in space (Carver and Brisson, 1984; Ong, and Yu, 1986; Homans *et al.*, 1987). The hydroxyl groups and anionic substituents (if present) on the saccharides tend to maximize their intermolecular distances. For example, the tetrasaccharide gangliotetraose (Fig. 1) has a molecular weight of ~700 and a long dimension of >20 Å, while the protein myoglobin has a molecular weight 24-fold greater (~17,000) but a long dimension of only 36 Å. The result is that the overall three-dimensional structures of oligosaccharides are more sensitive to subtle changes in sequence and linkage. The closely related tetrasaccharides pictured in Fig. 1 exemplify this point. These similar carbohydrate sequences result in very distinct tertiary structures, especially at the nonreducing termini, the area of glycoconjugates most available for intermolecular interactions. The variations are especially significant when one considers that hydrogen bonding between the oxygens and hydrogens on carbohydrates and precisely placed amino acids in a binding pocket of a protein can dictate binding specificity (Quiocho, 1986). Biological evidence (see Section III) demonstrates that diversity in carbohydrate structure is used as a code for cellular recognition and regulation.

The complex carbohydrates of mammalian cells consist of oligosaccharide or polysaccharide chains that occur primarily in conjugated forms as glycoproteins, glycolipids, and proteoglycans. The disproportionate expression of glycoconjugates on cell surfaces, or as part of the extracellular matrix, has led to the hypothesis that they function in intercellular communication and regulation (Roseman, 1970; Hakomori, 1981; Brandley and Schnaar, 1986; Sharon and Lis, 1989). The discovery of carbohydrate-binding proteins on the surface of mammalian cells (Ashwell and Morell, 1974), and direct demonstrations that specific complex carbohydrate structures modulate important physiological processes, has confirmed this hypothesis. In some cases, the biological activities of glycoconjugates have been attributed to their oligosaccharide "epitopes," consisting of a specific arrangement of a small number (≤ eight) of monosaccharides. The concept that these epitopes can be identified and manufactured in a biologically active form is at the heart of the effort to exploit complex carbohydrates as pharmaceuticals.

This article addresses some salient features of mammalian glycocon-

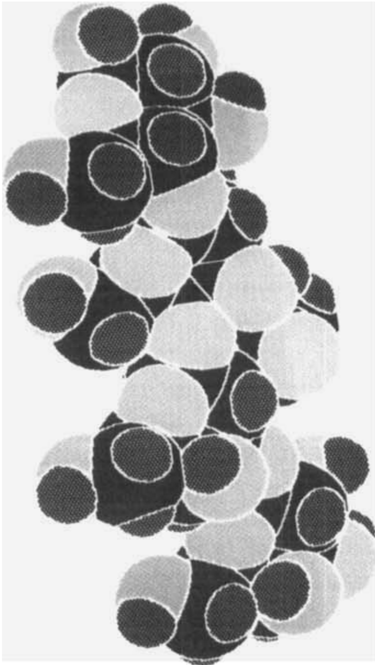
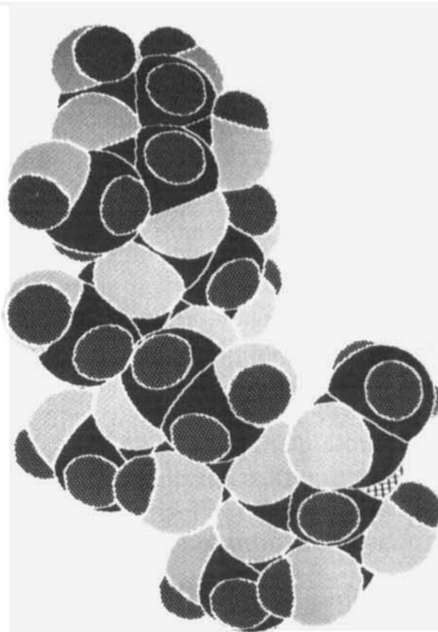
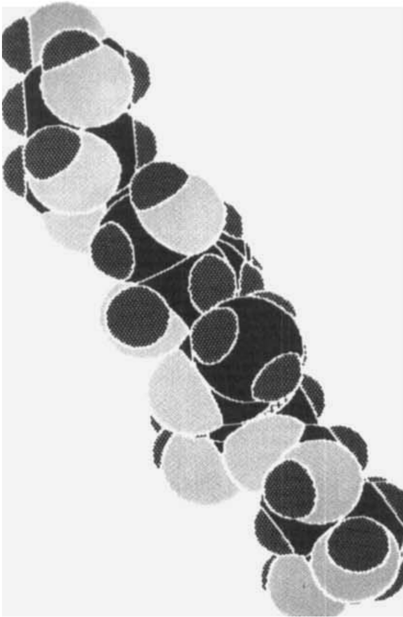
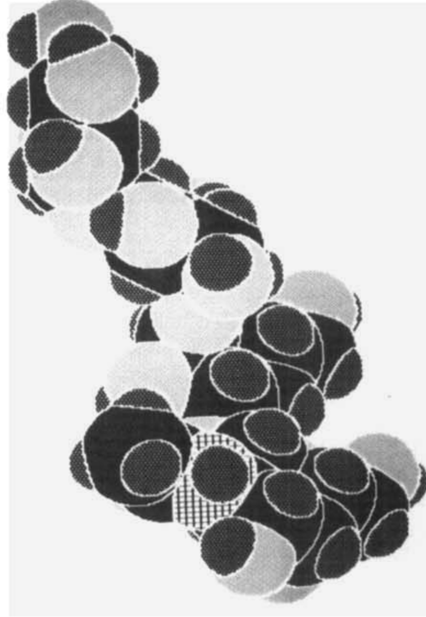
jugate structure and biosynthesis, and presents examples of the biological activities of complex carbohydrates. Although it would be impossible to review the complex carbohydrate literature in any comprehensive manner here, it is hoped that the selected examples will provide an accurate introduction to their pharmacological potential. The reader is referred to excellent recent reviews of complex carbohydrate analysis (Cummings *et al.*, 1989; Tarentino *et al.*, 1989; Lee *et al.*, 1990; Lee, 1990; Varki, 1991), biosynthesis (Elbein, 1987; Basu *et al.*, 1988; Lindahl *et al.*, 1989; Paulson and Colley, 1989; Schachter and Brockhausen, 1989; Schwarzmann and Sandhoff, 1990), chemical synthesis (Ferrier, 1990; Paulsen, 1990), and function (Bock and Harnett, 1989; Hakomori, 1990) for more detailed treatments of these areas.

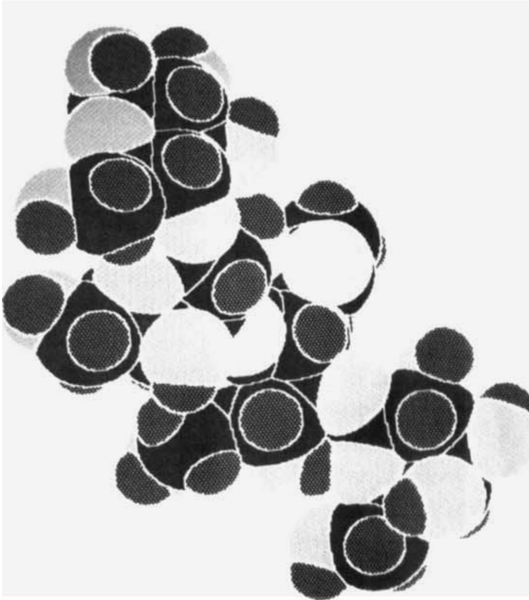
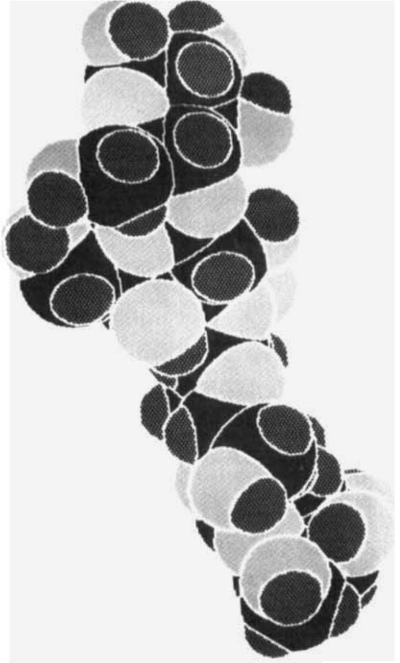
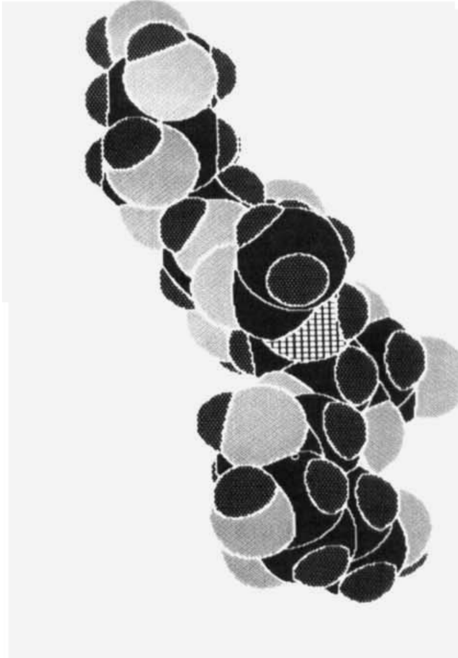
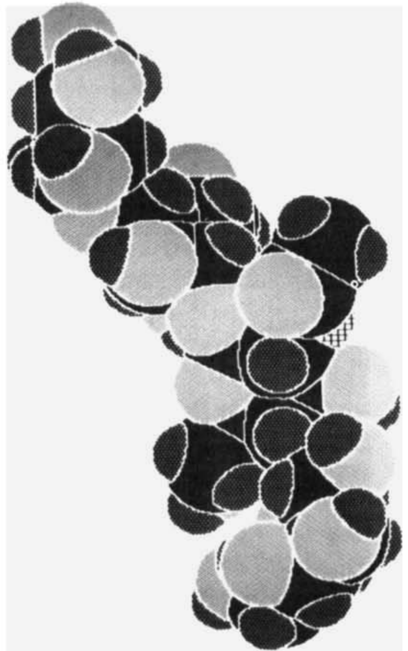
In addition to their independent functions, oligosaccharides can modify the activities of proteins to which they are covalently attached. Many glycoprotein enzymes and hormones require glycosylation for expression and function (Rademacher and Dwek, 1989). This ancillary role of carbohydrates, which is of great importance to the use of engineered glycoproteins as pharmaceuticals, is addressed at the end of the article.

II. Glycoconjugate Structure and Biosynthesis

The inherent complexity of complex carbohydrates, compared to polypeptides or nucleic acids, has been an impediment to their structural resolution. Unambiguous structural determination of any complex carbohydrate requires not only identification of the monosaccharides and their sequence, but their linkage positions, anomeric configurations, and any hydroxyl group modifications. Improvements in saccharide chromatographic resolution and detection (Kannagi *et al.*, 1987; Lee, 1990), mass

Fig. 1 Molecular models of glycosphingolipid tetrasaccharides. The four tetrasaccharides shown are commonly found attached via their reducing glucose (rightmost ring in each structure) to lipids in the cell membrane (see Section II,B). Two perspectives of each energy-minimized oligosaccharide conformation are presented, one with the reducing glucose viewed on edge (left) and one facing (right). For each perspective, the glucose is in the same orientation for each of the different tetrasaccharides to allow molecular shape comparisons. The structures are (top to bottom): Gangliotetraose, Gal β3 GalNAc β4 Gal β4 Glc; globotetraose, GalNAc β3 Gal α4 Gal β4 Glc; lactotetraose, Gal β3 GlcNAc β3 Gal β4 Glc; and neolactotetraose, Gal β4 GlcNAc β3 Gal β4 Glc. Modeling was performed using the computer program "Desktop Molecular Modeller" (Oxford University Press, Oxford, England). Carbons are shown in black, oxygens in light gray, hydrogens in dark gray, and nitrogens are crosshatched.

spectroscopy (Dell, 1987; Laine, 1989), and nuclear magnetic resonance (NMR) (Yu *et al.*, 1986; Dabrowski, 1989) have greatly enhanced carbohydrate structural analysis. Biological reagents such as exo- and endoglycosidases (Tarentino *et al.*, 1989; Lee *et al.*, 1990), carbohydrate-specific antibodies (Magnani, 1987), and carbohydrate-binding proteins (Lis and Sharon, 1984; Sharon and Lis, 1989) have also provided important tools for determining carbohydrate structure. Novel techniques, such as oligosaccharide electrophoresis (Jackson, 1990) and lectin chromatography (Merkle and Cummings, 1987; Green and Baenziger, 1989; Smith and Torres, 1989) promise to make oligosaccharide structural determination more accessible to individual investigators. As analytical methods improve, there has been an explosion in the discovery of novel complex carbohydrates. This is evidenced by the recent development of a complex carbohydrate structural database (Doubet *et al.*, 1989) currently containing >4000 structures.

Mammalian glycoconjugates are classified as glycoproteins, glycolipids, or proteoglycans. They carry carbohydrate chains assembled primarily (but not exclusively) from the eight monosaccharides shown in Fig. 2. Each monosaccharide is also found in modified forms. For example, in humans, *N*-acetylneuraminic acid (NeuAc,[1] Fig. 2) also occurs with one or more additional acetyl groups on the 4-, 7-, 8-, or 9-hydroxyl, or as the *N*-glycolyl derivative (Manzi *et al.*, 1990). The more general term, *sialic acid,* refers to all *N*-acyl derivatives of neuraminic acid and their O-acylated forms. Glucosamine, which is most commonly found as its *N*-acetyl derivative (GlcNAc, Fig. 2), is also found as the N-sulfated derivative (Lindahl *et al.*, 1989) and (rarely) as the free amine (Thomas *et al.*, 1990). Monosaccharide modifications can be essential for molecular recognition. A case in point is the addition of a phosphate ester to the 6-position of a terminal mannose on a glycoprotein, a modification which targets it for transport to the lysosome (Kornfeld, 1990; see Section III, C,3).

Glycoconjugates within a particular class share major structural features that often provide a valid starting point for structural analysis. Subtle modifications in sequence, linkage, anomeric configuration, and hydroxyl

[1] The following abbreviations are used: AT, antithrombin III; EGF, epidermal growth factor; aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; Fuc, fucose; GAG, glycosaminoglycan (unconjugated chain); Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; GSL, glycosphingolipid; HSPG, heparan sulfate proteoglycan (protein-conjugated chains); IdoA, iduronic acid; Man, mannose; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolyneuraminic acid; SMC, smooth muscle cells; Xyl, xylose. Ganglioside abbreviations are those of Svennerholm (1980).

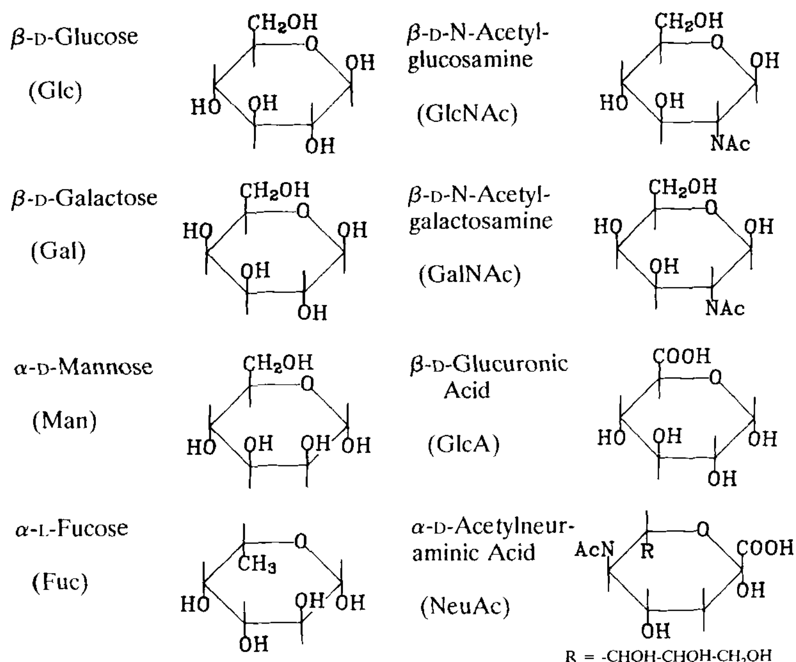


Fig. 2 Common monosaccharide constituents of mammalian complex carbohydrates. Each monosaccharide is presented in its most abundant anomeric form.

group modification lend the specificity required for biological activity. In this section, the major structural themes for each glycoconjugate class will be presented, as well as some of the more common variations.

Since sequence, anomeric configuration, and linkage position are all critical to oligosaccharide three-dimensional structure, a nomenclature has been devised to briefly but unambiguously express these features (IUPAC-IUB Joint Commission on Biochemical Nomenclature, 1987). Throughout this article, a "short-form" nomenclature is used. Each monosaccharide in an oligosaccharide chain is designated by its abbreviation (Fig. 2) followed by its anomeric configuration (α orβ) and its linkage position on the ring of the next innermost monosaccharide of the chain. For example, "Gal β4 Glc" designates galactose, in its β-anomeric form, linked from its 1-position to the 4-position of glucose. Although not explicitly designated, all saccharides in this article are in the pyranose (six-membered ring) form and are linked via their anomeric carbon (1-position for Glc, Gal, Man, Fuc, GlcNAc, GalNAc, and GlcA; 2-position for NeuAc) to the next saccharide in the chain. Branches are indicated by

parentheses, such as "Gal β4 (Fuc α3) GlcNAc," which designates galactose and fucose linked from their 1-positions to the *same* GlcNAc ring, but at different positions (4-position and 3-position, respectively).

A. Glycoproteins

Carbohydrate chains are attached to mammalian glycoproteins via the hydroxyl groups of serines and/or threonines as O-glycosidically linked chains, or at asparagines as N-glycosidically linked chains (Kornfeld and Kornfeld, 1976; Sharon and Lis, 1981). A notable exception is collagen, which carries galactosides on some of its hydroxylysines. Glycoproteins were once classified as "mucin type" or "serum type" based on the prototypes for O- and N-linked glycoproteins, respectively. These classifications became obsolete when many glycoproteins were found to carry both O- and N-linked chains on the same polypeptide (Kobata, 1984). Nevertheless, the prototype O- and N-linked structures described on mucins and serum glycoproteins remain the basis for subsequent structural analyses. The mucins are very large, highly glycosylated proteins that are major components of the epithelial mucosal layers of the respiratory, gastrointestinal, and reproductive tracts (Jentoft, 1990). The polypeptides of human mucus glycoproteins are 25–50% serine or threonine and can carry hundreds of short O-linked carbohydrate chains. In contrast, one or just a few N-linked chains are typically found on any one glycoprotein. Since N- and O-linked carbohydrate chains differ in basic structure and biosynthesis, they are discussed separately. Proteoglycans, in which the serines or threonines of polypeptides are glycosylated with long chains of repeating disaccharides, constitute a complex family of bioactive glycoconjugates that is treated as a separate class (Section II,C).

1. N-Glycosidically Linked Oligosaccharides

N-Linked glycosylation is marked by a branched $(Man)_3(GlcNAc)_2$ "core" pentasaccharide attached in N-glycosidic linkage to an asparagine (Fig. 3). The two outermost mannoses of the core are typically substituted with additional mannoses to generate "high mannose" chains or with a terminal trisaccharide (e.g. NeuAc α6 Gal β4 GlcNAc β2) to generate "complex" chains. Since each of the core mannoses can be glycosylated at multiple positions, branching is common. While most branched structures have either all high-mannose or all complex termini, in some cases one of the core mannoses carries a high mannose chain, while another carries a complex chain, generating a "hybrid" structure (Kobata, 1984).

Although the common themes outlined above are helpful in understanding N-linked oligosaccharide structure, diversity is the rule (Rademacher

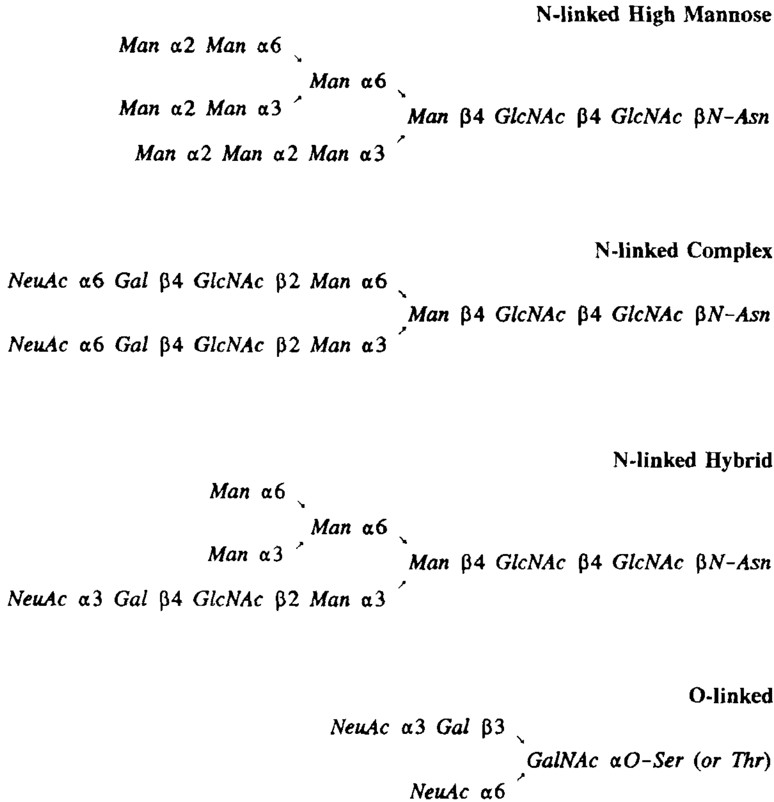


Fig. 3 Classes of glycoprotein oligosaccharide structures. Representative structures from each highly varied class are presented.

et al., 1988). In the core, a fucose (Fuc) is often found attached to the innermost GlcNAc, or an additional "bisecting" GlcNAc may be attached to the innermost mannose, creating a triple branch. Much more variability occurs in the outermost termini, regions that are most accessible to interactions with other molecules (Parekh *et al.*, 1989a). While complex chains (Fig.3) often terminate as (NeuAc α6 Gal β4 GlcNAc-), they may also terminate with (NeuAc α3 Gal β4 GlcNAc-), (Gal α3 Gal β4 GlcNAc-), (3-SO_3-Gal β4 GlcNAc-), or (4-SO_3-GalNAc β4 GlcNAc-) among others (Green and Baenziger, 1989). The latter structure is found on the glycoprotein hormones LH (luteinizing hormone), FSH (follicle-stimulating hormone), and TSH (thyroid-stimulating hormone) (Green and Baenziger, 1988a). Multiple "glycoforms" of each hormone coexist, some

with the typical NeuAc-Gal termini, but others with the 4-SO_3-GalNAc terminus. The most common glycoforms of human LH and TSH are hybrid structures with one arm of a "biantennary" branched terminus carrying a NeuAc-Gal-GlcNAc- and the other an SO_3-GalNAc-GlcNAc- structure (Green and Baenziger, 1988b).

N-Linked chains can sometimes be quite large, terminated by repeating *N*-acetyllactosamine (-Gal β4 GlcNAc β3-$)_n$ groups, where n may be $\geq$6, or chains of (-NeuAc α8-) residues, which may number >50/chain (Finne, 1989).

N-Linked carbohydrate chains are added to growing proteins during translation in the endoplasmic reticulum. Unlike most other glycoconjugates, which are synthesized by stepwise addition of each saccharide from activated nucleotide sugars to the growing carbohydrate chain, N-linked chains are transferred as a tetradecasaccharide precursor *en bloc* to an appropriate asparagine (Kornfeld and Kornfeld, 1985). The precursor is a branched $(Glc)_3(Man)_9(GlcNAc)_2$- oligosaccharide (which contains the N-linked pentasaccharide core) attached via a high-energy pyrophosphate bond to a carrier lipid, dolichol. After the tetradecasaccharide is transferred to protein, it is "trimmed" by specific glucosidases and mannosidases (Elbein, 1987). As the protein passes through the Golgi apparatus, it may be further modified by glycosyltransferases, which add the terminal sugar chains in stepwise fashion from nucleotide sugar precursors (Shaper *et al.*, 1988; Paulson and Colley, 1989; Schachter *et al.*, 1989). The oligosaccharide structure at any one asparagine varies with the particular polypeptide (or site on the polypeptide if more than one asparagine is glycosylated), and with the particular cell type in which the glycoprotein is synthesized (Sweidler *et al.*, 1985; Parekh *et al.*, 1989a). The consensus primary sequence around a glycosylated asparagine is Asn-X-Ser/Thr (Kornfeld and Kornfeld, 1985). However, a minority of such sequences are actually glycosylated and the mechanisms by which polypeptide sequences dictate oligosaccharide biosynthesis have not been determined.

2. O-Glycosidically Linked Oligosaccharides

Carbohydrate chains attached to mammalian glycoproteins at serines or threonines fall into three major categories, the "mucin-type" chains, O-linked GlcNAc, and proteoglycans (discussed in Section II,C). Mucin-type chains have a single *N*-acetylgalactosamine (GalNAc) core saccharide glycosidically linked to Ser or Thr (Fig. 3). Gal, NeuAc, Fuc, GlcNAc, and additional GalNAc residues are commonly found on short (di- to pentasaccharide) linear or branched chains, although large multibranched O-linked chains are sometimes expressed (Kornfeld and Kornfeld, 1976;

Sharon and Lis, 1981; Kobata, 1984). Terminal saccharide groups on mucins include the branched fucosylated structures responsible for A/B/H and Lewis a, b, and x blood groups specificities (Schachter and Brockhausen, 1989).

O-Linked carbohydrates are attached to proteins primarily in the Golgi apparatus, starting with transfer of GalNAc from UDP-GalNAc directly to a serine or threonine hydroxyl. Chain elongation occurs via stepwise addition of monosaccharides from their respective nucleotide sugars (Jentoft, 1990). As with N-linked oligosaccharide chains, the structure of O-linked chains on a particular polypeptide is dependent on the cell type in which that glycoprotein is synthesized (Carlsson *et al.*, 1986).

Recently, monosaccharide GlcNAc residues were found attached to clusters of serines or threonines on cytoplasmic and nuclear proteins (Torres and Hart, 1984). Up to 10% of GlcNAc residues in the cell may be intracellular O-GlcNAc, and their potential role(s) in molecular regulation are being pursued (Hart *et al.*, 1989).

B. Glycolipids

A structurally diverse family of oligosaccharides occurs on cell surfaces glycosidically attached to lipids embedded in the plasma membrane. The past few years have witnessed an explosion of interest in these molecules with a concomitant increase in the number and diversity of glycolipid structures identified (Stults *et al.*, 1989). The most prevalent glycolipids in mammalian tissues are the glycosphingolipids (GSLs), which are characterized by an oligosaccharide chain O-glycosidically linked to the 1-position hydroxyl of ceramide (Cer), a lipid with two long hydrophobic "tails" that anchor the GSL to the cell membrane (Table I) (Hakomori, 1983a). Ceramide contains a long-chain amino alcohol (sphingosine) with a fatty acid chain in amide linkage to the 2-position amine. Like other membrane lipids, variation in chain length and saturation (of the sphingosine or the fatty acid) generate a large number of naturally occurring ceramide structures, the significance of which are, as yet, unclear.

The oligosaccharide chains of GSLs are remarkably varied, with >250 distinct carbohydrate structures reported in the literature, over half of which were discovered in the past decade (Stults *et al.*, 1989). They are found predominantly in the outer leaflet of the plasma membrane (Hansson *et al.*, 1977) and are expressed (qualitatively and quantitatively) with anatomical and cell-type specificity and in developmentally regulated patterns (Svennerholm, 1974; Seyfried *et al.*, 1982; Kracun *et al.*, 1984; Yu *et al.*, 1988). Particular GSLs have been identified as major antigenic determinants, notably on certain human tumors (Hakomori, 1986; Sell, 1990)

Table I
Common Glycosphingolipid Core Structures

Structure	Name	Abbreviation
Gal β3 GalNAc β4 Gal β4 Glc β Cer	Gangliotetraosylceramide	Gg_4Cer
GalNAc β3 Gal α4 Gal β4 Glc β Cer	Globotetraosylceramide	Gb_4Cer
Gal β3 GlcNAc β3 Gal β4 Glc β Cer	Lactotetraosylceramide	Lc_4Cer
Gal β4 GlcNAc β3 Gal β4 Glc β Cer	Neolactotetraosylceramide	nLc_4Cer

and associated with certain human peripheral neuropathies (Chou *et al.*, 1986; Ariga *et al.*, 1987; Quarles, 1989).

The complexity of GSL structures has foiled attempts to develop a simple systematic nomenclature (IUPAC-IUB Commission on Biochemical Nomenclature, 1978). Nevertheless, several characteristic "core" tetrasaccharides have been defined, and many other GSL structures can be considered as derivatives and/or truncations of these core structures, some of which are shown in Fig. 1 and Table I. Nearly all mammalian GSL structures have a lactose group (Gal β4 Glc) glycosidically attached to the ceramide [the notable exceptions are galactosylceramide and its 3-O-sulfated form, sulfatide, which are major glycolipids in the brain and elsewhere (Stults *et al.*, 1989)]. In addition to the lactose, the most common saccharides found in GSLs are Gal, GalNAc, GlcNAc, NeuAc, and Fuc (others occur only rarely in mammals). GSL oligosaccharides can be found as complex branched arrays or in long chains, especially of repeating (-Gal β4 GlcNAc β3-) units. GSLs containing one or more sialic acid residue are classified as *gangliosides*, which are ubiquitous in the plasma membranes of mammalian cells, but are found in much higher concentrations in neural tissues (Ledeen, 1979; Yu and Saito, 1989). Gangliosides have generated considerable excitement for their potential in promoting neural repair, for their role in neutrophil cell adhesion, and as pathogen receptors (see Section III). Fucose-containing GSLs are of particular interest as oncofetal antigens (Hakomori, 1983b), as blood group determinants (Hakomori and Young, 1983), and in inflammation (Brandley *et al.*, 1990).

Glycosphingolipid biosynthesis occurs in the Golgi apparatus by stepwise addition of monosaccharides from nucleotide sugars to the growing chain. A separate glycosyltransferase is responsible for addition of each type of monosaccharide and linkage, but may transfer to different acceptors or at different sites along a preexisting chain to give a variety of structures (Roseman, 1979; Basu *et al.*, 1988; Schwarzmann and Sandhoff,

1990). The GSLs expressed by a cell depend on the array of glycosyltransferases present. The factors controlling expression of these glycosyltransferases have yet to be determined.

In contrast to other mammalian tissues, the major glycolipid in testes is sulfogalactosylglycerolipid, a glycerol substituted at its 3-position hydroxyl with a 3-O-sulfated galactoside, a fatty acid ester at the 2-position, and a fatty ether at the 1-position (Lingwood *et al.*, 1990). This unusual glycolipid, and a complementary binding protein, have been implicated in gamete recognition (Law *et al.*, 1988).

C. Proteoglycans

Proteoglycans consist of long chains of repeating disaccharides, termed glycosaminoglycans (GAGs, Table II), which are classified by their carbohydrate constituents and sulfation patterns. Except for hyaluronic acid, they are found attached to core proteins, each of which may carry from 1 to >100 GAG chains (Roden, 1980; Hook *et al.*, 1984). Different classes of GAGs may coexist on the same core protein, which may also carry typical N-linked and mucin-type O-linked carbohydrate chains. Although the repeating disaccharide chains of GAGs are structurally rudimentary, postpolymerization modifications by sulfation and/or epimerization (inversion of the configuration at a single carbon atom) make glycosaminoglycans among the most complex and structurally diverse biopolymers (Casu, 1989: Conrad, 1989; Lindahl *et al.*, 1989). Heparin, the most widely administered complex carbohydrate drug, is a case in point.

Heparin preparations consist of a highly heterogeneous mixture of sulfated glycosaminoglycan chains that have been metabolically clipped from their core protein(s) by limited endoglycosidase cleavage and that range in size from ~5000 to ~30,000 Da (Lindahl *et al.*, 1989). As a member of the heparan sulfate class of GAGs, the basic repeating disaccharide of heparin is (-GlcA β4 GlcNAc α4-$)_n$. Postpolymerization enzymatic modifications include N-sulfation of the hexosamine, C-5 epimerization of glucuronic acid (GlcA) to iduronic acid (IdoA), O-sulfation at the 2-position hydroxyl of IdoA and/or GlcA, and O-sulfation of the 6- and/or 3-position hydroxyl of the hexosamine. Since each modification occurs on only a subset of available sites, 32 unique disaccharides can theoretically be generated, although biosynthetic restrictions limit the number of actual heparin disaccharides to <20 (Lindahl *et al.*, 1989). Even so, thousands of "epitopes" consisting of distinct hexa- to octasaccharides might exist along heparin chains, each with the unique potential to interact with specific protein-binding sites. To explain the wide range of biological effects of heparin, Jaques (1982) suggests that, in the lock-and-key model for biochemi-

Table II
Proteoglycans

Proteoglycan	Repeating disaccharide	Sulfation sites	Protein linkage
Hyaluronic acid	(GlcA β3 GlcNAc β4)$_n$	None	None proven
Heparin/heparan sulfates	(GlcA β4 GlcNR α4)$_n$ (IdoA α4 GlcNR α4)$_n$ R = Ac or SO_3	GlcNR-6-SO_3, GlcNR-3-SO_3, GlcNR-3,6-di-SO_3, IdoA-2-SO_3, GlcA-2-SO_3	GlcA β3 Gal β3 Gal β4 Xyl β-Ser
Chondroitin sulfates	(GlcA β3 GalNAc β4)$_n$	GalNAc-4-SO_3, GalNAc-6-SO_3	As above
Dermatan sulfates	(GlcA β3 GalNAc β4)$_n$ (IdoA α3 GalNAc β4)$_n$	GalNAc-4-SO_3, GalNAc-6-SO_3, Ido-2-SO_3	As above
Keratan sulfates	(Gal β4 GlcNAc β3)$_n$	Gal-6-SO_3, GlcNAc-6-SO_3	Typical N- and O-glycosidic linkages[a]

[a] See Fig. 3.

cal specificity, heparin should be viewed as " . . . a bag of skeleton keys which can fit many locks." The detailed structure of one such key, the precise heparin sequence responsible for antithrombin binding (a pentasaccharide) has been elucidated (see Section III,C,1). The basic structures and common modifications of the other GAGs are presented in Table II. While the same general considerations apply to each class of sulfated GAGs, less structural diversity has been found, to date, in the chondroitin, dermatan, and keratan sulfates than in the heparan sulfates.

Heparan, dermatan, and chondroitin sulfate chains are attached to proteins via an unusual linkage tetrasaccharide, GlcA β3 Gal β3 Gal β4 Xyl β Ser (where Xyl is xylose). Chain initiation occurs via direct transfer of Xyl from UDP-Xyl to a serine hydroxyl group on the core protein, presumably in the endoplasmic reticulum (Roden, 1980; Lindahl *et al.*, 1989). After addition of the two Gal residues and the first GlcA, the polymer is constructed (in the Golgi apparatus) by alternating addition of hexosamine and glucuronic acid residues from their nucleotide sugars. Postpolymerization modifications on heparan sulfates occur in an ordered fashion, with de-N-acetylation/N-sulfation occuring first, then GlcA epimerization, followed by O-sulfations. Remarkably, all of these postpolymerization modifications occur within 30 sec. An intriguing model to explain these findings has been proposed by Lindahl *et al.* (1986). They envision the extended polysaccharide chains traversing fixed arrays of modifying enzymes, with each modification "station" immobilized on a membrane scaffold. This model is supported by the observation that modifications (or their absence) are not random along each heparin chain but occur in blocks.

Unlike other GAGs, keratan sulfates, which contain the broadly distributed *N*-acetyllactosamine repeat (-Gal β4 GlcNAc β3-)$_n$, are found attached to proteins via the typical N- or O-glycosidic linkages described in Section II,A (Roden, 1980).

III. Bioactivity and Pharmaceutical Potential of Complex Carbohydrates

Discoveries in complex carbohydrate bioactivity have kept pace with the rapid advances in carbohydrate purification, analysis, and synthesis. From viral infection to mammalian fertilization, cell–cell interactions and cell regulation have been associated with complex carbohydrate recognition. For each physiological or pathological interaction in which oligosaccharides or glycoconjugates participate, there is an opportunity for pharmaco-

logical intervention. This section presents some examples of complex carbohydrate bioactivity and their associated pharmaceutical opportunities.

A. Toxin, Virus, and Bacterial Binding

Many pathogens require interaction with cell-surface complex carbohydrates as an essential part of the etiology of the diseases they cause. Among these are certain viruses, bacteria, and bacterial exotoxins. The complex carbohydrates to which the pathogens bind are often referred to as the pathogen *receptors*. This terminology can be confusing, in that one typically thinks of receptors as proteins having a binding site for a ligand, while pathogen ''receptors'' are the cell surface ligands to which pathogen proteins bind (Paulson, 1987). In this context, a receptor *determinant* is defined as the limited structure (e.g., the minimum oligosaccharide sequence) recognized by a pathogen. Since some carbohydrate determinants are carried by both proteins and lipids, a determinant can be identified experimentally without determining the glycoconjugate(s) responsible for binding *in vivo*. Determinants may be effective inhibitors of pathogen attachment, or provide lead compounds for developing such inhibitors. This section summarizes a few well-defined systems in which carbohydrate determinants are responsible for pathogen binding.

1. Bacterial Exotoxin Binding

Protein exotoxins are released from bacteria, bind to target cells, and alter cellular functions (Middlebrook and Dorland, 1984). Some exotoxins bind specifically to cell-surface complex carbohydrates. One of the best characterized of these carbohydrate-binding pathogens is cholera toxin, the protein exotoxin of *Vibrio cholerae* responsible for severe diarrhea. Cholera toxin consists of two functional subunits, A and B, in an A_1B_5 hetero-oligomer (Holmgren and Lonnroth, 1980). B subunits, which are not themselves toxic, bind avidly to the intestinal epithelial cell surface, specifically to the cell-surface ganglioside G_{M1} [Gal β3 GalNAc β4 (NeuAc α3) Gal β4 Glc β1′ Cer; see Section II,B) (Holmgren *et al.*, 1973; van Heyningen, 1974). B subunit/G_{M1} binding causes a local membrane perturbation, allowing the A subunit to traverse the membrane and enter the cytoplasm, where it causes persistent activation of adenylate cyclase by enzymatically ADP-ribosylating a regulatory G protein (Cassel and Pfeuffer, 1978). When the A subunit is dissociated from the B subunits it retains its enzymatic activity (in broken cell preparations) but is no longer toxic to intact cells. Cells that lack G_{M1} are resistant to cholera toxin, but become susceptible if the ganglioside is experimentally inserted into their plasma membranes

(Moss *et al.* 1976). Since cholera is so epidemiologically important, evolutionary pressures would be expected to select against expression of G_{M1} in the susceptible intestinal epithelium. In fact, among all human tissues tested the intestinal mucosa is the lowest in ganglioside expression (Svennerholm, 1980). The persistent expression of enough G_{M1} to allow cholera toxicity may suggest an essential role for the ganglioside in intestinal cell physiology, although that role has yet to be identified.

Experimental studies have tested gangliosides as competitive inhibitors of the toxin in human patients. Notably, treatment with a crude ganglioside mixture actually worsened symptoms, presumably since some of the administered G_{M1} spontaneously inserted in cell membranes, increasing toxin binding (Nalin, 1981). In contrast, administration of G_{M1} preadsorbed to charcoal resulted in elimination of free toxin in the intestinal lumen and reduced purging in infected individuals (Stoll *et al.*, 1980). While this is unlikely to be developed as a practical therapy, it is a paradigm for the therapeutic application of complex carbohydrates identified as pathogen receptors.

Other bacterial exotoxins appear to utilize gangliosides as cell-surface receptors. Some pathogenic *Escherichia coli* strains produce an exotoxin, LT, that is closely related to cholera toxin and causes "traveler's diarrhea." Like cholera toxin, LT binds ganglioside G_{M1}, which may be its intestinal receptor (Holmgren, 1973; Tsuji *et al.*, 1985). Another *E. coli*-elaborated toxin, verotoxin, binds the neutral determinant on globotriaosylceramide, Gal α4 Gal β4 Glc (Waddell *et al.*, 1990).

Gangliosides, while found in all vertebrate tissues, predominate in nervous tissue. It is not surprising, then, that certain neurotropic exotoxins bind to gangliosides. In particular, tetanus and botulinum toxins, both of which block neurotransmitter release, bind to gangliosides containing the "1b" substructure Gal β3 GalNAc β4 (NeuAc α8 NeuAc α3) Gal β4 Glc β1′ Cer, which is contained in the major neuronal gangliosides G_{T1b} and G_{D1b} (Habermann and Dreyer, 1986: Montecucco, 1986). Tetanus toxin, the protein exotoxin from *Clostridium tetani,* is the best studied of these neurotropic toxins. Early studies demonstrated that crude brain gangliosides could "fix" tetanus toxin *in vitro* (van Heyningen, 1980). Subsequently, purified 1b gangliosides were demonstrated to selectively inhibit high-affinity tetanus toxin binding to isolated brain membranes and to suppport toxin binding directly (Holmgren *et al.*, 1980b; Rogers and Snyder, 1981; Critchley *et al.*, 1986). Tetanus toxin has a molecular domain responsible for cell binding and a separable domain that, when injected intracellularly, blocks exocytosis (Penner *et al.*, 1986). Although the molecular mechanisms of tetanus toxicity are not fully resolved, and there remains controversy over the endogenous receptor for the toxin, a

reasonable hypothesis is that the cell-binding domain interacts specifically with gangliosides of the 1b family, leading to uptake followed by retrograde and transsynaptic transport. Subsequently, the exocytosis-blocking domain generates alterations in cell physiology that block neurotransmitter release (Habermann and Dreyer, 1986).

2. Viral Attachment

Membrane-enveloped viruses have surface attachment proteins responsible for target cell binding. The best defined viral attachment proteins are those of the influenza viruses (Paulson, 1987). In early experiments, influenza attachment proteins were found to agglutinate red blood cells *in vitro*, leading them to be termed *hemagglutinins*. A receptor-destroying enzyme was found associated with the hemagglutinins and was subsequently identified (in some strains) as a neuraminidase. The hemagglutinin mediates attachment of viruses to their target cells and initiates membrane fusion leading to release of the viral transcription complex into the cytoplasm. The neuraminidase is thought to facilitate subsequent budding of virus from the cell surface. The discovery of viral neuraminidase as the receptor-destroying enzyme focused attention on sialic acids as viral receptor determinants. In an elegant series of experiments J. Paulson and colleagues probed the fine structure of the target cell-surface sugars to which influenza viruses bind (Higa *et al.*, 1985). Removal of sialic acids from the red cell surface eliminated virus binding. Resialylation using highly purified sialyltransferases and activated sugar donors (CMP-sialic acids) reestablished virus binding and revealed the subtle carbohydrate specificity of the viral hemagglutinins. Some virus isolates required NeuAc α3 Gal for binding, while others required NeuAc α6 Gal and yet others bound to either linkage. Some isolates required the sialic acid to be N-acetylated (NeuAc), others to be N-glycolated (NeuGc), and some bound to either structure. There were even strains (influenza C) that bound only to sialic acids having acetyl groups on both the amino group and the 9-position hydroxyl (9-O-Ac-NeuAc) (Rogers *et al.*, 1986). The receptor-destroying enzyme of influenza C, notably, is a neuraminate O-acetylesterase. The binding selectivity based on saccharide fine structure demonstrates the ability of saccharides to encode very specific recognition. Because protein–saccharide binding affinity is largely based on hydrogen bonding between specific hydroxyl groups on the oligosaccharide and precisely positioned amino acid residues in the binding pocket (Quiocho, 1986), subtle alterations in protein primary structure can be reflected in marked changes in carbohydrate specificity. Since the primary and three-dimensional crystal structures of influenza virus hemagglutinins are

known, the molecular details of carbohydrate recognition in this system are now accessible (Weis *et al.*, 1988).

Since both glycoproteins and glycolipids containing terminal sialic acids bind influenza virus *in vitro* (Suzuki *et al.*, 1985a; Pritchett and Paulson, 1989), it is not clear which glycoconjugate(s) are responsible for infection *in vivo*. However, recent studies have revealed an additional key factor in high-affinity carbohydrate recognition: multivalent binding. Viral hemagglutinins are multimeric in the viral envelope, and bind most avidly to multiple optimally spaced carbohydrate determinants (Pritchett and Paulson, 1989). For example, equine α_2-macroglobulin is a high-affinity glycoprotein receptor for influenza virus while human α_2-macroglobulin supports only low-affinity binding. Surprisingly, oligosaccharides derived from these glycoproteins bind with equivalent (and relatively low) affinity. Evidently, the spacing between oligosaccharides on the equine α_2-macroglobulin results in optimal binding to the hemagglutinin. The importance of multivalency, which has been documented in detail for other protein–carbohydrate binding systems (Weigel *et al.*, 1979; Hardy *et al.*, 1985; Lee and Lee, 1987; Schengrund and Ringler, 1989), has important implications for the design of high-affinity carbohydrate-based drugs. In particular, appropriately spaced sialic acid residues synthesized on a low-molecular-weight carrier may provide the best opportunity for developing potent antivirals (Matrosovich *et al.*, 1990).

In addition to influenza, several other viruses have been reported to bind to glycoconjugates, including rabies virus, Sendai virus, Newcastle disease virus, coronaviruses, and reoviruses (Holmgren *et al.*, 1980a; Suzuki *et al.*, 1985b; Superti *et al.*, 1986; Vlasak *et al.*, 1988; Willoughby *et al.*, 1990). The carbohydrate determinants for these viruses have not been as fully characterized as those for influenza.

3. Bacterial Adherence

Unlike toxins and viruses, bacterial virulence does not require specific host cell binding per se. Nevertheless, adherence may protect bacteria from normal removal mechanisms such as fluid shear, increasing the probability of colonization. Experimental evidence suggests that bacterial lectins (carbohydrate-binding proteins) function in host adherence and the initiation of infection (Karlsson, 1989; Ofek and Sharon, 1990).

Sugar-specific adherence of bacteria to host cells was first detected as the ability of enterobacteria to agglutinate red blood cells. Early studies denoted bacterial strains as mannose sensitive for agglutination (inhibited by mannose and mannosides) or mannose resistant. Subsequently, several distinct carbohydrate specificities were detected, some of which are ex-

pressed concomitantly by the same cultures. The best characterized of theses are Man-specific and Gal $\alpha 4$ Gal-specific binding by pathogenic strains of *E. coli*. In both cases, the lectin is found at the tips of filamentous bacterial appendages termed fimbriae or pili.

Sharon and colleagues (Sharon, 1987) explored the carbohydrate determinants for several mannose-sensitive bacterial strains. They concluded that *E. coli* strains were specific for α-mannosides, and preferentially bound to naturally occurring structures containing a nonreducing terminal Man $\alpha 3$ Man. Of pharmaceutical interest, α-mannosides having a large apolar aglycon (e.g., *p*-methylumbelliferyl α-mannoside) were 10-fold more potent than naturally occuring oligosaccharides and 1000-fold more potent than α-methyl mannoside in inhibiting *E. coli* adherence to epithelial cells. These and other investigators demonstrated that high concentrations of mannose or mannosides specifically inhibited bacterial infections in a number of rodent experimental models (Ofek and Sharon, 1990). While these data are encouraging, two caveats should be noted. First, bacteria may express multiple saccharide-binding proteins with distinct determinant specificities. Second, recognition of mannosides on the surface of macrophages by bacteria may actually facilitate their phagocytosis, a process which has been termed *lectinophagocytosis* (Ofek and Sharon, 1988). Blocking this defense mechanism may be counterproductive. Only further experiments will determine the effectiveness of carbohydrate inhibitors as antibacterials.

Other forms of carbohydrate-directed bacterial adherence have been identified using a modification of the clever "overlay" technique introduced by Magnani *et al.* (1980). Glycolipids were extracted from tissues or cells and resolved by thin-layer chromatography (TLC). Subsequently, the TLC plate was dried, plastic impregnated, and overlaid with radioactively labeled bacteria (Hansson *et al.*, 1985). After incubation, nonadherent cells were removed by washing, then the TLC plate was dried and subjected to autoradiography. Only those glycolipids bearing the receptor determinant bound the bacteria. Bock *et al.* (1985) applied this technique to uropathogenic strains of *E. coli* and found that GSLs containing a Gal $\alpha 4$ Gal determinant, whether at the nonreducing terminus or internal in the oligosaccharide, bound the bacteria while GSLs lacking the determinant were nonsupportive of binding. Naturally occurring GSLs containing this determinant were apparent in extracts from human ureter epithelium. When a rapid screening method was used to detect bacterial binding to immobilized Gal $\alpha 4$ Gal, ~80% of *E. coli* isolates from urine of patients with pyelonephritis but only 13% of isolates from normal feces were positive for binding (Enerback *et al.*, 1987).

An interesting correlation between glycolipid fine structure and patho-

gen susceptibility was documented for certain *E. coli* strains (expressing the K99 antigen) that cause diarrhea in neonatal, but not adult, animals (Teneberg *et al.*, 1990). A component of the glycolipid extracts from piglet intestinal mucosa bound the bacteria, while adult extracts were devoid of binding activity. The receptor glycolipid was identified as NeuGc α3 Gal β4 Glc β1′ Cer (ganglioside G_{M3} having an N-*glycolyl* group on the sialic acid). G_{M3} having an N-*acetyl* group on the sialic acid, remarkably, was completely nonsupportive of binding. Apparently, the lectin of these bacteria distinguishes between the presence or absence of a single hydroxyl group on a glycolipid receptor of ~1200 Da. Of interest, the *N*-glycolyl form of G_{M3} is found in neonates, but is replaced by the *N*-acetyl form as the animals mature.

Many different bacterial species and strains bind to lactosylceramide and/or gangliotetraosylceramide *in vitro* (Karlsson, 1989; Ofek and Sharon, 1990). Normal flora and pathogenic strains with various tissue tropisms bind, and the target glycolipids are expressed on many different tissues. While recognition of these glycolipids may be important to colonization, the physiological and pathological significance of this binding has yet to be established. Among bacterial species that recognize these glycosphingolipids is *Neisseria gonorrhoeae,* which are responsible for one of the most commonly reported infectious diseases (Stromberg *et al.*, 1988; Deal and Krivan, 1990). Using a powerful combination of molecular cloning and glycolipid TLC overlay techniques, Paruchuri *et al.* (1990) directly isolated recombinant *E. coli* harboring the gene for the *N. gonnorrhoeae* surface protein responsible for glycolipid recognition. Molecular characterization of this lectin will provide the tools to establish its function, an important step toward developing carbohydrate-based inhibitors.

B. Cell–Cell Recognition and Adhesion

Cell-surface glycoconjugates have long been posited to act as cell–cell recognition molecules. Several possible modes for carbohydrate interactions on the cell surface have been envisioned (Rosemann, 1970; Sharon and Lis, 1989): (1) Specific binding between carbohydrates on one cell and protein receptors on an apposing cell; (2) cross-linking of surface carbohydrates on apposing cells by extracellular soluble lectins; and (3) specific interactions directly between carbohydrates on apposing cells (also see Eggens *et al.*, 1989; Kojima and Hakomori, 1989). As described below, new analytical and cell biological techniques have provided experimental evidence demonstrating carbohydrate-mediated cell–cell recognition, resulting in exciting new opportunities for carbohydrate-based drug development.

1. Leukocyte Adhesion Molecules

The hypothesis that cell-surface carbohydrates are involved in cell–cell recognition has received a remarkable boost from the discovery that a series of related proteins, previously demonstrated to mediate leukocyte adhesion, are lectins. Immunological techniques have identified proteins involved in leukocyte adhesion to vascular endothelium (Stoolman, 1989). Determination of their primary amino acid sequences (Bevilacqua *et al.*, 1989; Johnston *et al.*, 1989; Lasky *et al.*, 1989; Siegelman *et al.*, 1989) revealed that three of these, previously referred to as gp90^{mel}, ELAM1, and GMP-140, contained structural motifs related to well-characterized vertebrate lectins (Drickamer, 1988). Based on their molecular domains, they have been renamed LECAM1, LECAM2, and LECAM3, respectively (Brandley *et al.*, 1990). Previous data was consistent with the discovery of a carbohydrate-binding domain on LECAM1 (gp90^{mel}). This protein was detected on the surfaces of lymphocytes, where it is responsible for their binding to specialized peripheral lymph node vasculature termed high endothelial venules (Jalkanen *et al.*, 1987). Binding of isolated lymphocytes to lymph node tissue sections was potently inhibited by mannose phosphate and fucose sulfate polymers, and was neuraminidase sensitive (Stoolman *et al.*, 1984; Rosen *et al.*, 1985). While there are significant data to implicate anionic saccharides in the LECAM1-binding determinant, the endogenous structure responsible for binding has eluded characterization, primarily because of the difficulty in obtaining sufficient quantities of high endothelial venule target tissue.

Identification of a lectin motif in LECAM2 and LECAM3 was unexpected, and led investigators to search for complementary carbohydrate ligands. Within months, several groups using a variety of techniques independently identified a carbohydrate structure that bound LECAM2 (see below), and a report of carbohydrate-specific binding by LECAM3 has also appeared (Larsen *et al.*, 1990). The following discussion focuses on the binding determinant for LECAM2, since it is under intense scrutiny as a potential carbohydrate-based antiinflammatory.

Inflammation involves the specific binding of leukocytes to activated endothelium and their extravasation into the surrounding tissue. LECAM2 is expressed on human endothelial cells after activation, and a monoclonal antibody to LECAM2 inhibits leukocyte adhesion to activated endothelial cells (Bevilacqua *et al.*, 1987). Discovery of a lectin domain in LECAM2 (Bevilacqua *et al.*, 1989) led investigators to the leukocyte cell-surface glycoconjugates responsible for leukocyte-endothelial adhesion. Two indirect experimental approaches proved informative: (1) Transfection of nonmyeloid cells with cDNA for an $\alpha(1,3)$fucosyltransferase converted

them from LECAM2 nonbinders to binders. Among fucosylated determinants that could be identified by previously established monoclonal antibodies, LECAM2 binding correlated best with the expression of the sialyl Lewis x (sialyl-Le^x) structure: NeuAc α3 Gal β4 (Fuc α3) GlcNAc (Lowe *et al.*, 1990); and (2) cell adhesion to activated endothelium was inhibited by established anti-sialyl-Le^x antibodies or by liposomes carrying purified glycolipids with the sialyl-Le^x determinant (Phillips *et al.*, 1990; Walz *et al.*, 1990).

Similar results were obtained by Tiemeyer *et al.* (1991), who used a direct approach to identify endogenous glycoconjugate ligands for LECAM2. Glycosphingolipids extracted from neutrophil-like human myelogenous leukemia cells (Fukuda *et al.*, 1986) were resolved by TLC. The plates were placed in specially designed cell adhesion chambers (Swank-Hill *et al.*, 1987), overlaid with radiolabeled cells expressing LECAM2, and adherent cells detected by autoradiography. Cells adhered to positions on the TLC plate corresponding to the migration of only a subpopulation of the glycolipids. The supportive glycolipid species were isolated using specific cell adhesion as an assay to direct their purification. Mass spectroscopy and carbohydrate compositional and linkage analysis of the purified glycolipids revealed the sialyl-Le^x determinant and a related structure, NeuAc α3 Gal β4 GlcNAc β3 Gal β4 (Fuc α3) GlcNAc. Concordance with other reports gives considerable confidence that sialyl-Le^x-related structures form the minimum binding determinant for LECAM2, and will provide excellent lead compounds for drug development. More significantly, these studies establish the physiological importance of carbohydrate-mediated cell recognition and demonstrate several technologies that can rapidly identify the relevant carbohydrate determinants.

2. Fertilization

The first cell–cell interaction in mammalian development is the specific binding between complementary molecules on the surfaces of sperm and egg. In particular, avid binding of sperm to the egg extracellular coat, the zona pellucida, is required for penetration and fertilization to occur. Compelling experimental evidence identifies specific zona pellucida glycoprotein oligosaccharide determinants as the targets for sperm binding (Wassarman, 1990).

Of the three glycoproteins that make up the mass of the mouse zona pellucida, ZP3 is responsible for specific sperm binding. Purified ZP3 not only supports binding by the adhesive portion of the sperm head, but blocks sperm–egg association *in vitro* and can induce the acrosome reaction (see below), while other glycoproteins (including ZP1 and ZP2)

are ineffective. Sperm binding to ZP3 was not compormised by enzymatic removal of its N-linked carbohydrate chains, but was blocked by β-elimination, which removes O-linked carbohydrate chains. A portion (~10%) of the size-fractionated O-linked oligosaccharides isolated after β-elimination inhibited sperm–egg binding (Bleil and Wassarman, 1988). Pretreatment of these oligosaccharides with α-galactosidase (but not several other glycosidases, including β-galactosidase) eliminated inhibitory activity, as did treatment with galactose oxidase, an enzyme that converts the 6-position hydroxyl on galactose to an aldehyde. Furthermore, α-galactose residues were identified immunochemically on intact ZP3, but not on ZP1 or ZP2. These data strongly implicate an α-Gal residue on an O-linked ZP3 oligosaccharide as part of the relevant determinant for sperm binding.

Successful fertilization requires not only sperm binding, but induction of the acrosome reaction (AR), in which a lysosome-like organelle in the sperm head fuses with its plasma membrane and exposes proteases required for penetration. Purified ZP3 induces the AR. However, glycopeptides prepared from ZP3 do not, although they do block sperm–egg binding. When ZP3 glycopeptides were bound to sperm, then cross-linked with an anti-ZP3 antibody, the AR proceeded. Successive treatment of sperm with ZP3 glycopeptides and monovalent Fab fragments from anti-ZP3 antibody were without effect. However, further cross-linking of the Fab fragments with intact anti-IgG antibody initiated the AR. These data suggest that it is the multivalent nature of ZP3, and cross-linking of sperm-binding proteins, that induce the AR (Leyton and Saling, 1989). The observation that sperm binding and induction of the AR can be induced by isolated zona pellucida glycoproteins has been extended to other mamalian species, including man (Cross *et al.*, 1988). Further structural details of the ZP3 sperm-binding determinant are eagerly awaited, and may lead to information helpful in understanding and modulating human fertility.

A second protein–carbohydrate interaction, the binding of a sperm cell surface β-galactosyltransferase to nonreducing terminal GlcNAc residues on the egg surface, has been suggested to mediate sperm–egg binding in the mouse (Lopez *et al.*, 1985). However, the presence of galactosyltransferase on the intact sperm membrane remains controversial (Chan *et al.*, 1990).

An unusual sulfogalactosylglycerolipid is the major testicular glycolipid in vertebrates (see Section I,B). A prominent testicular protein, SLIP1, which binds to sulfogalactosylglycerolipid, colocalizes with the glycolipid early in spermatogenesis, but segregates from it prior to sperm maturation (Law *et al.*, 1988). Although SLIP1 is also found on oocytes and may be involved in sperm–egg recognition, a role for sulfogalactosylglycerolipid-SLIP1 binding in fertilization has yet to be established.

3. Neural Cell Interactions

The most complex network of cell–cell interactions is the vertebrate nervous system, where $>10^{11}$ neurons connect with topographic and cellular specificity to numerous targets and support cells. Among the multiple systems for cell recognition operating in the nervous system, the prominence and diversity of cell surface carbohydrates strongly implicate their involvement. Sensitive analytical and immunochemical techniques reveal that cell-surface carbohydrate determinants vary among distinct neural cell populations, in different anatomical regions, and during development (Kracun *et al.*, 1984; Regan *et al.*, 1986; Yu *et al.*, 1988). A particularly fascinating example of this is the ganglioside G_{D3} (NeuAc α8 NeuAc α3 Gal β4 Glc β1′ Cer). While G_{D3} is evenly distributed across the developing mammalian retina, the same structure having a 9-*O*-acetyl group on the terminal sialic acid is distributed in a marked dorsoventral gradient and may be involved in topographical recognition (Blum and Barnstable, 1987; Sparrow and Barnstable, 1988). Other examples of carbohydrate determinants that may be involved in neural recognition are detailed below.

a. Carbohydrates and Complementary Lectins in Dorsal Root Ganglia and Spinal Cord. Primary sensory neurons in the dorsal root ganglion receive somatosensory input from the periphery and send the signals onward via projections to the spinal cord. Several distinct classes of dorsal root ganglion neurons, each of which extends axons to particular regions (lamina) of the spinal cord, have been identified by anatomic and functional criteria (Brown, 1983).

Studies with monoclonal antibodies that recognize discrete carbohydrate determinants revealed distinct patterns of glycoconjugate expression by different classes of dorsal root ganglion neurons (Dodd and Jessell, 1985). For example, cells expressing a particular sialylated globoseries structure (recognized by antibody SSEA-4) make up ~10% of the dorsal root ganglion neurons, and project to deep (laminae III and IV) spinal cord targets, while cells expressing neolactoseries determinants (see Table I) with nonreducing Gal termini (recognized by antibody A5) are morphologically distinct, make up about half of the dorsal root ganglion neurons, and project to the more superficial dorsal spinal cord (laminae I and II).

Although a direct role for glycoconjugate expression in this system has not been established, a fascinating correlation in the distribution of Gal-terminated determinants and Gal-specific lectins was reported (Regan *et al.*, 1986). Soluble Gal-specific lectins are found in extracts from many vertebrate tissues, including the nervous sytem (Barondes, 1984), and are located both intra- and extracellulary. Antibodies to Gal-specific lectins revealed differences in expression by subsets of dorsal root ganglion neurons. For example, a lectin designated RL-29 colocalized with >90% of

A5-positive cells project, but not in the deeper laminae where SSEA-4 cells recognized by SSEA-4 (expressing a sialylated globoseries determinant). The same lectin was found in the superficial dorsal spinal cord where A5-positive cells project, but not in the deeper laminae where SSEA-4 cells project. Based on this correlation, it has been proposed that extracellular Gal-specific lectins might facilitate cell–cell recognition by cross-linking membranes expressing the appropriate determinants (Jessell *et al.*, 1990).

b. Sulfated Glucuronyl Glycosphingolipids and the HNK-1 Adhesion Determinant. Some patients with plasma cell neoplasms expressing monoclonal IgM antibodies have associated weakness and sensory loss characteristic of peripheral neuropathy. In a large proportion of such patients having neuropathies characterized by disruption of myelin (the membranes of Schwann cells, which insulate axons projecting to or from the central nervous system) IgMs bind to carbohydrate determinants shared by myelin glycoproteins and glycolipids that are also recognized by a mouse monoclonal antibody, HNK-1 (Quarles *et al.*, 1986). Glycosphingolipids expressing this determinant were purified and their structure characterized (Chou *et al.*, 1986; Ariga *et al.*, 1987). They have the unusual nonreducing terminal saccharide 3-SO_3-GlcA $\beta3$ attached to the outermost Gal of a neolactosylceramide core (see Table I). It is assumed (although not demonstrated) that similar structures are carried by immunoreactive glycoproteins, including some forms of well-characterized cell adhesion molecules (Schachner, 1989).

Although HNK-1-reactive lipids are found on peripheral axons and Schwann cells (Kohriyama *et al.*, 1987), the role of the HNK-1 determinant in peripheral nerve demyelination remains speculative. Rabbits immunized with lipids carrying the HNK-1 determinant showed some muscle weakness and sciatic nerve damage (Kohriyama *et al.*, 1988; Yu *et al.*, 1990), and plasmapheresis reduced neuropathy in a patient expressing antibodies against this epitope (Sherman *et al.*, 1984). Furthermore, HNK-1 determinants may act as adhesive ligands, in that cell–cell and substratum adhesion of neural cells *in vitro* was perturbed by HNK-1 antibody and by HNK-1-reactive glycolipids and oligosaccharides (Kunemund *et al.*, 1988). These observations are consistent with a role for HNK-1-reactive glycolipids in the maintenance of peripheral nervous system myelin.

Direct evidence that HNK-1-reactive glycosphingolipids support Schwann cell adhesion *in vitro* was recently reported (Needham and Schnaar, 1990). When peripheral nerve glycolipids were resolved by TLC and overlaid with radiolabeled Schwann cells, the HNK-1 reactive glycosphingolipids supported cell adhesion while gangliosides and neutral glycosphingolipids did not. The adhesion was cell type specific, in that liver,

retina, and fibroblast cells did not adhere to the HNK-1-reactive glycolipids. These data raise the hope of identifying receptor molecules on Schwann cell membranes complementary to the HNK-1 determinant that might mediate cell recognition in the peripheral nervous system.

c. A Ganglioside-Binding Protein in the Central Nervous System. Gangliosides are ubiquitous in vertebrate tissues, but are found at especially high concentrations in the brain (Ledeen, 1979). While much is known about their structure, anatomical and cellular distribution, and developmental expression, their physiological functions remain elusive. The recent discovery of sialyl-Le^x ganglioside binding by the protein LECAM2 in neutrophil–endothelium adhesion (see Section III,B,1) strengthens the theory that gangliosides and ganglioside-binding proteins mediate neural cell recognition. Complementary binding proteins for gangliosides in the central nervous system were recently probed using the major brain ganglioside G_{T1b} [NeuAc α3 Gal β3 GalNAc β4 (NeuAc α8 NeuAc α3) Gal β4 Glc β1′ Cer] as ligand (Tiemeyer *et al.*, 1989). By covalently linking multiple G_{T1b} molecules, via their ceramide portion, to a radiolabeled protein carrier (albumin), a high-affinity ligand for ganglioside-binding proteins was synthesized. Radioligand binding to isolated rat brain membranes revealed a high-affinity binding activity selective for G_{T1b} and related structures. Subcellular and anatomical studies localized the binding activity specifically to central nervous system myelin (Tiemeyer *et al.*, 1990), which is structurally much like peripheral nervous system myelin, but is elaborated by oligodendroglial cells rather than Schwann cells and contains different cell-surface proteins. These findings were of interest, since G_{T1b} and related structures are absent from oligodendroglial membranes, but are dominant species on the axons which they ensheathe. This raises the possibility that a myelin ganglioside receptor may be involved in oligodendroglial–axon recognition or in myelin stabilization.

C. Regulatory Carbohydrates

Through noncovalent or covalent association with proteins, enzymes, and intact cells, specific complex carbohydrates act as potent physiological regulators. Identification of the bioactive carbohydrate structures and their mechanisms of action hold significant promise for drug development.

1. Heparin

Heparin was one of the first carbohydrate-based drugs (Jaques, 1978). While much is known about its molecular structure and the mechanism of its anticoagulant activity, it remains one of the most complex and function-

ally diverse mixtures in the pharmacopeia (Jaques, 1982). This is because, as described in Section I,C, postpolymerization modifications result in thousands of epitopes within the glycosaminoglycan chains. Compelling evidence demonstrates that biological systems take advantage of the subtle structural variations in heparin, and hold out the hope that many of its biological effects can be traced to specific defined oligosaccharide determinants. We are just beginning to uncover the medically relevant substructures hidden within the heparin polymers.

The best known effect of heparin is its anticoagulant activity. Coagulation is the result of a cascade of proteases, each of which activates another protease, until ultimately thrombin cleaves fibrinogen to fibrin, which is cross-linked to form an insoluble clot (O'Reilly, 1985). Heparin blocks this process at several steps, but only in the presence of another serum component, antithrombin III (AT), a protein that binds to and reacts with several of the clotting factors, including thrombin, inactivating their protease activities (Marcum *et al.*, 1987). The rate of AT reaction with thrombin (and other clotting factors) is markedly enhanced by heparin, which binds directly to free AT at a specific binding site. Although heparin binds to AT in a 1:1 complex, after the AT–thrombin heterodimer is formed, heparin is rapidly released and can bind to and accelerate the reaction of a second AT molecule, thus acting in a catalytic fashion.

The studies that revealed the carbohydrate determinants in heparin responsible for AT binding stand as a prototype for elucidating the diverse functions of heparin. Lam *et al.* (1976) fractionated whole heparin based on its ability to bind to purified AT. The heparin chains that bound AT constituted approximately one-third of the total heparin and carried essentially all of the anticoagulant activity. Random cleavage of the active heparin chains resulted in smaller AT-binding oligosaccharides amenable to structural analysis. Chemical modification and synthetic studies, in conjunction with AT-binding assays, helped define the minimum binding determinant as the naturally occuring pentasaccharide 6-SO_3-GlcNAc α4 GlcA β4 3,6-$(SO_3)_2$-GlcNSO_3 α4 2-SO_3-IdoA α4 6-SO_3-GlcNSO_3 (Atha *et al.*, 1985). High-affinity binding is particularly dependent on the arrangement of *O*-sulfate groups, in that removal of either the nonreducing terminal sulfate (from GlcNAc) or the 3-sulfate on the center residue reduces AT-binding affinity ~1000-fold. These studies raise the hope that each of the diverse biological activities of heparin can be identified with a unique small binding determinant. It should be noted, however, that while small determinants (5–8 saccharides) display high-affinity binding to AT and enhance inhibition of some proteases, longer chains (>16 saccharides) are required to elicit all of the anticoagulant activities of heparin (Choay, 1989). This suggests that the binding site on AT is larger and more complex

than the binding studies indicate, or that a multivalent ligand is required. Such considerations may complicate the potential use of synthetic heparin determinants as pharmaceuticals.

Heparins are members of the family of heparan sulfates, which are broadly distributed as components of proteoglycans found on cell surfaces or in the extracellular matrix, where they are well placed to mediate cell physiology. Anticoagulant heparan sulfate-containing proteoglycans (HSPG) have been demonstrated on vascular endothelium, where they may help protect these surfaces against the formation of thrombi (Marcum *et al.*, 1986).

Heparin also plays a role in vascular smooth muscle proliferation. Normally, blood vessels are lined with a continuous monolayer of quiescent endothelial cells that cover a medial layer of quiescent smooth muscle cells. When the endothelium is removed (experimentally, during surgical procedures, or by pathogens) the underlying smooth muscle cells (SMC) begin to proliferate (perhaps in response to platelet growth factors released at the injured site), a process that can lead to pathological stenosis (Austin *et al.*, 1985). Heparin (but not other GAGs) sharply reduced SMC proliferation after removal of the endothelial layer *in vivo,* and retarded SMC proliferation *in vitro* (Karnovsky *et al.*, 1989). Growth of cell types besides SMC is sensitive to heparin or heparan sulfate, although the effect is not universal (Fedarko *et al.*, 1989; Wright *et al.*, 1989b).

After the endothelial cell lining is stripped from the blood vessel wall, reestablishment of a confluent endothelial layer halts SMC proliferation. Castellot *et al.* (1981) tested conditioned medium from confluent endothelial cell cultures for SMC growth inhibition *in vitro* and found an antiproliferative heparan sulfate that may function normally in vascular wall homeostasis. It is thought that the antiproliferative activity of commercial heparin is due to its structural relation to endogenous endothelial heparan sulfates. Coagulant (AT-Sepharose-binding) and noncoagulant (AT-nonbinding) heparin fractions were equally potent antiproliferative agents both *in vivo* and *in vitro* (Marcum *et al.*, 1987; Karnovsky *et al.*, 1989). Further structure/function studies, of the type performed for AT binding, demonstrated that heparin chains of ≥ 10 saccharides were required for maximal SMC antiproliferative activity, although significant activity was retained by natural hexasaccharides and the synthetic (AT-binding) pentasaccharide described above (tetra- and disaccharides were devoid of activity). Within a size class, more highly charged fragments had higher antiproliferative activity, and synthetic oversulfation enhanced the activity of any size class (Wright *et al.*, 1989a). Unlike the case for AT binding, no particular saccharide residue or set of residues has proven essential for the SMC antiproliferative activity of heparin. While this lessens enthusiasm

for isolating a high-potency "growth control determinant" from heparin, antiproliferative heparin fragments devoid of anticoagulant activity hold some promise for clinical utility.

The mechanisms of the antiproliferative effects of heparin are unknown, and multiple molecular sites of action may exist (Castellot *et al.*, 1989). Three possible mechanisms have been the focus of experimentation.

1. Heparin or heparan sulfate may bind to specific cell surface receptors capable of mediating negative growth-modulatory signals. In support of this hypothesis, evidence has appeared demonstrating a 78-kDa heparin-binding protein from uterine smooth muscle (Lankes *et al.*, 1988). Although antibodies to the 78-kDa protein slow SMC cell division, direct evidence for its role in the antiproliferative activity of heparin has not appeared. High-affinity binding of heparin (Castellot *et al.*, 1985) is followed by rapid internalization, which may deliver bioactive sequences to intracellular sites (see below).
2. Heparin binds to certain growth factors that are known to be mitogenic for SMC and are present in the serum used to culture the cells. While heparin binding to growth factors may reduce their mitogenic effectiveness, it may also stabilize and/or enhance their actions (see below). It is more likely that heparin exerts its antiproliferative effect downstream of growth factor binding, since cell proliferation induced by growth factors that do not bind heparin and by phorbol esters (which directly activate protein kinase C) is inhibited by heparin (Castellot *et al.*, 1989). Recent studies suggest that heparin blocks a protein kinase C-dependent pathway for mitogenesis, although the complexity of its inhibition suggests multiple sites of action (Castellot *et al.*, 1989; Wright *et al.*, 1989b).
3. A fascinating potential mechanism for the antiproliferative action of heparin was suggested by the discovery of free heparan sulfate chains in nuclei isolated from hepatic cells (Fedarko and Conrad, 1986). Nuclear heparan chains could be distinguished from other cellular heparans based on their disaccharide content, and the concentration of nuclear heparan increased as cells reached confluence. Exogenously added intact HSPG isolated from radiolabeled confluent hepatoma cells inhibited cell growth of log-phase cells. Concomitantly, radiolabeled free heparan sulfate chains appeared in the nucleus (Ishihara *et al.*, 1986; Fedarko *et al.*, 1989). Whether such heparan sulfate chains exist in the nuclei of intact cells and how they might modulate nuclear events has yet to be determined.

The most notable interaction between heparin and growth factors involves both the acidic and basic forms of fibroblast growth factor, aFGF and bFGF (Burgess and Maciag, 1989; Klagsbrun, 1990). These single-

chain nonglycosylated 18-kDa polypeptides share >50% primary sequence identity and are widely distributed in the body. Their mitogenic effects are mediated by the same high-affinity ($K_D \sim 10^{-10}$ *M*) receptors found on a variety of cell types, including fibroblasts, endothelial cells, SMC, chondrocytes, etc. The therapeutic effects of FGF in wound healing and other pathologies has made them the focus of considerable pharmaceutical interest (Editorial, 1990). The remarkably high affinity of aFGF and bFGF for heparin has been used as a means for their rapid isolation (on heparin-Sepharose columns), and led to their classification as "heparin-binding growth factors" (Burgess and Maciag, 1989). The interaction of FGF with heparin goes far beyond convenient isolation and clearly impacts on physiological function. Heparin or HSPG potentiates the mitogenic activity of aFGF by ~2 to >10-fold, depending on the system under investigation (Gordon *et al.*, 1989; Mueller *et al.*, 1989). Although the mechanism for the potentiation has not been established, binding to heparin protects both aFGF and bFGF from proteolytic digestion or denaturation (Mueller *et al.*, 1989; Sommer and Rifkin, 1989). In addition, heparin may increase the affinity of aFGF for its receptor (Schreiber *et al.*, 1985).

The positive effects of heparin on FGF activity are thought to be due to its structural similarity to cell-surface HSPGs, which act as endogenous FGF "sinks." FGF-binding studies have identified endogenous binding sites with high affinity ($K_D \sim 10^{-10}$; 80,000 sites/cell) and "low" affinity ($K_D \sim 10^{-9}$; 600,000 sites/cell) for FGF (Moscatelli, 1987). Only the high-affinity sites mediate signal transduction, while binding to the low-affinity sites can be blocked by heparin or destroyed by heparinases. HSPGs that bind to and protect FGF can be isolated from FGF-responsive cells (Saksela *et al.*, 1988). Recently, a clever panning technique was used to isolate cDNA encoding an FGF-binding HSPG (Kiefer *et al.*, 1990). Cells that normally do not bind FGF were stably transfected with a cDNA expression library from FGF-responsive cells, and the subpopulation expressing FGF-binding proteins was selected by adhesion to plastic dishes coated with bFGF. The binding of stably transfected cell lines to FGF-coated plates was blocked by bFGF [but not epidermal growth factor (EGF)], by heparin (but not by other GAGs), and by pretreatment of the cells with heparinase. The primary structure of the transfected cDNA from FGF-binding clones revealed a single polypeptide having several potential GAG glycosylation sites. Whether FGF binding *in vivo* is a common feature of all HSPGs, or requires expression of a particular HSPG species, has been addressed in fascinating studies by Gordon *et al.* (1989). $^{35}SO_4$-Labeled HSPGs from human endothelial cells in culture were isolated and fractionated on an aFGF-affinity column. Half of the radiolabel bound to the

column and the other half was unretarded. When the bound HSPG was recovered by salt elution, it enhanced the mitogenic activity of aFGF on endothelial cells with a potency 100-fold that of heparin. Remarkably, the unbound HSPG potently *inhibited* the same mitogenic effect. These data raise the possibility that responsiveness to mitogens could be controlled, both positively and negatively, by the balance of particular cell-surface HSPGs expressed. Only half of the carbohydrate chains released from the aFGF-binding HSPG by β-elimination could rebind to the aFGF column. Analysis of the binding and nonbinding chains may reveal specific determinants essential for aFGF binding. Such determinants would be good candidates for developing drugs to stabilize and enhance growth factor activity. Studies on fractionated heparin fragments suggest that aFGF-stimulatory activity generally increases with increasing size and degree of sulfation (Sudhalter *et al.*, 1989).

2. Gangliosides in Cell Growth and Differentiation

Cell-surface gangliosides may influence cell physiology via two general mechanisms (Hakomori, 1990; Tiemeyer and Schnaar, 1990). Ganglioside receptors on one cell surface may recognize and bind to gangliosides on a second cell surface in a trans configuration (e.g., Section III,B). Alternatively, gangliosides may interact laterally with proteins in the same membrane and influence their activities in a cis configuration. The latter mechanism appears to be involved in the regulation of cell growth by gangliosides. The mitogenic response of cells to EGF is significantly reduced by exogenous addition of ganglioside G_{M3} (NeuAc α3 Gal β4 Glc β1′ Cer), but not other gangliosides (Bremer *et al.*, 1986). G_{M3} affects neither the number nor affinity of EGF receptors; rather it inhibits the receptor *response* to EGF by inhibiting its hormone-dependent tyrosine kinase activity. The effect appears direct, since purified EGF receptors reconstituted in phospholipid vesicles are as sensitive to inhibition by G_{M3} as the receptors in intact membranes. Exogenously added G_{M3} may spontaneously incorporate into cell membranes, associate laterally with the EGF receptor, and modulate its activity (although direct biophysical evidence for this model has not appeared, and other possibilities remain viable). The observation that a neuraminidase inhibitor, 2-deoxy-2,3-dehydro-NeuAc, inhibits both G_{M3} catabolism and cell growth in primary fibroblasts suggests that modulation of endogenous G_{M3} levels may regulate cell division (Usuki *et al.*, 1988). This notion is supported by experiments with a mutant cell line that requires exogenously added galactose to synthesize G_{M3} and other Gal-containing glycoconjugates. Addition of galactose essentially blocks the response of these cells to EGF, although their response to other

mitogens remains normal (Weis and Davis, 1990). Remarkably, the de-*N*-acetyl form of G_{M3}, discovered in small quantities in cultured cells, is a strong promoter of EGF-receptor kinase activity (Hanai *et al.*, 1988). Together, these data support important roles for gangliosides in cis regulation of growth hormone responsiveness.

Gangliosides have also been reported to mediate cell differentiation. A bipotent human promyelocytic luekemia cell line, HL60, can be induced to differentiate along either a monocytic or granulocytic pathway by addition of phorbol ester or dimethylsulfoxide respectively (Collins, 1987). Monocytic differentiation results in a specific increase in ganglioside G_{M3}, while granulocytic differentiation is accompanied by an increase in neolactoseries gangliosides (Nojiri *et al.*, 1986, 1988). Exogenous addition of the gangliosides themselves induced differentiation, with G_{M3} (but not G_{M1}) generating monocytic characteristics and a mixture of neolactoseries gangliosides generating granulocytic characteristics. The mechanism and specificity of these differentiating effects of gangliosides are under investigation (Nakamura *et al.*, 1989; Xia *et al.*, 1989).

Because gangliosides are found in unusually high concentrations in the membranes of neural cells, there has been much interest in their effects on neuronal differentiation and function. Many effects of gangliosides on nerve cells and tissues have been documented, including induction of neurite outgrowth (Doherty *et al.*, 1985; Cannella *et al.*, 1988; Tsuji *et al.*, 1988; Ferreira *et al.*, 1990), modulation of specific protein kinases (Chan, 1987, 1988; Goldenring *et al.*, 1985; Kreutter *et al.*, 1987; Nagai and Tsuji, 1989), and sparing of neurons after injury (Cuello, 1990). In fact, a purified ganglioside preparation from bovine brain [Cronassial (Fidia)] has been approved for use in peripheral neuropathies in several countries. To date, clinical trials of gangliosides have generated mixed results, with recent double-blind studies showing little or no therapeutic (or toxic) effect of high doses administered to patients with diabetic neuropathy, amyotrophic lateral sclerosis, or other chronic neuromuscular diseases (Hallett *et al.*, 1987; Bradley *et al.*, 1988; Lacomblez *et al.*, 1989). With over 300 million single doses dispensed in the 1980s (Letter, 1988), distribution of gangliosides as a drug has outpaced mechanistic and clinical studies, leading to some skepticism about their clinical potential (Editorial, 1988). Nevertheless, a compelling body of experimental evidence continues to accumulate to suggest that gangliosides act as neurotrophic and neuroregulatory factors both *in vitro* and *in vivo*. As carefully controlled studies elucidate the mechanisms and specificity of ganglioside effects in the nervous system, opportunities to exploit this large and complex family of glycoconjugates to develop therapeutics may arise. An intriguing recent development along these lines is the discovery that gangliosides and gan-

glioside derivatives spare neurons from glutamate-induced toxicity *in vitro* (Favaron *et al.*, 1988; Manev *et al.*, 1990), a finding that may find clinical relevance in treatment of ischemic brain injury. For more details on the actions of gangliosides on nerve growth and repair both *in vivo* and *in vitro*, the reader is referred to the excellent review in this series by A. Claudio Cuello (1990), whose careful work has helped refocus attention on the potential of gangliosides as neurotrophic factors.

3. Glycoprotein Regulation by Glycosylation

While many glycoprotein oligosaccharide structures and the general steps in their biosynthesis have been elucidated, their specific functions remain largely enigmatic. To an extent, this is due to their inherent complexity. Elucidation of the glycosylation patterns of several well-defined polypeptides has revealed some important general principles (Rademacher *et al.*, 1988). A particular glycoprotein often occurs in many glycoforms, each with the identical polypeptide backbone but carrying different carbohydrate chains. Single polypeptides with multiple carbohydrate chains usually have distinct oligosaccharide structures at each glycosylation site. Glycosylation pattern "microheterogeneity" is not random, but is cell type specific and reproducible for a given cell type under defined physiological or experimental conditions (Swiedler *et al.*, 1985; Parekh *et al.*, 1989a). The result is a stable family of glycoforms defined by the percent of each oligosaccharide structure at each glycosylation site. Different cell types may produce nonoverlapping sets of glycoforms, presumably because of differences in glycosyltransferase expression. Cell lines with glycosylation mutations and cells stably transfected with glycosyltransferases are under intense scrutiny as factories for glycoform control and production (Stanley, 1989; Smith *et al.*, 1990). Interest in this area has burgeoned, since oligosaccharide alterations can modulate the intrinsic biological activity and pharmacodynamics of glycoproteins of therapeutic interest, such as tissue plasminogen activator (Parekh *et al.*, 1989b; Lucore *et al.*, 1988) and erythropoietin (Dubé *et al.*, 1988; Takeuchi *et al.*, 1990).

The biological consequences of protein glycosylation can be classified as biophysical or biochemical based on structural specificity. In general, glycosylation has been reported to stabilize the conformations of some polypeptides and to protect them against proteolysis (Paulson, 1989). When N-linked protein glycosylation is blocked, some glycoproteins aggregate in the endoplasmic reticulum and are degraded, while the expression and functions of others appear unaffected. O-Linked glycosylation can physiclly block protease access to nearby peptide sequences. In cell

mutants lacking O-glycosylation (but with normal N-glycosylation), certain plasma membrane glycoproteins are expressed normally, but are then rapidly degraded (Kozarsky *et al.*, 1988). Furthermore, O-glycosylation keeps polypeptides in extended conformations (Jentoft, 1990). This is especially relevant in the very large (10^7 Da) and heavily O-glycosylated mucins, which form intertangled networks on mucous membranes, physically protecting them from pathogens and toxins.

An important biochemical consequence of glycosylation is protein targeting to intracellular compartments or to specific tissues. Many lysosomal hydrolases are biosynthetically marked for lysosomal targeting by phosphorylation of mannose residues on their N-linked oligosaccharides (Kornfeld, 1990). A genetic defect in an enzyme responsible for the phosphate transfer results in I-cell disease, in which hydrolases are missing from lysosomes, but appear in excess in extracellular fluids. Evidently, when they are not appropriately marked for lysosomal targeting, the default pathway for these glycoprotein hydrolases is secretion. A binding protein specific for mannose-phosphate residues and responsible for transport of the relevant hydrolases to the lysosome has been purified (Lobel *et al.*, 1987) and occurs both intracellularly and on cell surfaces, where it may mediate Man-P-glycoprotein endocytosis.

Of particular importance to the survival of serum glycoproteins, both endogenous and administered, are "clearance" receptors on hepatocytes and macrophages (Neufeld and Ashwell, 1980). This field of study was initiated by the serendipitous discovery by Morell *et al.* (1968) that desialylation of ceruloplasmin decreased its *in vivo* serum half life from 56 hr to <15 min! Elegant studies subsequently identified a hepatocyte cell-surface galactose-binding protein responsible for the rapid clearance (Ashwell and Harford, 1982; Drickamer *et al.*, 1984; Spiess, 1990). While the biological role(s) of the "hepatic lectin" are still under study, it is likely that the exposure of Gal residues on endogenous circulating proteins targets them for uptake and degradation in the liver. Other lectins capable of binding glycoproteins via their oligosaccharides have been characterized on mammalian cell surfaces, including reticuloendothelial cell receptors that bind terminal Man or GlcNAc residues on serum glycoproteins (Haltiwanger and Hill, 1986; Stahl, 1990). Although endogenous cell surface lectins can confound the therapeutic use of engineered glycoproteins, they may also be used to target appropriate glycoforms to particular tissues.

Some glycoprotein oligosaccharides may code for specific cell–cell interactions. The sialyl-Le^x structure involved in leukocyte–endothelial adhesion (see Section III,B,1) is found on both glycolipids and glycoproteins (Fukuda *et al.*, 1984, 1986). Epitopes of the HNK-1 antigen, which have been implicated in neural cell recognition (see Section III,B,3)

have been detected on glycolipids (Chou *et al.*, 1986; Ariga *et al.*, 1987) and on a minority of glycoforms of known cell adhesion molecules (Schachner, 1989), although the glycoprotein oligosaccharide structures have not been characterized. Identical oligosaccharide determinants carried on glycoproteins and glycolipids may serve different functions. An interesting experimental example of this was reported by Pacuszka and Fishman (1990), who artificially attached the cholera toxin receptor oligosaccharide from G_{M1} (see Section III,A,1) to endogenous cell-surface proteins. Although equivalent toxin binding to cell surfaces was obtained, the toxin was unable to mediate biochemical changes deleterious to the cells. When the same oligosaccharide on a lipid carrier was introduced into the cells the toxin was able to both bind and mediate biochemical alterations. Whether endogenous carbohydrate epitopes shared by glycoproteins and glycolipids mediate different biological functions has not been explored.

IV. Conclusions and Perspectives

Major advances in the purification, analytical characterization, and biological functions of glycoconjugates have greatly broadened the potential of glycobiology. Therapeutic opportunities that were unimagined a few years ago are being enthusiastically attacked in university and industrial laboratories. From glycoprotein regulation to pathogen–target recognition to neuronal repair, we are challenged to identify the specific carbohydrate structures generating biological effects and the detailed mechanisms responsible, from which carbohydrate-based drugs can be designed and developed. Lead compounds are already apparent in several cases, such as the heparin pentasaccharide that binds antithrombin, sialyl glycosides that bind influenza virus, and sialyl-Le^x-related structures involved in leukocyte–endothelium adhesion. Major remaining hurdles for development of any of these into pharmaceuticals include the design of high-affinity analogs and their economical synthesis. Improvements in selective glycoside bond formation, both chemical and enzymatic (via cloned glycosyltransferases) will certainly play an important role in advancing these goals. With encouraging recent discoveries by many laboratories on the vital biological roles of complex carbohydrates, confidence and enthusiasm for their pharmaceutical application are high. In the next decade, the infrastructure to move carbohydrate research from the laboratory to the clinic will continue to develop and that confidence will be put to the test.

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