Chapter 2

Biochemistry and Role of Sialic Acids

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1. INTRODUCTION

Sialic acids mainly occur as terminal components of cell surface glycoproteins and glycolipids, playing as such a major role in the chemical and biological diversity of glycoconjugates. Cell-type-specific expression of glycosyltransferases, particularly of sialyltransferases (Paulson and Colley, 1989; van den Eijnden and Joziasse, 1993), leads to specific sialylation patterns of oligosaccharides which can be considered as key determinants in the makeup of cells. Striking differences have been found in the sialoglycosylation patterns of cells during development, activation, aging, and oncogenesis. Research on the structures, metabolism, and molecular biology, as well as on the biological and clinical importance of sialic acids as components of these glycoconjugates, has therefore intensified during the past several years.

Structurally, sialic acids comprise a family of 36 naturally occurring derivatives of neuraminic acid that are all *N*-acylated to form *N*-acetylneuraminic acid (Neu5Ac) or *N*-glycoloylneuraminic acid (Neu5Gc) as fundamental molecules (Figure 1). Most additional modifications result from *O*-acetylation at one or several of the hydroxyl functions, at C-4, -7, -8, or -9, or to the introduction of a double bond between C-2 and C-3 (Table I) (Rosenberg and Schengrund, 1976a;

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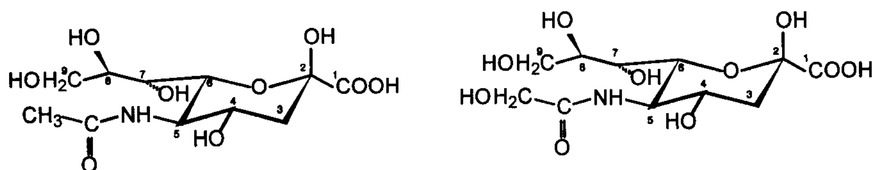


FIGURE 1. $^2C_5$-conformation of *N*-acetyl-neuraminic acid (left) and *N*-glycoloylneuraminic acid (right).

Corfield and Schauer, 1982a; Reuter *et al.*, 1983; Schauer *et al.*, 1984a,b; Reuter and Schauer, 1988, 1994; Varki, 1992a). Sialic acids in these various structural forms are found on some viruses, microorganisms such as bacteria and protozoa, in a large number of higher animals, and on cell membranes and in body fluids of all mammals. The saturated sialic acids usually occupy terminal, nonreducing positions of oligosaccharide chains of complex carbohydrates on outer and inner (e.g., lysosomal) membrane surfaces in various linkages mainly to galactose, *N*-acetylgalactosamine, and to sialic acid itself. Sialic acids are among the first molecules encountered by other cells or by compounds coming into contact with the cell, a feature that is important in the expression of their biological role (Schauer, 1982a,b, 1991). The 2,3-didehydro-sialic acids and 2,7-anhydro-*N*-acetylneuraminic acid have been found only as free sugars in body fluids and secretions, since they lack the linkage-forming glycosidic hydroxyl group. In Sections 2 and 3, some new aspects of the occurrence and analysis of sialic acids will be discussed.

In the past decade, little progress has been made in elucidating the enzymatic and regulatory mechanisms involved in the long pathway of Neu5Ac biosynthesis starting from glucose (Corfield and Schauer, 1982b). However, further insight has been gained into the biosynthesis and role of modified sialic acids, especially enzymatic *N*-acetyl-hydroxylation, *O*-acetylation, and *O*-methylation. This will be discussed in Section 4. Numerous studies have been carried out on the transfer of sialic acids onto oligosaccharides, polysaccharides, glycoproteins, and glycolipids mediated by sialyltransferases, which in most cases result in the completion of their oligosaccharide chain extensions (see Chapter 3). Such studies have led to the elucidation of the acceptor specificity of at least ten sialyltransferases (Paulson and Colley, 1989; van den Eijnden and Joziasse, 1993). By now, eight cDNA clones of sialyltransferases have been obtained (Weinstein *et al.*, 1987; Grundmann *et al.*, 1990; Gillespie *et al.*, 1992; Wen *et al.*, 1992; Kitagawa and Paulson, 1993, 1994; Lee *et al.*, 1993; Kurosawa *et al.*, 1994). All of these cDNA clones contain a stretch of about 50 amino acids displaying high homology, the sialyl motif possibly involved in substrate binding (Wen *et al.*, 1992). Using polymerase chain reactions with degenerate primers deduced from this motif, additional cDNA clones were obtained (Livingston and Paulson, 1993; Kurosawa *et al.*, 1993) for which no sialyltransferase activity could be assigned as yet.

Table I
Naturally Occurring Sialic Acids and Suggested Abbreviations[a,b]

N-acetylneuraminic acid	Neu5Ac
N-acetyl-4-*O*-acetylneuraminic acid	$Neu4,5Ac_2$
N-acetyl-7-*O*-acetylneuraminic acid	$Neu5,7Ac_2$
N-acetyl-8-*O*-acetylneuraminic acid	$Neu5,8Ac_2$
N-acetyl-9-*O*-acetylneuraminic acid	$Neu5,9Ac_2$
N-acetyl-4,9-di-*O*-acetylneuraminic acid	$Neu4,5,9Ac_3$
N-acetyl-7,9-di-*O*-acetylneuraminic acid	$Neu5,7,9Ac_3$
N-acetyl-8,9-di-*O*-acetylneuraminic acid	$Neu5,8,9Ac_3$
N-acetyl-7,8,9-tri-*O*-acetylneuraminic acid	$Neu5,7,8,9Ac_4$
N-acetyl-9-*O*-acetyl-8-*O*-methylneuraminic acid*	$Neu5,9Ac_28Me$
N-acetyl-9-*O*-lactoylneuraminic acid	Neu5Ac9Lt
N-acetyl-4-*O*-acetyl-9-*O*-lactoylneuraminic acid	$Neu4,5Ac_29Lt$
N-acetyl-8-*O*-methylneuraminic acid	Neu5Ac8Me
N-acetylneuraminic acid 9-phosphate	Neu5Ac9P
N-acetylneuraminic acid 8-sulfate	Neu5Ac8S
2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid	Neu5Ac2en
2-deoxy-2,3-didehydro-*N*-acetyl-9-*O*-acetylneuraminic acid*	$Neu5,9Ac_22en$
2-deoxy-2,3-didehydro-*N*-acetyl-9-*O*-lactoylneuraminic acid*	Neu5Ac2en9Lt
2,7-anhydro-*N*-acetylneuraminic acid*	2,7-anhydro-Neu5Ac
N-glycoloylneuraminic acid	Neu5Gc
N-glycoloyl-4-*O*-acetylneuraminic acid	Neu4Ac5Gc
N-glycoloyl-7-*O*-acetylneuraminic acid	Neu7Ac5Gc
N-glycoloyl-9-*O*-acetylneuraminic acid	Neu9Ac5Gc
N-glycoloyl-7,9-di-*O*-acetylneuraminic acid	$Neu7,9Ac_25Gc$
N-glycoloyl-8,9-di-*O*-acetylneuraminic acid	$Neu8,9Ac_25Gc$
N-glycoloyl-7,8,9-tri-*O*-acetylneuraminic acid	$Neu7,8,9Ac_35Gc$
N-glycoloyl-9-*O*-acetyl-8-*O*-methylneuraminic acid*	Neu9Ac5Gc8Me
N-(*O*-acetyl)glycoloylneuraminic acid*	Neu5GcAc
N-glycoloyl-9-*O*-lactoylneuraminic acid*	Neu5Gc9Lt
N-glycoloyl-8-*O*-methylneuraminic acid*	Neu5Gc8Me
N-glycoloylneuraminic acid 8-sulfate	Neu5Gc8S
2-deoxy-2,3-didehydro-*N*-glycoloylneuraminic acid*	Neu2en5Gc
2-deoxy-2,3-didehydro-*N*-glycoloyl-9-*O*-acetylneuraminic acid*	Neu9Ac2en5Gc
2-deoxy-2,3-didehydro-*N*-glycoloyl-9-*O*-lactoylneuraminic acid*	Neu2en5Gc9Lt
2-deoxy-2,3-didehydro-*N*-glycoloyl-8-*O*-methylneuraminic acid*	Neu2en5Gc8Me
Ketodeoxynonulosonic acid*	Kdn

[a]Reuter and Schauer (1988).
[b]Asterisks identify those sialic acids discovered since the publication of the last book review (Schauer, 1982a).

Research has also been focused on the enzymes of sialic acid catabolism, mainly the sialidases, which may initiate the degradation of sialoglycoconjugates and thus are involved in regulation of the turnover of these substances (Corfield *et al.*, 1981a; Corfield and Schauer, 1982b) (see Chapter 8). Sialidases are important enzymes, since they may destroy the sialic acid-mediated biological

functions of many glycoconjugates (Rosenberg and Schengrund, 1976a; Corfield, 1992; Corfield *et al.*, 1992). This will be further elucidated in Section 6, where sialic acid-specific cellular recognition and adhesion are described. Furthermore, desialylation can lead by unmasking to the formation of new recognition sites, frequently galactose or novel antigens (see below).

In addition to the common exo-α-sialidases (EC 3.2.1.18; Cabezas, 1991), a rare enzyme has been found, so far only in the leech (Li *et al.*, 1993), yielding 2,7-anhydro-Neu5Ac as its reaction product. This compound may also be a product of an as yet unknown microbial sialidase, since it was found in human cerumen (Suzuki *et al.*, 1985).

An extraordinary sialidase is expressed in several trypanosomal species, *Trypanosoma cruzi* (Zingales *et al.*, 1987; Schenkman *et al.*, 1991), *T. brucei* (Engstler *et al.*, 1993), and *T. congolense* (Engstler *et al.*, 1994). This enzyme transfers sialic acids (Neu5Ac or Neu5Gc) from oligosaccharide chains either onto water, which constitutes a normal sialohydrolase reaction, or with greater preference, onto nonsialylated glycan chains with terminal galactose residues, leading to the formation of α2,3 sialic acid linkages. The existence of this trans-sialidase explains the occurrence of sialic acids on the surface of *T. cruzi*, since this parasite is not able to synthesize sialic acid (Schauer *et al.*, 1983). The biological significance of this unusual sialylation mechanism is to protect these pathogens from the host's immune or proteolytic defense systems (reviewed by Engstler and Schauer, 1993).

One particular exosialidase, from the loach *Misgurnus fossilis*, is specialized for cleaving 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid (Kdn) residues (Section 2), and can therefore be described as a Kdn'ase (Li *et al.*, 1993). It also acts slowly on sialic acid glycosidic linkages.

An endo-α-sialidase (EC 3.2.1.129; Cabezas, 1991) exists in bacteriophages, which hydrolyzes α2,8 bonds within the polysialyl chains of bacterial colominic acid and mammalian "cell adhesion" molecules (Long *et al.*, 1993).

Since sialidases are extremely important in the pathogenesis of microbial diseases, much research on their diagnosis and inhibition has been carried out. Influenza A and B viruses contain sialidases as receptor-destroying enzymes, which are necessary for the propagation of the viruses, possibly for the release of virus particles from inhibitory mucins or during budding of new viruses (see Chapter 9). Based on earlier observations that an *O*-acetyl group at C-4 of Neu5Ac completely prevents release of the corresponding sialic acid by bacterial and mammalian sialidases, but not by the influenza virus enzyme (Kleineidam *et al.*, 1990), inhibitors for viral sialidases were designed. The rationale for this was the observation of a pocket in the active center of influenza virus sialidases, elucidated by X-ray crystallography, into which the 4-*O*-acetyl group of sialic acid fits and thus allows the (slow) hydrolysis of this substrate. (Nonviral sialidases do not seem to have this pocket.) Correspondingly, Neu5Ac2en, known as an inhibitor of most sialidases including the viral ones, could be

transformed into a much more potent inhibitor (inhibitor constants up to 10^{-9} M) by the addition of substituents at C-4, as in 4-amino- and 4-guanidino-Neu5Ac2en (Corfield, 1993; von Itzstein *et al.*, 1993).

In *Clostridium* infections, the bacterial sialidases can be identified in wound fluids or blood serum by immunological methods (T. Roggentin *et al.*, 1993). In this way, infecting bacterial species can be recognized at an early stage of the disease, thus enabling a prompt and specific therapy of lethal diseases such as gas edema, which is most frequently caused by *Clostridium perfringens*. Studies on these bacterial enzymes led us to the investigation of their gene structures, which revealed a sialidase gene family, thus giving support to theories about the molecular relationship between microbial and animal sialidases. Section 5 is devoted to these studies.

Investigations on the biological functions of sialic acids are rapidly growing fields of research. Several aspects have already been mentioned and more aspects, such as the significance of sialic acid *O*-acetylation, *N*-acetyl-hydroxylation, and sialic acid-binding proteins, will be discussed in the following sections. Here, only an introduction to the main aspects of sialobiology can be given, based on numerous experiments performed in many laboratories. (For reviews, see Rosenberg and Schengrund, 1976a; Schauer, 1982a,b; and Varki, 1992a,b, 1993.)

The functions of sialic acids may be grouped into three sections: one is a less specific, more general role, related to the fact that sialic acids are relatively large, hydrophilic and acidic molecules that exert physicochemical effects on the glycoconjugates to which they are bound, and on the environmental molecules *in situ*, e.g., in cell membranes. Therefore, sialyl groups influence and stabilize the conformation of both the glycan and the protein parts of glycoconjugates, resulting in modified properties, e.g., higher thermal and proteolytic stability of glycoproteins (Schauer, 1982a,b). These oligosaccharide moieties have therefore been considered to "mimic the effect of a molecular chaperone" (Jaenicke, 1993). Mucin glycoproteins of vertebrates are highly sialylated (the name sialic acid is derived from the Greek "sialos," meaning saliva) which gives them a high viscosity in solution because of their negative charges. The protective and lubricative effects of mucins are well known (Schauer, 1992).

Two other functions of the sialic acids deal with molecular and cellular recognition. Here, sialic acids have dual and opposite effects. On the one hand, sialic acids act as masks to prevent biological recognition, e.g., of subterminal galactose residues, and on the other they serve as recognition sites (Schauer, 1982a,b, 1985; Schauer *et al.*, 1984b). The best studied example of the masking function is the binding and uptake of desialylated serum glycoproteins by hepatocytes (Harford *et al.*, 1984).

In a similar way, sialidase-treated erythrocytes, lymphocytes, and thrombocytes are bound and partly phagocytized by macrophages, mediated by a galactose-specific lectin (Müller *et al.*, 1983; Fischer *et al.*, 1991; Kluge *et al.*,

1992). It was observed during the programmed cell death (apoptosis) of rodent thymocytes that these cells are phagocytized after the loss of cell surface sialic acids during this process (Savill *et al.*, 1993). These studies show the role of sialic acids in maintaining the life span of molecules and cells which, together with demasked galactose residues, regulate these and many more biological processes, as well as pathological events like the spreading of cancer.

In contrast, it has long been known that influenza viruses recognize sialic acids and bind to them on cell surfaces via hemagglutinin (see Chapter 9). Similar receptors are also involved in the infection mechanism of many bacteria and protozoa (see Table V in Section 6). Many physiological processes also are regulated by sialic acid recognition, and this field of research is rapidly expanding. Section 6 is devoted to reviewing characteristic features of sialic acid-dependent receptors focusing on those mammalian proteins that are well defined as molecules, in terms of specificity and possible functions. Further aspects discussed are the nature of ligands for these receptors and the potential modulatory role of sialic acid modifications.

The intention of this chapter is to give the reader a feeling for the great chemical and biological diversity of sialic acids, which is unique in glycobiology. It may also contribute to an understanding of the vulnerability of sialobiology to misuse by pathogens and transformed cells, and to the possibility that microbial sialidases from infectious agents can severely disturb normal sialobiological function.

2. OCCURRENCE OF SIALIC ACIDS

Since publication of the last book dealing with various aspects of sialic acids (Schauer, 1982a), 12 new derivatives have been structurally identified, the 9-*O*-acetylated and 9-*O*-lactoylated derivatives of the unsaturated 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid, 2-deoxy-2,3-didehydro-*N*-glycoloylneuraminic acid together with the corresponding 9-*O*-acetylated, 9-*O*-lactoylated, and 8-*O*-methylated derivatives, and the 9-*O*-lactoylated derivative of *N*-glycoloylneuraminic acid (Schauer *et al.*, 1984a,b; Shukla *et al.*, 1987) (Table I). In the starfish *Asterias rubens*, in which Neu2en5Gc8Me was detected, Neu5,9$Ac_2$8Me and Neu9Ac5Gc8Me were also found (Bergwerff *et al.*, 1992), thus extending the series of 8-*O*-methylated sialic acids discovered in echinoderms. Finally, a derivative of *N*-glycoloylneuraminic acid was isolated from rat thrombocytes, in which the OH group of the *N*-glycoloyl function is acetylated. This sialic acid is very labile and seems not to occur on other rat blood cells (Kluge *et al.*, 1992). A substitution of the *N*-glycoloyl OH function in Neu5Gc8Me by another Neu5Gc8Me has been characterized from *A. rubens* as sialo-di- and trisaccharide (Bergwerff *et al.*, 1992, 1993) (Figure 2).

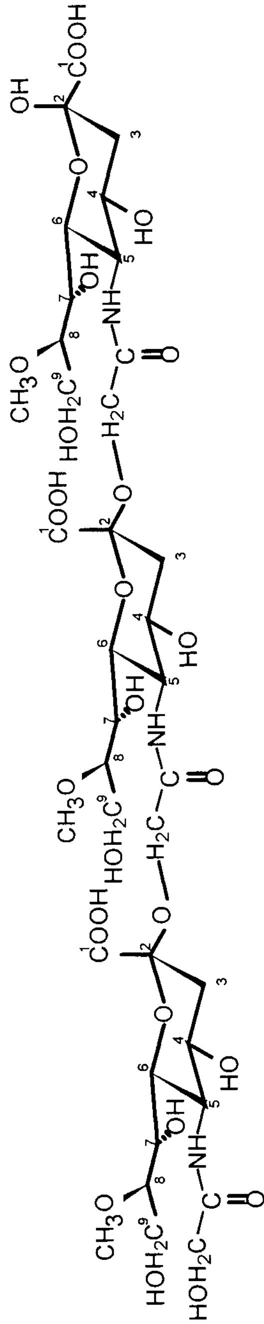


FIGURE 2. Structure of the Neu5Gc8Me-trisaccharide identified from *Asterias rubens*.

With the 5-deaminated derivative of neuraminic acid, 3-deoxy-D-*glycero*-D-*galacto*-nonulosonic acid (ketodeoxynonulosonic acid, Kdn) (Figure 3), an unusual modification was found in the eggs of rainbow trout (Inoue, 1993) and the egg jelly coat from the newt *Pleurodeles waltlii* (Strecker *et al.*, 1992a), from *Axolotl mexicanum* (Strecker *et al.*, 1992b), and from *Xenopus laevis* (Strecker *et al.*, 1993). In trout, it is a component of gangliosides (Song *et al.*, 1991) and glycoproteins, where it can terminate long sialic acid chains consisting of Neu5Gc (Nadano *et al.*, 1986) or form poly-Kdn chains (Kitazume *et al.*, 1992; Inoue et al., 1992). In some cases its 9-*O*-acetylated derivative was also found (Strecker *et al.*, 1993). The metabolism of Kdn is beginning to be elucidated (Terada *et al.*, 1993). Recently, an enzyme releasing Kdn from its glycosidic linkage has been found in the loach *Misgurnus fossilis* (Li *et al.*, 1993). Although Kdn is not structurally a neuraminic acid, it is denominated as a sialic acid.

In *Shigella boydii* and *Pseudomonas aeruginosa*, other rare neuraminic acid derivatives ("pseudaminic acids") were identified with a different stereochemistry at C-5, -7, and -8 and a 3-hydroxybutyramido or formamido substituent at C-7 (Knirel *et al.*, 1985, 1986, 1987a,b). A further unusual derivative was found in the *O*-specific chain of *Legionella pneumophila* serogroup 1 lipopolysaccharide having an α2,4 homopolymer of 5-acetamidino-7-acetamido-8-*O*-acetyl-3,5,7,9-tetradeoxy-D-*glycero*-L-*galacto*-nonulosonic acid (Knirel *et al.*, 1994).

More and more bacteria have been found to contain sialic acids. Only Neu5Ac (Moran *et al.*, 1991; Krauss *et al.*, 1988, 1992) and in few cases Neu5,9Ac$_2$ (Varki and Higa, 1993) were identified. The position of sialic acids within the oligosaccharide chain of bacterial lipopolysaccharides is unusual since nonterminal linkages were found such as that in Galβ1,3GalNAcβ1,4Gal-α1,4Neu5,9Ac$_2$ (Gamian *et al.*, 1991) or Galα1,6Glcβ1,7Neu5Ac (Krauss *et al.*, 1992) in addition to the polysialic acid chains of *Escherichia coli, Neisseria meningitidis, Pasteurella haemolytica,* and *Moraxella nonliquefaciens* (Vann *et al.*, 1993).

Recently, the first proof for the occurrence of sialic acids in insects has been published (Roth *et al.*, 1992). Neu5Ac was identified by mass spectrometry only

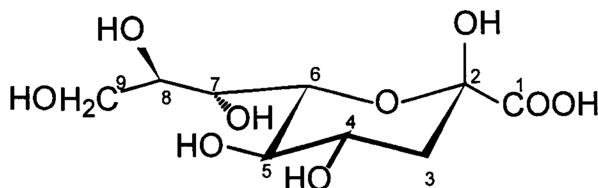


FIGURE 3. Structure of 3-deoxy-D-*glycero*-D-*galacto*-nonulosonic acid (ketodeoxynonulosonic acid, Kdn).

Table II
Sialic Acid Composition of Various Mouse Erythrocytes

Strain	%Neu5Ac	%Neu5Gc	%Neu5,9Ac$_2$	μg sialic acid per 10^{10} cells
DBA2	86	7	7	58
C57BL6	73	13	14	60
Swiss	57	7	36	6
Balb c	82	0	18	61
C3H	50	45	5	56
CBA	81	7	12	71

in certain developmental stages of *Drosophila* larvae, where it exists most probably in the form of a polysialic acid chain, as detected with corresponding antibodies (see Chapter 4).

In man, the spectrum of identified sialic acids is still restricted to Neu5Ac, Neu5,9Ac$_2$, Neu5Ac9Lt, and Neu5Ac2en as previously described (Corfield and Schauer, 1982a). In tumors such as colon and mammary carcinoma, gastric and liver cancer, malignant lymphoma and teratoma, Neu5Gc has been detected in low amounts (e.g., Higashi *et al.*, 1985; Devine *et al.*, 1991; Kawai *et al.*, 1991; Hanisch *et al.*, 1992). Only in the latter two reports was a chemical identification of this sialic acid type carried out; rather, most authors use antibodies raised against certain Neu5Gc-containing structures for detection.

In human melanoma, a tumor-associated expression of the gangliosides GD3 and GD2, each with one 9-*O*-acetylated sialic acid residue, was identified (Thurin *et al.*, 1985; Sjoberg *et al.*, 1992), so adding to other reports about modified sialic acid *O*-acetylation in some tumors (Muchmore *et al.*, 1987; Hutchins *et al.*, 1988). The finding of Neu5,9Ac$_2$ on human T lymphocytes only in cancer patients (Stickl *et al.*, 1991) was not confirmed by Suguri *et al.* (1993) who reported approximately 5% Neu5,9Ac$_2$ in the total sialic acid pool isolated from normal human B as well as T cells.

Many more recent findings not only provide further evidence for the long-established species-specific pattern of sialic acids (Corfield and Schauer, 1982a; Varki, 1992a), but also indicate a strain, tissue, or cell specificity within a given species and a developmental regulation of the expression of certain sialic acids. The sialic acid composition of mouse erythrocytes, for example, shows great variation among the strains analyzed (Klotz *et al.*, 1992; Table II).

A comparative analysis of 13 different adult and fetal bovine tissues (Schauer *et al.*, 1991) revealed a concentration of 0.1–3.1 mg total sialic acids/g wet weight in adult bovine tissues, while in the corresponding fetal tissues the sialic acid concentration was higher and ranged from 1.1 to 12.5 mg/g wet tissue. The percentage of Neu5Gc in the total sialic acid pool was always greater in the adult

than in the fetus, ranging from 9 to 66% and from 0 to 49%, respectively. Chickens were found to acquire the full, i.e., adult, level of Neu5,9Ac_2 on their erythrocytes only on day 21 after hatching, whereas freshly hatched chickens do not have detectable amounts of this sialic acid on their red blood cells (Herrler *et al.*, 1987). Similar examples of developmentally regulated expression of Neu5Gc or of sialic acid *O*-acetylation were reported for rat small intestine (Bouhours and Bouhours, 1983, 1988), human and rat colon (Muchmore *et al.*, 1987), transgenic mice (Varki *et al.*, 1991), and for hibernating animals (Rahmann *et al.*, 1987).

3. SIALIC ACID ANALYSIS

Since a number of biological phenomena depend not only on the general presence of sialic acids, but also on a specific sialic acid type, the exact analysis of these sugars within a biological system is of great importance. The tools formerly described for sialic acid analysis (Schauer, 1978a, 1982b, 1987a,b; Schauer and Corfield, 1982; Vliegenthart *et al.*, 1982; Kamerling and Vliegenthart, 1982; Reuter *et al.*, 1983; Reuter and Schauer, 1986) are still successfully used for this purpose. Over the past 10 years, however, a number of improvements have been made in the various techniques (Reuter and Schauer, 1994).

One of the major problems in sialic acid analysis is still the liberation of this sugar from its glycosidic linkage. Although complete release requires milder conditions than for hexoses, there is no method available that can prevent the simultaneous cleavage of sialic acid ester substituents. The two-step acid hydrolysis using formic acid (pH 2, 1 hr, 70°C) followed by hydrochloric acid (pH 1, 1 hr, 80°C) completely releases sialic acids from sialoglycoconjugates but will result in a loss of 20–50% of *O*-acyl groups together with some degradation of sialic acid itself (Schauer, 1978a, 1987a,b; Reuter and Schauer, 1994). Other conditions used for acid hydrolysis (Varki and Diaz, 1984) did not improve the recovery, especially of *O*-acetylated sialic acids, in our hands. The use of sialidases for liberation of sialic acids does not result in significant losses of these ester substituents. However, only a portion of all sialic acids present is often released (Corfield and Schauer, 1982b; Reuter and Schauer, 1994), though this depends on the sialidase type used for hydrolysis.

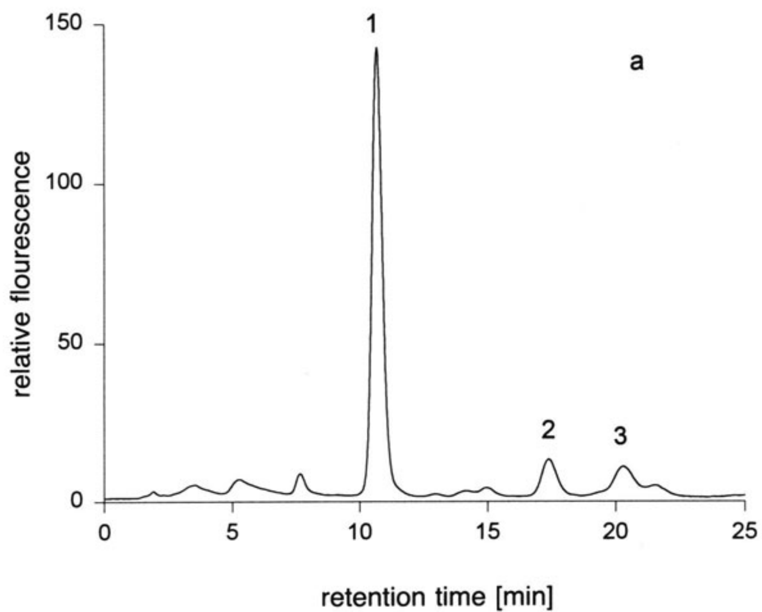
These problems in the analysis of free sialic acids demonstrate the need to investigate intact sialoglycoconjugates that can often be isolated from biological material without significant modification of sialic acids. By ^{1}H-NMR spectroscopy, which has become a routine application for some laboratories using machines with high magnetic fields, and various two-dimensional techniques, even complex sialoglycoconjugates can be analyzed. The information obtained in-

cludes structural details like the type of monosaccharides, including sialic acid substitution, sequence, anomeric configuration, and the type of linkage as well as conformational data and dynamic behavior of the whole molecule (Vliegenthart *et al.*, 1982, 1983; Dabrowski, 1989).

In addition to the more conventional electron impact and chemical ionization mass spectrometric techniques that have been applied to the analysis of free sialic acids (Kamerling and Vliegenthart, 1982; Reuter *et al.*, 1983; Reuter and Schauer, 1986), fast-atom bombardment mass spectrometry (FAB-MS) allows the analysis of whole glycoconjugates (Egge *et al.*, 1985; Dell, 1987). FAB-MS requires about 5 μg of substance and gives information about the molecular weight, type of monosaccharide unit in terms of hexose, *N*-acetylhexosamine, deoxyhexose, etc., and about branching of an oligosaccharide chain. However, it does not allow the localization of the position of a substituent within a given residue; thus, a discrimination between, e.g., 7- and 9-*O*-acetylated sialic acids is not possible using this technique.

Both methods, NMR and FAB-MS, require preparation and purification of individual sialylated oligosaccharides, glycopeptides, or gangliosides before analysis, making this approach less attractive if only low amounts of the corresponding materials are available. Therefore, sensitive methods for isolation and analysis of sialic acids and sialoglycoconjugates are required. Several HPLC methods have been developed that fulfill this need.

Derivatization of free sialic acids with 1,2-diamino-4,5-methylenedioxybenzene (DMB) yields fluorigenic derivatives that can be separated on reversed phase columns with a detection limit in the femtomole range (Hara *et al.*, 1986, 1987, 1989). Since the derivatization reaction is specific for α-keto acids, Kdo and Kdn will also be detected by this method (Reuter and Schauer, 1994). Even with this specificity, a structural identification by HPLC is not possible. To ascertain the preliminary assignment made on the basis of retention times, treatment with specific reagents or enzymes is very helpful (Shukla and Schauer, 1986; Schauer, 1987b; Reuter and Schauer, 1994). Incubation with sialate 9-*O*-acetylesterase (EC 3.1.1.53), e.g., from influenza C virus (Schauer *et al.*, 1988a,c), will result in a decrease of the peak for 9-*O*-acetylated sialic acids with a corresponding increase of unmodified Neu5Ac or Neu5Gc. Alkali treatment (Reuter and Schauer, 1994) hydrolyzes all esters, not only 9-*O*-acetyl groups, again accompanied by an increase of Neu5Ac and Neu5Gc. These latter sialic acids are cleaved by incubation with *N*-acylneuraminate-pyruvate lyase (EC 4.1.3.3, aldolase) that also acts on *O*-acetylated derivatives but significantly slower. Thus, a combination of various treatments of the sialic acid pool followed by fluorimetric HPLC analysis indicates the presence of this sugar even in very low quantities (Figure 4). In addition, the use of sialic acid-specific enzymes in this assay allows identification of sialic acids, which is not possible by HPLC alone.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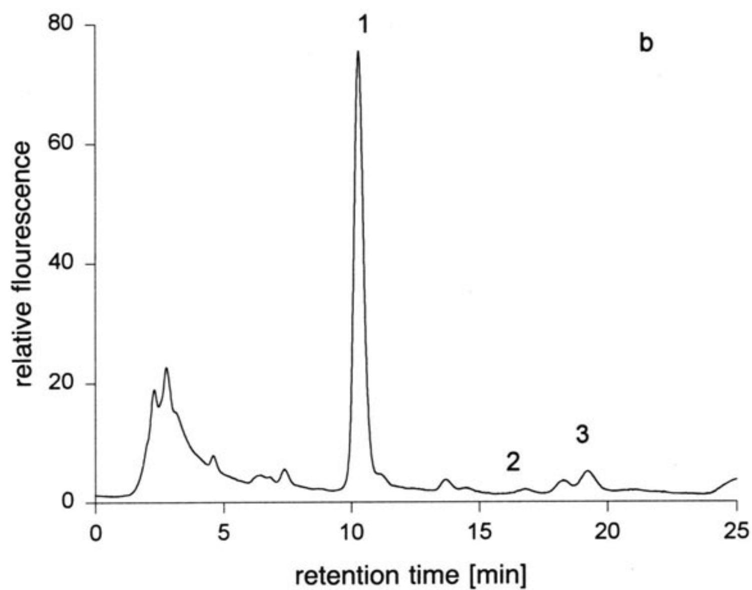
20
25
retention time [min]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25
retention time [min]

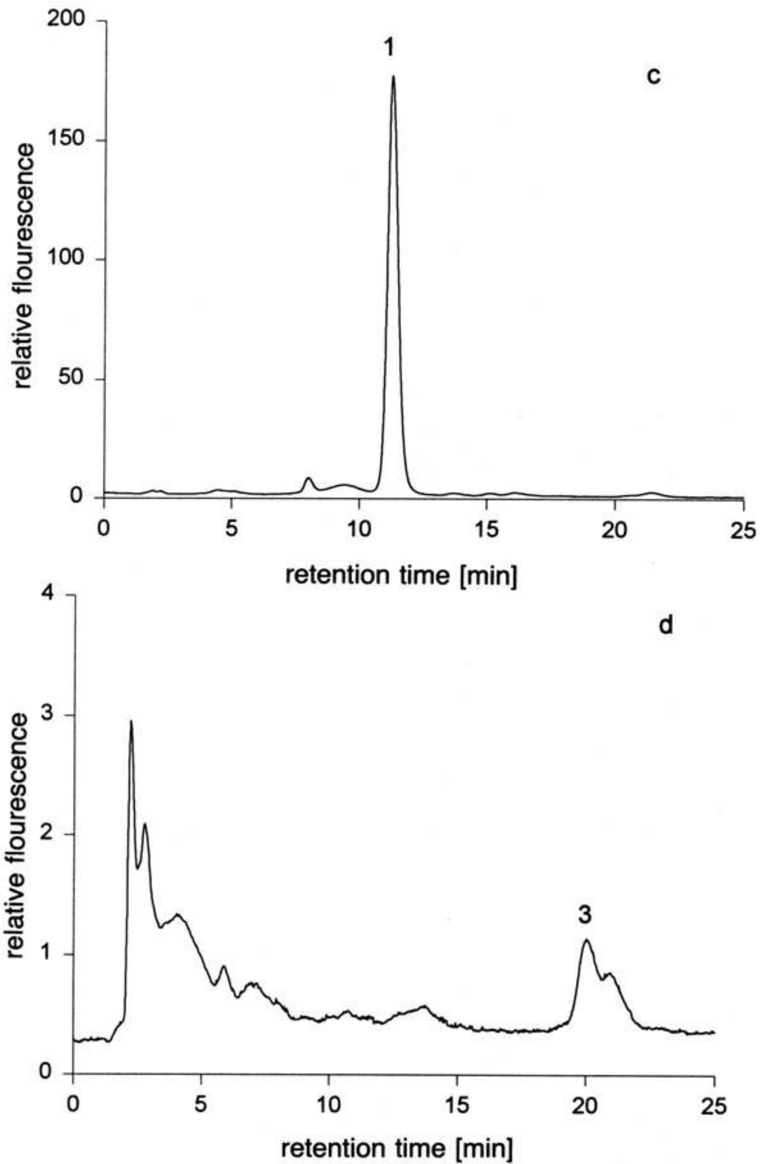
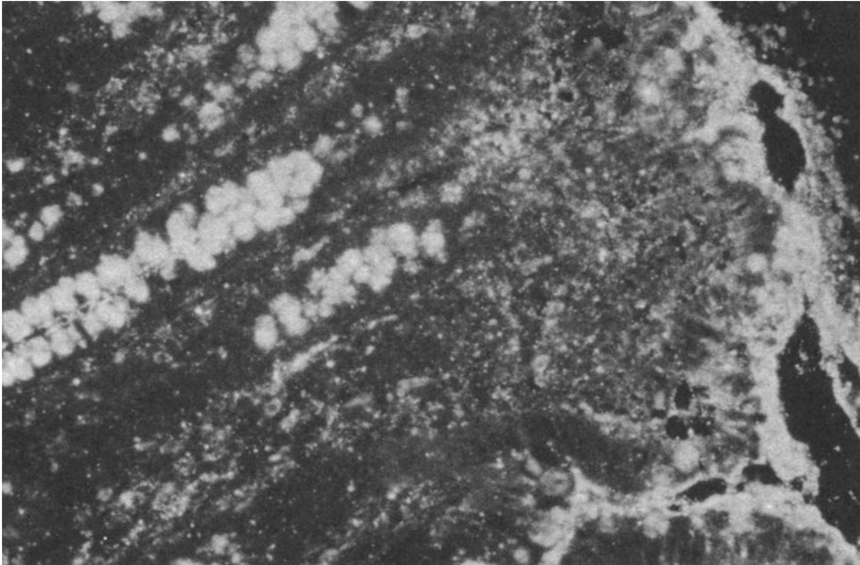


FIGURE 4. Analysis of sialic acids from human colon as DMB derivatives by fluorimetric HPLC on RP-18. (a) Sialic acids after direct derivatization with DMB; (b) after treatment with influenza C virus; (c) after treatment with 0.1 M NaOH; (d) after treatment with aldolase. The following sialic acid peaks were identified by comparison with known standards: 1, Neu5Ac; 2, Neu5,9Ac_2; 3, higher *O*-acetylated sialic acids.



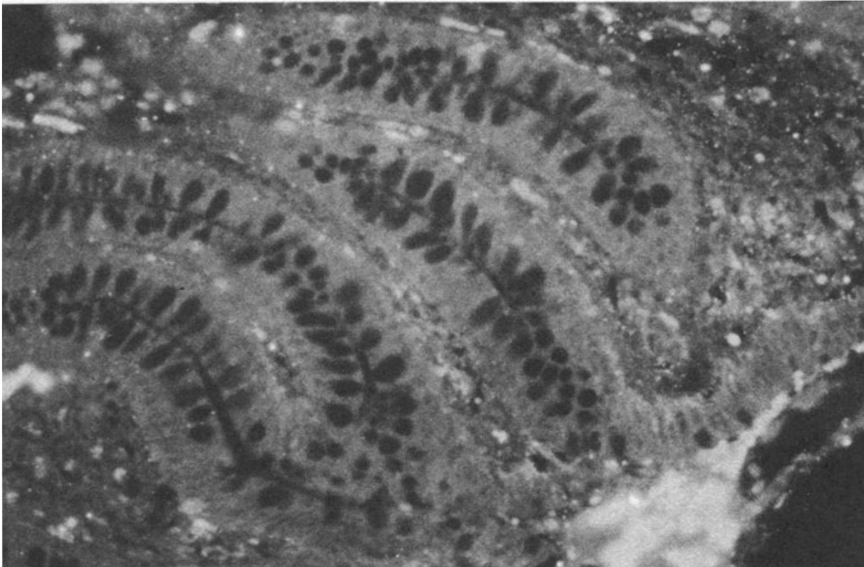


FIGURE 5. Thin section of human colon overlaid with influenza C virus. Bound virus was visualized by an immunoassay with FITC-conjugated anti-influenza antibodies. (Top) Normal staining; (Bottom) same as a after sialidase treatment.

A separation of neutral or sialylated oligosaccharides or glycopeptides can be achieved by HPLC on anion-exchange resins using eluents with strong alkali followed by pulsed amperometric detection (Townsend *et al.*, 1988), allowing analysis of these compounds at the picomole level. Because of the high pH of the eluent, analysis of *O*-acetylated sialic acids is not possible. For separation of unmodified gangliosides, a new HPLC method has been established using anion-exchange resins (Unland and Müthing, 1993).

In addition to these methods, histochemical techniques may be used. Various lectins, antibodies, and viruses are known to specifically detect certain sialylated epitopes in glycoconjugates (summarized by Reuter and Schauer, 1994, and see Section 6.1). Recently, influenza C virus, which is well suited for detection of glycosidically linked terminal Neu5,9Ac_2 residues via its hemagglutinin (Zimmer *et al.*, 1992; see Chapter 9), has been used for histochemical analysis of thin sections (Harms *et al.*, 1993) (Figure 5).

4. ENZYMATIC MODIFICATIONS OF SIALIC ACIDS

4.1. *O*-Acylation of Sialic Acids

Over the past several years, the modification of sialic acids by *O*-acetylation has been found to be of great importance (Reuter and Schauer, 1987; Schauer, 1991; Varki, 1992a). Probably the best established example is the binding of influenza viruses to sialic acids. Whereas influenza A and B viruses exclusively bind to non-*O*-acetylated sialic acids, influenza C virus only recognizes Neu5,9Ac_2 (see Chapter 9). Furthermore, sialic acid *O*-acetylation reduces or even prevents the action of sialidases and *N*-acylneuraminate-pyruvate lyase (Schauer, 1982b; Corfield and Schauer, 1982b). The biological significance of this influence is illustrated by the observation that sialic acid de-*O*-acetylation followed by sialidase treatment of rat erythrocytes significantly increases the binding rate of these cells to peritoneal macrophages when compared to sialidase treatment alone (Kiehne and Schauer, 1992). An increased level of sialic acid *O*-acetylation was discovered in human melanoma gangliosides that are considered to be tumor-associated antigens (Thurin *et al.*, 1985; Sjoberg *et al.*, 1992). For human colon cancer, however, a reduction of sialic acid *O*-acetylation was found (Hutchins *et al.*, 1988). The effect of Neu5,9Ac_2 on complement activation has also been described (Varki and Kornfeld, 1980). Thus, this type of molecular modification seems to be a very important tool for the organism to regulate recognition and cellular interaction.

Although 9-*O*-acetylation of sialic acids is the most frequent modification found in many species from bacteria to humans (Corfield and Schauer, 1982a; Schauer, 1982b, 1991), the mechanism of the acetylation reaction is not clear.

The data available up to now indicate an enzymatic transfer of the acetyl function from acetyl-CoA onto glycoconjugate-bound sialic acid and not onto free sialic acids or their CMP-glycosides (Schauer, 1982b, 1987b, 1991; Varki, 1992a). The complete pattern of sialic acid side chain *O*-acetylation may require several sialate *O*-acetyltransferases with a specificity for only one position. However, the primary insertion site for the *O*-acetyl function may be the 7-OH alone from where the ester group can migrate even under physiological conditions to the 9-position, presumably via C-8 (Kamerling *et al.*, 1987; Schauer, 1987b), leaving the 7-OH ready for a new transfer. Thus, for complete side chain *O*-acetylation, only one enzyme, acetyl-CoA:sialate 7-*O*-acetyltransferase (EC 2.3.1.45), may be necessary together with nonenzymatic migration of this substituent. In rat liver Golgi vesicles 7- and 9-*O*-acetylation of glycoconjugates were found (Diaz *et al.*, 1989; Higa *et al.*, 1989a,b), 7-*O*-acetylation primarily in lysosomal membranes and 9-*O*-acetylation in plasma membranes (Butor *et al.*, 1993a). In bovine submandibular glands, sialate *O*-acetyltransferase was detected in a cytosolic fraction as well as associated with smooth and mitochondrial membranes (Schauer *et al.*, 1988a) using de-*O*-acetylated bovine submandibular gland mucin or immobilized fetuin as substrates. Sialic acid *O*-acetyltransferase has been reported to be specific for Neu5Ac in *N*-glycans in rat liver (Butor *et al.*, 1993a) and for Neu5Ac in gangliosides in human melanoma cells (Sjoberg *et al.*, 1992), suggesting that *O*-acetyltransferase may also exhibit substrate specificity for the oligosaccharide to which the sialic acid is bound. So far, however, sialate *O*-acetyltransferases have not been purified to homogeneity, which may be a prerequisite for a detailed characterization of these enzymes.

4-*O*-Acetylation has been found in horse, donkey, guinea pig, and *Echidna* (Corfield and Schauer, 1982a) and requires a different *O*-acetyltransferase, acetyl-CoA:sialate 4-*O*-acetyltransferase (EC 2.3.1.44), which has been described for equine submandibular gland as a membrane-associated enzyme (Schauer, 1978b, 1987b).

Little is known about sialic acid *O*-lactoylation, which has been found as Neu5Ac9Lt in human, cow, horse, and trout (Corfield and Schauer, 1982a; Schmelter *et al.*, 1993; Corfield *et al.*, 1993). Recent investigations with a particulate fraction from horse liver suggested that this modification occurs enzymatically, although the exact mechanism and type of lactoyl donor are unknown (Kleineidam *et al.*, 1993).

4.2. De-O-Acetylation of Sialic Acids

Sialate 9-*O*-acetylesterases (EC 3.1.1.53) have been isolated and characterized from bovine brain, horse and rat liver, and influenza C virus (Schauer *et al.*, 1988b,c, 1989; Butor *et al.*, 1993b). These enzymes specifically release 9-*O*-acetyl groups from free and glycosidically bound sialic acids; 9-*O*-lactoyl

groups, Neu5,7Ac_2, or sialic acid methyl esters are not substrates. An additional specificity for 4-*O*-acetyl groups was discovered for the esterase from horse liver (Schauer *et al.*, 1988b), which may render this type of sialic acid susceptible to sialidases, since a 4-*O*-acetyl function in sialic acids prevents their release from glycoconjugates by mammalian and bacterial sialidases (Schauer, 1982b; Corfield and Schauer, 1982b), including as well the sialidase from horse liver. Deesterification of side-chain *O*-acetylated sialic acids also increases the catabolism of these sugars (Schauer, 1987b). These esterases, which, however, also attack *O*-acetyl groups of nonphysiological substances, e.g., naphthylacetate, belong to the few esterases that have been recognized to have a physiological function (Schauer *et al.*, 1988c).

Influenza C viruses express a surface glycoprotein (HEF) consisting of three different domains, two of which have been found to recognize *O*-acetylated sialic acids, the receptor-destroying enzyme that was characterized as sialate 9-*O*-acetylesterase (Herrler *et al.*, 1985), and the hemagglutinin that binds to 9-*O*-acetylated sialic acids of glycoproteins and glycolipids (Zimmer *et al.*, 1992), as well as to Neu5,7Ac_2-GD3 (Harms *et al.*, 1993). Whereas the enzymatic activity is optimal at 37°C and hardly detectable at 4°C, the hemagglutinin is fully capable of binding at the lower temperature. On the basis of this temperature difference in the two activities, a test was developed for the detection of glycoconjugate-bound 9-*O*-acetylated sialic acids on thin-layer chromatograms, SDS gels after blotting, microtiter plates, and tissue sections (Zimmer *et al.*, 1992; 1994; Harms *et al.*, 1993). The third domain of the HEF is involved in fusion of the virus with the cell membrane. The primary sequence of HEF has been elucidated by molecular cloning (Vlasak *et al.*, 1987).

4.3. *O*-Methylation of Sialic Acids

Sialic acid *O*-methylation is a modification that seems to be restricted to *Echinodermata* (Corfield and Schauer, 1982a). *O*-Methyl groups have only been found at the 8-position of Neu5Gc and in much lower quantities on Neu5Ac (Bergwerff *et al.*, 1992, 1993). As outlined in Section 2, 9-*O*-acetylated derivatives of 8-*O*-methylated sialic acids were also detected. The existence of a sialate 8-*O*-methyl transferase needed for the biosynthesis of this sialic acid was first demonstrated to occur in the starfish *Asterias rubens* (Schauer and Wember, 1985; Bergwerff *et al.*, 1992). The enzyme has now been further characterized from a crude preparation of starfish gonads as a membrane-associated protein that transfers methyl groups from *S*-adenosyl methionine preferably onto glycosidically linked Neu5Gc residues. Neither free Neu5Gc nor CMP-Neu5Gc are substrates for this enzyme. Horse erythrocytes containing almost exclusively Neu5Gc residues are excellent substrates for the sialate *O*-methyltransferase (de Freese *et al.*, 1993).

4.4. *N*-Acetyl-Hydroxylation of Sialic Acids

4.4.1. Occurrence of *N*-Glycoloylneuraminic Acid

N-Glycoloylneuraminic acid (Neu5Gc) is formally derived by the addition of a hydroxyl group onto the *N*-acetyl function of Neu5Ac. Neu5Gc is present in essentially all animal groups possessing sialylated glycoconjugates, i.e., spanning the deuterostomate lineage from the echinoderms up to mammals (Warren, 1963; Corfield and Schauer, 1982a; Varki, 1992a). The extent of glycoconjugate sialylation with Neu5Gc is very much dependent on the species (Warren, 1963; Corfield and Schauer, 1982a), tissue (Reuter *et al.*, 1988), stage in development (Muchmore *et al.*, 1987; Sherblom *et al.*, 1988; Schauer *et al.*, 1991; Budd *et al.*, 1992), and the presence of certain pathogenic conditions (Higashi *et al.*, 1985). Humans and chickens are, however, notable since they lack Neu5Gc in healthy tissues but do express small amounts in certain tumors (Gottschalk, 1960; Haverkamp *et al.*, 1976; Ledeen and Yu, 1976; Corfield and Schauer, 1982a; Higashi *et al.*, 1985; Kawai *et al.*, 1991). The significance of this is discussed later in this section. Although several bacterial species incorporate Neu5Ac and even Neu5,9Ac_2 into surface lipopolysaccharides (Dutton *et al.*, 1987; Gibson *et al.*, 1993) and capsular polysialic acid (Higa and Varki, 1988; Troy, 1992), the existence of Neu5Gc in bacteria has never been reported. The pathogenic protozoan *Trypanosoma cruzi* has been shown to contain Neu5Gc (Schauer *et al.*, 1983). This, however, most probably originates from sialoglycoconjugates in the culture medium. The transfer reaction may be catalyzed by a trans-sialidase (Schenkman *et al.*, 1991), though activity with a Neu5Gc-containing sialyl donor has only been demonstrated for the trans-sialidase from *Trypanosoma brucei* (Engstler and Schauer, 1993).

Neu5Gc is present in gangliosides and glycoproteins, exhibiting no pronounced preference for a particular oligosaccharide type. However, in porcine submaxillary gland mucin Neu5Gc was found to occur preferentially in α2,6 linkage to GalNAc (Savage *et al.*, 1986). Furthermore, in *N*-glycans of porcine vitronectin, Neu5Gc residues are located predominantly on the Man α1,6 arm (Yoneda *et al.*, 1993). Like Neu5Ac, Neu5Gc is also derivatized in a variety of positions, including *O*-acetylation in positions 4, 7, 8, and 9 (Corfield and Schauer, 1982b), *O*-lactoylation in position 9 (Reuter *et al.*, 1988), and a rather unusual 8-*O*-methylation occurring in certain starfish (Warren, 1964; Corfield and Schauer, 1982a; Bergwerff *et al.*, 1992). The additional C-5 hydroxyl group in Neu5Gc may also be acetylated to yield Neu5GcAc (Kluge *et al.*, 1992) present in rat thrombocytes, or may be glycosylated, as demonstrated in certain starfish gangliosides (Smirnova *et al.*, 1987) and oligosaccharides (Bergwerff *et al.*, 1992, 1993). While oligomers of Neu5Gc have been detected in the polysialic acid on the vitelline coat glycoproteins of several fish eggs (see Section 2;

Kitajima *et al.*, 1988; Kanamori *et al.*, 1990), the presence of Neu5Gc in mammalian NCAM-associated polysialic acid has yet to be demonstrated (Troy, 1992). (See Chapter 4.)

4.4.2. Biological Role of Neu5Gc

Since the sialic acid residues of glycoconjugates play a variety of roles, as discussed elsewhere in this chapter, it is unlikely that Neu5Gc has one specific function. However, it seems to modulate the functions exerted by sialic acids. For example, cell surface glycoconjugates may help to protect tissues from attack by certain pathogen-derived toxic enzymes. In order to aid the digestion of cell surface glycoconjugates, several microorganisms secrete a sialidase, which may facilitate spreading such as that of bacteria during infection (Ezepchuk *et al.*, 1973; Corfield, 1992). Since the cleavage rate of Neu5Gc by bacterial and viral sialidases is generally lower than that of Neu5Ac, the expression of Neu5Gc may be an adaptation to retard the effects of sialidases (Corfield *et al.*, 1981b). The further degradation of Neu5Gc by the acylneuraminate-pyruvate lyase also occurs at a slower rate than for Neu5Ac (Corfield and Schauer, 1982b). It is therefore conceivable that Neu5Gc may mask subterminal galactose residues of oligosaccharide chains thus preventing their recognition and phagocytosis by macrophages more potently than does Neu5Ac (Schauer, 1985).

Over the last few years, certain sialic acid-containing oligosaccharide structures have been found to function as ligands for a number of receptors involved in important cell–cell recognition phenomena (Varki, 1992b). Such receptors include the selectins (Cummings and Smith, 1992), the B-lymphocyte-associated glycoprotein CD22 (Sgroi *et al.*, 1993), and the sialoadhesin associated with mouse stromal macrophages (Crocker *et al.*, 1991), as will be discussed in detail in Section 6. In addition, several sialic acid-binding lectins have been described, which exhibit specificity for either Neu5Ac or Neu5Gc (Section 6, Table IV).

Although several bacterial (Karlsson, 1989; Liukkonen *et al.*, 1992) and viral (Higa *et al.*, 1985) pathogens exploit glycoconjugate-bound sialic acid residues for adherence to a cell surface prior to infection (Table V), only the enterotoxic *E. coli* strain K99, which infects young pigs, is specific for Neu5Gc (Kyogashima *et al.*, 1989).

4.4.3. Biosynthesis of Neu5Gc

4.4.3.a. *N*-Acylation with Activated Glycolic Acid. The mechanism of the biosynthesis of Neu5Gc has been the subject of several investigations. The possibility that the *N*-glycoloyl group might arise by hexosamine acylation with glycoloyl-CoA at an early step in sialic acid biosynthesis was advanced by Jourdian and Roseman (1962). However, later studies on glycoprotein biosynthe-

sis in sheep colon largely excluded this pathway (Allen and Kent, 1968). Nevertheless, in a recent publication it was put forward that glycoloyl-CoA derived from fatty acid by ω-oxidation with subsequent β-oxidation might give rise to Neu5Gc (Vamecq *et al.*, 1992).

4.4.3.b. Oxidative Hydroxylation of Neu5Ac: Specificity of a Sialic Acid Hydroxylase. Several studies on the production of mucin-bound Neu5Gc in pig submandibular glands indicated that the *N*-glycoloyl group results from the hydroxylation of the amino-sugar-bound *N*-acetyl moiety at some point during Neu5Ac biosynthesis (Schauer *et al.*, 1968). Subsequent experiments with cell-free extracts of this tissue suggested that free (Schauer, 1970) and glycoconjugate-bound Neu5Ac (Buscher *et al.*, 1977) are the substrates for an NAD(P)H-dependent Neu5Ac hydroxylase, which could exist in soluble and membrane-bound forms.

However, a number of later investigations using fractionated homogenates of pig submandibular gland and mouse liver unequivocally established that Neu5Gc is synthesized exclusively by the hydroxylation of the sugar nucleotide sialic acid metabolite CMP-Neu5Ac, giving CMP-Neu5Gc as the product (Shaw and Schauer, 1988, 1989). The isolation of CMP-Neu5Gc from pig submandibular glands is consistent with the suggested substrate specificity (Buscher *et al.*, 1977). Moreover, pulse–chase experiments using mouse myeloma cells incubated in culture with [6-^{3}H]mannosamine revealed that labeled Neu5Gc was first detected in the CMP-sialic acid pool, confirming that the enzyme exhibits the same substrate specificity in living cells (Muchmore *et al.*, 1989).

Interestingly, the existence of CMP-Neu5Ac hydroxylase activity was also demonstrated in the starfish *Asterias rubens* (Bergwerff *et al.*, 1992; Schlenzka *et al.*, 1993a). Since this is one of the evolutionarily least advanced organisms known to possess Neu5Gc-containing glycoconjugates, the pathway leading to Neu5Gc is evidently highly conserved throughout evolution.

4.4.3.c. Properties of CMP-Neu5Ac Hydroxylase. In all mammalian tissues and cell lines so far tested, CMP-Neu5Ac hydroxylase is extracted in a particle-free fraction, suggesting that this enzyme is cytosolic (Shaw and Schauer, 1988, 1989; Bouhours and Bouhours, 1989; Shaw *et al.*, 1991). Similarly, the CMP-Neu5Ac hydroxylase from gonads of *A. rubens* was also predominantly found in a high-speed supernatant fraction, though some membrane-associated activity was also detected (Schlenzka *et al.*, 1993a).

The hydroxylase in supernatants of various tissues exhibits an apparent K_M for CMP-Neu5Ac ranging from 18 μM for the enzyme from *A. rubens* (Schlenzka *et al.*, 1993a) through to 2.5 μM for the hydroxylase in pig submandibular glands (Muchmore *et al.*, 1989), 1.3 μM for the mouse liver enzyme (Shaw and Schauer, 1989), and 0.6 μM for the hydroxylase from rat small intestine (Bouhours and Bouhours, 1989). Although the complex substrate and cofactor

requirements of this enzyme (see later) only permit the measurement of an apparent K_M, the values cited above indicate that the hydroxylase has a very high affinity for its CMP-Neu5Ac substrate. The fact that the K_M of the Golgi membrane-associated CMP-sialic acid antiporter from mouse and rat also lies in the low micromolar range, is consistent with both the hydroxylase and the transporter being exposed to the same cytosolic pool of CMP-Neu5Ac (Carey *et al.*, 1980; Lepers *et al.*, 1989, 1990).

CMP-Neu5Ac hydroxylase exhibits a high level of substrate specificity. Thus, neither glycoprotein-bound Neu5Ac nor free Neu5Ac is hydroxylated by this enzyme (Shaw and Schauer, 1988, 1989). Moreover, relatively high concentrations of free Neu5Ac and a variety of cytidine nucleotides are unable to inhibit the hydroxylase from mouse (Shaw *et al.*, 1992) and starfish (Schlenzka *et al.*, 1993a) to any significant degree. Even CMP-Neu5Gc, the product of the hydroxylase reaction, is only a weak inhibitor (Shaw *et al.*, 1992). A number of sugar-nucleotides (i.e., GDP-Man, UDP-Glc, UDP-GlcNAc, UDP-Gal, and UDP-GalNAc), relevant to glycoconjugate biosynthesis, also have little influence on the activity of the hydroxylase from mouse (Shaw *et al.*, 1992). These results show that the hydroxylase has stringent structural requirements for its CMP-sialic acid substrate and also suggest that this enzyme is unlikely to be regulated by any of the above-mentioned substances.

Experiments with the hydroxylase in high-speed supernatants of several tissues revealed that this enzyme requires a number of substrates and coenzymes for activity. The removal of oxygen from reaction media causes severe inhibition of the hydroxylase, suggesting that the enzyme is a monooxygenase (Shaw and Schauer, 1989; Schlenzka *et al.*, 1993a). This conclusion, however, requires confirmation using the purified enzyme. A reducing cofactor is also essential for the turnover of CMP-Neu5Ac hydroxylase. Although several reducing agents such as ascorbate, dithiothreitol, and tetrahydrobiopterin support a low level of activity, reduced pyridine nucleotides are by far the most effective cofactors, NADH generally being the preferred coenzyme (Shaw and Schauer, 1988, 1989; Kozutsumi *et al.*, 1990; Schlenzka *et al.*, 1993a).

Most NAD(P)H-dependent monooxygenases usually consist of two or three loosely associated protein components. These form an electron transport chain where an NAD(P)H oxidoreductase passes electrons to a terminal acceptor which catalyzes the reductive activation of oxygen and the subsequent hydroxylation of the substrate (Hayaishi, 1974). The inhibitory effect of increased ionic strength and dilution on the activity of the hydroxylase from mammalian sources points to the involvement of protein–protein interactions during catalysis (Shaw *et al.*, 1992). This has been confirmed in a number of studies in which the participation of cytochrome b_5 and cytochrome b_5 reductase in the hydroxylase reaction of mammalian tissues was demonstrated (Kozutsumi *et al.*, 1990; Shaw *et al.*,

1992; Kawano *et al.*, 1993a). The postulated scheme for the interaction between the various components of this system is depicted in Figure 6. On the basis of these properties, the EC number 1.14.13.45 has been suggested.

In mammalian cells, cytochrome b_5 and its reductase occur in membrane-bound forms associated with the endoplasmic reticulum, where they serve as a source of reducing equivalents for several membrane-bound redox enzymes, such as cytochrome P450 (Chiang, 1981) and stearoyl-CoA desaturase (Strittmatter *et al.*, 1974). The majority of cytochrome b_5-dependent enzymes are integral membrane proteins bound to the endoplasmic reticulum, functioning in conjunction with the cytochrome b_5 system associated with this membrane compartment (Arinç, 1991). CMP-Neu5Ac hydroxylase, however, is a soluble protein and only draws electrons from the microsomal cytochrome b_5 system at a very low rate *in vitro*. The source of reducing equivalents *in vivo* is still unclear, though some evidence points to the involvement of an amphiphilic cytochrome b_5 system present in the cytosol (Shaw *et al.*, 1994). Although cytochrome b_5 is present in starfish (den Besten *et al.*, 1990), it is not known whether it participates in the activity of CMP-Neu5Ac hydroxylase in this organism. Indeed, the insensitivity of the enzyme from starfish to high ionic strength and dilution suggests that this enzyme may consist of more firmly associated components (Schlenzka *et al.*, 1993a), contrasting with the mouse enzyme system.

All cytochrome b_5-dependent enzymes so far described possess an iron-containing prosthetic group and several lines of evidence point to the participation of an iron cofactor in the reaction of the hydroxylase. For example, several iron-binding compounds are potent inhibitors of this enzyme (Kozutsumi *et al.*, 1991; Shaw *et al.*, 1992). Furthermore, the addition of iron salts can stimulate the hydroxylase (Shaw and Schauer, 1988, 1989; Schlenzka *et al.*, 1993a). Although no firm conclusions can yet be made, the above observations rule out the presence of a heme group, a suggestion that is supported by preliminary spectroscopic measurements on the purified enzyme (Kawano *et al.*, 1993b).

The characterization of CMP-Neu5Ac hydroxylase described above was, for the most part, carried out using unfractionated supernatants and many of the investigations need refining using the purified hydroxylase as well as purified

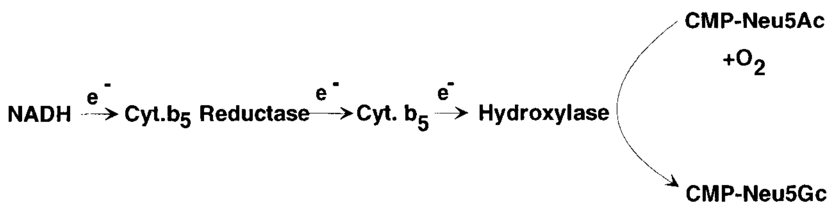


FIGURE 6. Redox protein components involved in the hydroxylation of CMP-Neu5Ac.

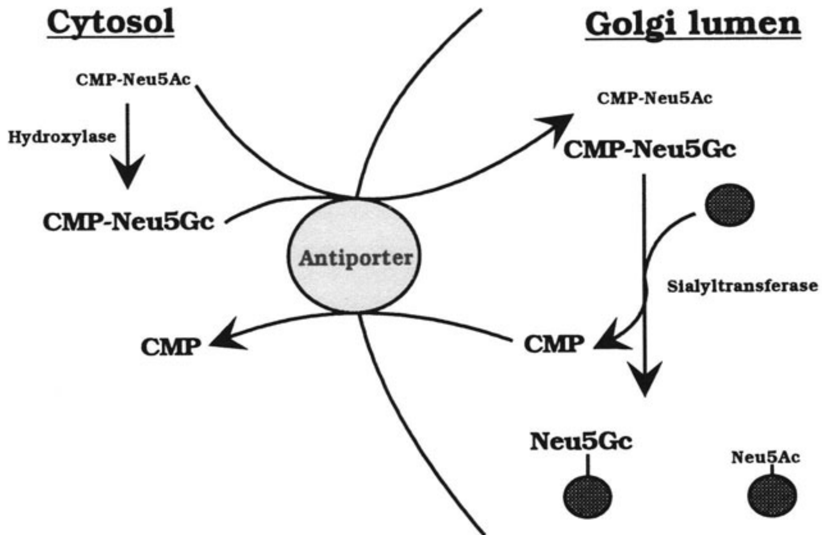


FIGURE 7. Cellular compartmentalization and regulation of Neu5Gc biosynthesis and incorporation into glycoconjugates.

cytochrome b_5 and reductase. The purification of the hydroxylase from mouse liver (Kawano *et al.*, 1993b; Schneckenburger *et al.*, 1993) and pig submandibular glands (Schlenzka *et al.*, 1993b) has been reported recently. The enzyme from both sources was found to be a monomer with a molecular mass of 65 kDa.

4.4.3.d. Role of CMP-Neu5Ac Hydroxylase in Regulating the Incorporation of Neu5Gc into Glycoconjugates. The activity of CMP-Neu5Ac hydroxylase is probably the main factor influencing the level of sialylation with Neu5Gc. Using rat small intestine mucosal cells, Bouhours and Bouhours (1989) showed that the increase in Neu5Gc-GM3 content occurring in the period 15–20 days after birth correlates with an increase in the activity of the hydroxylase, though other tissues did not exhibit such a clear correlation (Muchmore, 1992). Similarly, the relative level of Neu5Gc and Neu5Ac in rat and mouse liver (Lepers *et al.*, 1990), as well as in several related mouse lymphoma cell lines, correlates with the activity of CMP-Neu5Ac hydroxylase (Shaw *et al.*, 1991). Neither the Golgi CMP-sialic acid antiporter nor the sialyltransferases exhibit a pronounced preference for CMP-Neu5Ac or CMP-Neu5Gc, and thus have little or no role in regulating the relative incorporation of Neu5Gc or Neu5Ac into glycoconjugates (Figure 7) (Lepers *et al.*, 1990). The activity of the hydroxylase may thus be tuned so that the ratio of Neu5Gc and Neu5Ac required in the resulting glycoconjugates is generated in the form of CMP-glycosides in the cytosol. Since the cytochrome b_5 system has a multiplicity of functions and the hy-

droxylase is not apparently influenced by any metabolites, the rate of production of CMP-Neu5Gc is presumably regulated at the level of expression of the 65-kDa monooxygenase component. The tissue-specific and developmental factors affecting the expression of this protein still remain to be elucidated.

Although *O*-acetyl modifications of sialic acids can be enzymatically removed (see Section 4.2), no mechanism for the dehydroxylation of Neu5Gc is known. Neu5Gc from recycled glycoconjugates may thus augment the hydroxylase-mediated Neu5Gc biosynthesis (Muchmore *et al.*, 1989).

4.4.3.e. Neu5Gc as a Human Oncofetal Antigen. Glycoconjugates sialylated with Neu5Gc have not been detected in normal human and chicken tissues and are in fact antigenic in both species, inducing the formation of heterophile antibodies historically referred to as Hanganutziu–Deicher antibodies (Fujii *et al.*, 1982; Schauer, 1988; Higashi, 1990). Antigens reacting with Hanganutziu–Deicher antibodies have been detected in a variety of human tumor types, including colon cancers (Higashi *et al.*, 1985; Hirabayashi *et al.*, 1987a), retinoblastoma (Ohashi *et al.*, 1983; Higashi *et al.*, 1988), melanoma (Hirabayashi *et al.*, 1987b; Saida *et al.*, 1990), and breast cancer (Hanisch *et al.*, 1992). Cancerous tissue from chickens has also been shown to contain Neu5Gc (Kawai *et al.*, 1991). The majority of these antigens have been identified as gangliosides sialylated with Neu5Gc. Tumor-associated glycoproteins sialylated with Neu5Gc have also been reported in a human gastric cell line grown in serum-free medium (Fukui *et al.*, 1989) and in a mucin from breast cancer (Devine *et al.*, 1991). The frequency of these antigens is variable and depends on the type of tumor, but generally varies from 30 to 50% of all cancers (Higashi *et al.*, 1984, 1985). In all human tumors so far tested, the amount of Neu5Gc, as a proportion of total sialic acid, is extremely low, usually less than 1% and frequently in the range 0.01–0.1% (Higashi *et al.*, 1985; Kawai *et al.*, 1991; Hanisch *et al.*, 1992). Such low levels of Neu5Gc are generally detected with immunological methods rather than with chemical techniques. Some authors have, however, identified Neu5Gc in tumors by gas chromatography and mass spectrometry and the amounts of Neu5Gc determined are in the same range as those estimated by immunological methods (Kawai *et al.*, 1991; Hanisch *et al.*, 1992).

To date one can only speculate on the origin of Neu5Gc in human tumor tissues. Neu5Gc observed in HeLa cells grown in the presence of fetal calf serum could have arisen by incorporation of Neu5Gc present in the serum (Carubelli and Griffin, 1968). However, in the majority of investigations, pathological tissue removed directly from patients was analyzed. The abnormal uptake or incorporation of dietary Neu5Gc or *N*-glycoloylated amino-sugar metabolites is a possible source, though in animal experiments, the main part of orally administered Neu5Gc is excreted (Nöhle *et al.*, 1982). The possibility that abnormal glycoloyl-CoA metabolism could give rise to Neu5Gc must also be considered (Vamecq *et al.*, 1992). The simplest mechanism leading to Neu5Gc formation in

human tumors is the anomalous expression of an otherwise dormant or repressed CMP-Neu5Ac hydroxylase gene. However, no evidence has been obtained for this assumption.

5. RELATIONSHIP AND EVOLUTIONARY DISTRIBUTION OF MICROBIAL SIALIDASES

Sialidases (EC 3.2.1.18) are essential tools in sialic acid catabolism (see Chapter 8). These enzymes cleave the *O*-glycosidic linkages between the terminal sialic acids and the subterminal sugars of free and glycoconjugate-bound oligosaccharides as one of the first steps in sialoglycoconjugate degradation. Sialidase, as well as its substrate, is common in metazoan animals of the deuterostomate lineage from echinoderms to mammals (Rosenberg and Schengrund, 1976b; Corfield *et al.*, 1981a; Corfield and Schauer, 1982b). Diverse viruses and microorganisms, like fungi, protozoa, and bacteria, also produce sialidases (Müller, 1974; Corfield *et al.*, 1981a; Corfield, 1992; Engstler *et al.*, 1993), although they mostly lack sialic acids. A common property of these organisms is their close contact to animals as commensals or pathogens (Schauer and Vliegenthart, 1982), whereby sialidase is used primarily for nutritional purposes (Corfield, 1992; Müller, 1992). However, this enzyme may additionally be employed for adhesion to host cells (Gabriel *et al.*, 1984) and as a spreading or virulence factor in invasive infections (Ezepchuk *et al.*, 1973; Godoy *et al.*, 1993). Close interactions between animals and microorganisms may have led to the exchange of sialidase genes between these groups and also between the microorganisms involved.

It is remarkable that the occurrence of sialidase in microorganisms is frequently not in accordance with the phylogenetic relationship of bacterial species or strains. Highly related species, e.g., *Clostridium sordellii* and *C. bifermentans* (Roggentin *et al.*, 1985), or even strains of one species, e.g., *C. butyricum* (Popoff and Dodin, 1985) or *Salmonella typhimurium* (Hoyer *et al.*, 1992), differ with respect to sialidase production. This irregular distribution of the enzyme indicates that sialidases evolved by other mechanisms than their producers in the bacterial kingdom. It is therefore of interest to gain insight into the relatedness and the directions of propagation of the factor sialidase, the possession of which possibly is the result of an adaptation of the microorganisms to a "new" sugar presented by their animal hosts.

A comparison of the properties of the sialidases from a variety of microbial sources revealed that these enzymes are highly diverse with respect to molecular mass, number of subunits, isoelectric point, temperature optimum, influence of Ca^{2+} on activity, substrate specificity, and specific activity (Nees *et al.*, 1975; Uchida *et al.*, 1979; Reuter *et al.*, 1987; Roggentin *et al.*, 1987; Teufel *et al.*,

Table III
Summary of Cloned and Sequenced Sialidases from Eukarya and Bacteria

Organism	Type of sialidase	Reference
Rattus rattus	Cytosolic	Miyagi *et al.* (1993)
Trypanosoma cruzi	Cell surface, trans-sialidase	Pereira *et al.* (1991)
Trypanosoma rangeli	Secreted (sialidase-like sequence)	Buschiazzo *et al.* (1993)
Actinomyces viscosus	Cell surface	Henningsen *et al.* (1991), Yeung (1993)
Bacteriodes fragilis	Secreted (partial sequence)	Russo *et al.* (1990)
Clostridium perfringens	"Large" secreted isoenzyme	Traving *et al.* (1993)
	"Small" cytosolic isoenzyme	Roggentin *et al.* (1988)
Clostridium septicum	Secreted	Rothe *et al.* (1991)
Clostridium sordellii	Secreted	Rothe *et al.* (1989)
Micromonospora viridifaciens	Secreted	Sakurada *et al.* (1992)
Salmonella typhimurium	Cytosolic	Hoyer *et al.* (1992)
Vibrio cholerae	Secreted	Galen *et al.* (1992)

1989; Aisaka *et al.*, 1991; Heuermann *et al.*, 1991; Hoyer *et al.*, 1991; Tanaka *et al.*, 1992; Engstler *et al.*, 1992, 1994; Zenz *et al.*, 1993). The only common property is the acidic pH optimum (pH 5.0–6.1), though the trypanosomal trans-sialidases exhibit maximum activity at a neutral pH (Engstler *et al.*, 1993). As these properties did not reveal any relationship between microbial sialidases, the enzymes were further investigated on the DNA level, from which interesting information became available.

In the first primary structures of microbial sialidases, obtained by cloning and sequencing of the respective genes from *Clostridium perfringens* (Roggentin *et al.*, 1988), *Vibrio cholerae* (Galen *et al.*, 1992), *Clostridium sordellii* (Rothe *et al.*, 1989), and *Salmonella typhimurium* (Hoyer *et al.*, 1992), an amino acid sequence motif was detected, which is repeated fourfold in each protein: S-X-D-X-G-X-T-W (Roggentin *et al.*, 1989). This motif, named Asp-box, was found in all sialidases of animals and bacteria that have been sequenced (Table III). From the alignment of more and more sialidase sequences, it became obvious that the Asp-boxes II and IV are more degenerate than I and III, whereby box III appears to be virtually complete in each protein (Figure 8). In viral sialidases, however, the motif was rarely detectable (e.g., only an N9 influenza A virus strain exhibits the complete motif) (Air *et al.*, 1987), and has probably undergone mutational alterations.

The function of this conserved and repeated motif is as yet unknown. In the N9 influenza A virus sialidase (Air *et al.*, 1987), the single Asp-box is located as part of a β-pleated sheet polypeptide at the connections between the four protein

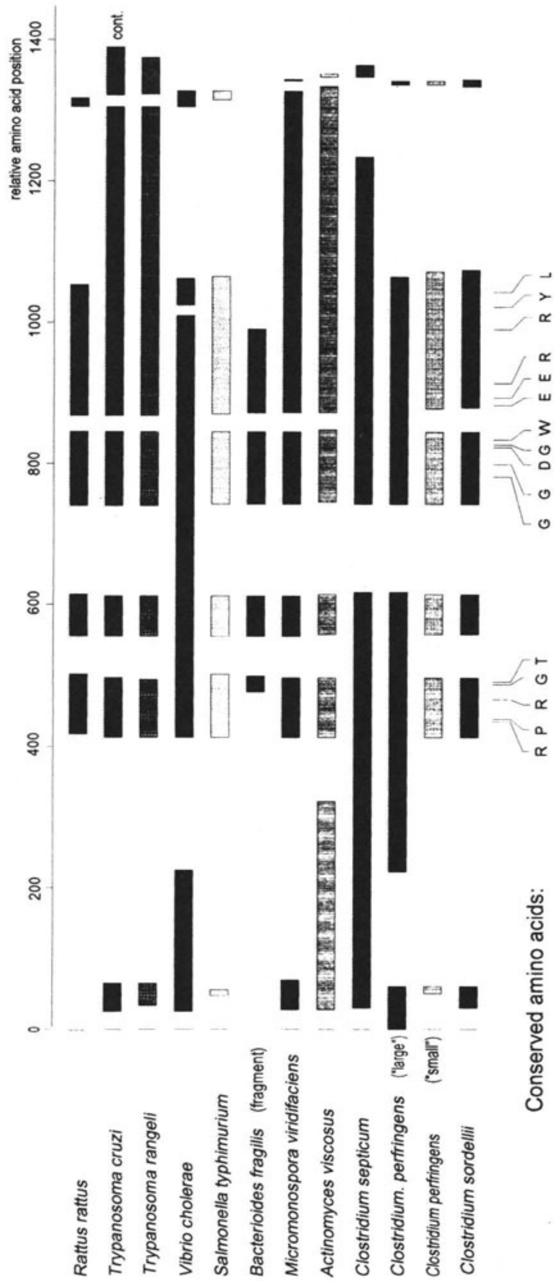


FIGURE 8. Schematic survey of an alignment of 12 sialidase primary structures and the location of 16 conserved amino acids.

subunits. Immunological studies revealed that the Asp-box I of *Trypanosoma cruzi* sialidase is inaccessible to antibodies, which has been taken as an indication that it is not part of the external catalytic domain (Prioli *et al.*, 1992). Site-directed mutagenesis experiments of the "small" sialidase isoenzyme of *Clostridium perfringens* resulted in only small alterations of enzyme activity by changing some of the Asp-box amino acids (Roggentin *et al.*, 1992), while the exchange of other highly conserved amino acids drastically reduced enzyme activity and increased the K_M value, e.g., by replacement of the N-terminal conserved arginine (Figure 8) with lysine.

In addition to the Asp-boxes, further identical motifs, or single amino acids, became evident by an alignment of sialidase protein sequences, which is schematically demonstrated in Figure 8. Gaps had to be introduced as a consequence of the differences in protein sizes. The central regions of these proteins are especially homologous and exhibit most of the conserved amino acids. A further motif, the FRIP-region, which is located N-terminally from the first Asp-box, is highly conserved in clostridial sialidases, but was found to be degenerated to X-R-X-P, when further bacterial and animal sialidase sequences were included in the alignment. Nevertheless, 16 amino acids have been found to be conserved. The presence of conserved motifs and single amino acids indicates that the enzymes are interrelated and originate from one source.

By a pairwise comparison of the sequences, further amino acids were found to be identical at certain positions, which allows a calculation of similarity values. This gives the percentage of identical amino acids (excluding the conserved amino acids mentioned in Figure 8) from the total number of amino acids situated at the same points after alignment as 100%. A dendrogram (Figure 9) based on the average linkage method (Anderberg, 1973) combines the values obtained from the pairwise comparisons. It shows that some of the sialidases are related in accordance with the phylogenetic distances of their producers, e.g., *Micromonospora viridifaciens* and *Actinomyces viscosus* sialidase, or the "large" (72 kDa) isoenzyme of *Clostridium perfringens* and the *Clostridium septicum* sialidase. On the other hand, the three "small" sialidases of lower molecular mass (42–44 kDa) produced by *Salmonella typhimurium, Clostridium perfringens,* and *Clostridium sordellii* exhibit a higher similarity than is expected from the relationship of the bacterial species. These gram-positive (clostridia) or gram-negative (salmonella) bacteria are quite distinct from an evolutionary point of view, but are found at the same ecological location, e.g., the intestine of vertebrates. Here, an exchange of genes even between unrelated microorganisms might be possible via phages, transposons, or plasmids by mechanisms of transfection or conjugation. The participation of phages is indicated by typical sequence motifs up- and downstream from sialidase genes of *Salmonella typhimurium* and *Micromonospora viridifaciens* (Hoyer *et al.*, 1992; Sakurada *et al.*, 1992), and from the observation that the "small" sialidase gene is located

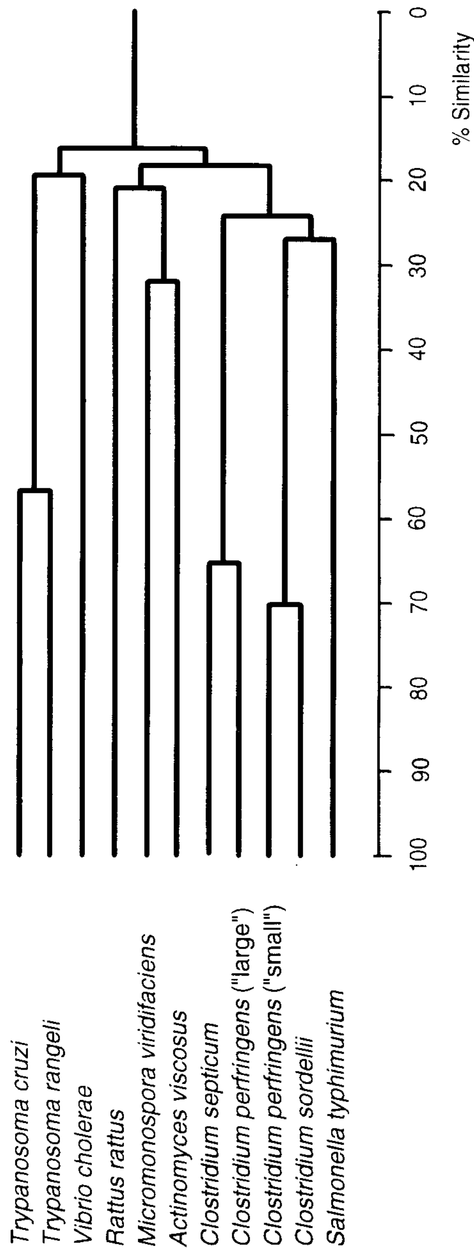


FIGURE 9. Dendrogram of similarities among sialidase primary structures based on identical amino acids.

near a phage attachment site on the chromosome of *Clostridium perfringens* (Canard and Cole, 1990).

Such a horizontal gene transfer would explain the cytoplasmic location (Table III) of active sialidases, thus hindering them from contact with their extracellular substrate, because of the absence of signal peptides. If a gene is acquired from a foreign source, it usually has to be adapted for bacterial purposes, or it is eliminated. The presence of unadapted but active gene products, such as the cytoplasmic sialidases in *Clostridium perfringens* and *Salmonella typhimurium,* therefore indicates that the gene transfer may have occurred recently. The sialidase of *Clostridium sordellii,* which is highly related to these sialidases, probably originated from the same source. In the meantime, it has been modified for secretion and exhibits a broader substrate specificity than the cytosolic enzymes, which are highly selective for α(2-3)-linked sialic acids of oligosaccharides, a structure that is regularly found in higher animals. In contrast, the well-adapted microbial sialidases cited in Table III, e.g., of *Actinomyces viscosus, Bacteroides fragilis, Clostridium perfringens* ("large" isoenzyme), *Clostridium septicum, Micromonospora viridifaciens,* or *Vibrio cholerae,* must have been acquired earlier in evolution, which is also indicated by a relatively poor similarity between the amino acid sequences of sialidase proteins produced by these different genera.

Investigations on the structural sialidase gene composition often revealed a percentage of the bases G + C which is atypical for the chromosomal DNA of the respective bacterial species (e.g., *Salmonella typhimurium:* 40.9/50–53 mol% G + C, or *Actinomyces viscosus:* 70.8/56–58 mol% G + C for the sialidase and the chromosome, respectively; P. Roggentin *et al.*, 1993). Summing up the mol% G + C data available, the origin of sialidase is expected in an organism exhibiting genes that contain about 45 mol% G + C. This value is not uncommon in higher animals.

In conclusion, the hypothesis put forward by Schauer and Vliegenthart (1982) is supported, that sialidase as well as its substrate originated in deuterostomate animals, and that its gene was possibly captured by cell-lysing microorganisms as a reaction to the expression of a "new" sugar.

6. SIALIC ACID-DEPENDENT RECEPTORS AND THEIR LIGANDS

6.1. Occurrence

Many functional roles for the carbohydrate chains of glycoconjugates have been proposed in cellular recognition events, as extensively reviewed by Varki (1993). Whereas for a long time the main function of terminal sialic acid residues was considered to be that of a mask for recognition sites on cell surfaces, e.g., galactose residues or other antigens (Schauer, 1985), only recently have recep-

tors been described that seem to be necessary to connect the structural diversity of sialylated glycoconjugates to specific functions in cellular interaction.

Many microorganisms, plants, and animals express proteins that bind to sialic acids occurring mostly as components of glycoconjugates. A list of such sialic acid-recognizing proteins or lectins from plants and invertebrates is given in Table IV. They show more or less specific binding to different sialic acids or even specific sialic acid linkages and oligosaccharide sequences, as reviewed by Zeng and Gabius (1992b). Since these organisms do not express sialic acids themselves, it is unlikely that these lectins play roles as sialic acid-binding proteins in their own cellular functions. However, they may function in the defense of sialic acid-containing microorganisms. Some of the sialic acid-binding lectins have proved to be useful tools in the analysis and histochemistry of glycoconjugates. Most frequently used are the agglutinins from wheat germ

Table IV
Sialic Acid-Binding Lectins from Plants and Invertebrates[a]

Source	Specificity
Plants	
Wheat germ *Triticum vulgare*	Neu5Ac < GlcNAc
Elderberry *Sambucus nigra*	Neu5Acα2,6Gal/GalNAc
Maackia amurensis	Neu5Acα2,3Galβ1,4GlcNAc
Invertebrates	
Snail *Dolabella*	Neu5Ac
Slug *Limax flavus*	Neu5Ac > Neu5Gc
Snail *Cepaea hortensis*	Neu5Ac > Neu5Gc
Snail *Achatina fulica*	NeuAcα2,3Gal > Neu5Acα2,6Gal
Snail *Pila globosa*	Neu5Gc
Oyster *Crassostrea gigas*	Neu5Ac
Horseshoe crab *Limulus polyphemus*	Neu5Ac
Lobster *Homarus americanus*	Neu5Ac, Neu5Gc
Horseshoe crab *Tachypleus tridentatus*	Neu5Ac, Neu5Gc
Scorpion *Androctonus australis*	Sialyllactose
Horseshoe crab *Carcinoscorpius rotunda*	Neu5Acα2,6Gal > Neu5Acα2,3Gal
Scorpion *Centruroides sculpturatus*	Neu5Ac, Neu5Gc
Prawn *Macrobrachium rosenbergii*	Neu5Ac
Scorpion *Masticoproctus giganteus*	Neu5Ac
Spider *Aphonopelma cepaeahortensis*	Sialoglycoproteins
Scorpion *Heterometrus granulomanus*	Neu5Acα2,3Lac
Prawn *Peneaus monodon*	Neu5Ac
Scorpion *Paruroctonus mesaenis*	Sialoglycoproteins

[a]Zeng and Gabius (1992b, and references therein).

Table V
Pathogenic Microorganisms and Toxins Binding to Sialic Acids on Host Cells

Pathogen	Specificity	Reference
	Viruses	
Influenza A and B	Neu5Ac (some strains prefer Neu5Acα2,3Gal or Neu5Acα2,6Gal, dependent on host specificity)	Paulson (1985)
Influenza C	Neu5,9Ac$_2$	Rogers *et al.* (1986)
Corona virus	Neu5,9Ac$_2$	Vlasak *et al.* (1988)
Sendai virus	Neu5Ac	Markwell and Paulson (1980)
Polyoma virus	Neu5Acα2,3Galβ1,3GalNAc	Cahan and Paulson (1980)
Rotavirus group C	Neu5Ac	Svensson (1992)
	Mycoplasma	
Mycoplasma pneumoniae	Neu5Acα2,3Gal on polylactosamine chains	Loomes *et al.* (1984)
	Bacteria	
Streptococcus sanguis	*O*-linked sialylated tetrasaccharides	Murray *et al.* (1982)
Escherichia coli K99	Neu5Gc-containing glycolipids	Ouadia *et al.* (1992)
Escherichia coli, S-fimbriae	Neu5Acα2,3Galβ1,3GalNAc	Parkkinen *et al.* (1986)
(newborn human meningitis)	Neu5Gc-GM3, GD3, GD1b	Hanisch *et al.* (1993)
Bordetella bronchiseptica	Neu5Ac	Ishikawa and Isayama (1987)
Pseudomonas aeruginosa	Neu5Ac	Ko *et al.* (1987)
Helicobacter (*Campylobacter*) *pylori*	Neu5Acα2,3Lac > Neu5Acα2,6Lac	Evans *et al.* (1988)
Streptococcus suis	Neu5Acα2,3Galβ1,4GlcNAcβ1,3Gal	Liukkonen *et al.* (1992)
	Protozoa	
Malaria (MSA-1) *Plasmodium falciparum*	Neu5Ac	Perkins and Rocco (1988)
Chagas disease *Trypanosoma cruzi*	Neu5Ac	Schenkman and Eichinger (1993)
	Toxins	
Vibrio cholerae toxin	GM1	Schengrund and Ringler (1989)
Pertussis toxin	Neu5Ac	Brennan *et al.* (1988)
Tetanus toxin	Sialoglycolipids	Schiavo *et al.* (1991)

(WGA), *Limax flavus* (LFA), *Sambucus nigra* (SNA), and *Maackia amurensis* (MAA).

It has been known for many years that microbial pathogens, i.e., viruses, mycoplasma, bacteria, and protozoa, take advantage of cell surface sialic acids to adhere to their respective host cells (Table V). In Chapter 9 the role of sialic acids in infection by myxoviruses will be discussed. Sialic acid-specific adhesion

Table VI
Mammalian Sialic Acid-Binding Proteins (for Selectins See Table VII)

Source	Specificity	Reference
Frog egg	Sialylated glycoproteins	Titani *et al.* (1987)
Rat uterus	Neu5Ac	Chakraborty *et al.* (1993)
Rat brain	Neu5Ac, Neu5Gc	Popoli and Mengano (1988)
Rat brain myelin	Gangliosides, preferentially GT1b, GQ1b, GD1b	Tiemeyer *et al.* (1989)
Human placenta (IgG)	*O*-acetylated sialic acids	Ahmed and Gabius (1989)
Blood (factor H of alternative complement pathway)	Sialylated glycoconjugates, other polyanionic molecules	Meri and Pangburn (1990)
Murine macrophages (sialoadhesin)	Neu5Acα2→3Galβ1→3GalNAc on glycoproteins and glycolipids	Crocker *et al.* (1991)
Bovine heart (calcyclin)	Neu5Ac, Neu5Gc	Zeng and Gabius (1991)
Human placenta (sarcolectin)	Neu5Ac, Neu5Gc	Zeng and Gabius (1992a)
B lymphocytes (CD22)	Neu5Acα2→6Galβ1→4GlcNAc	Sgroi *et al.* (1993), Powell *et al.* (1993)

of bacteria is a phenomenon drawing an increasing level of interest, since it often is a critical step in infectious diseases. Examples are the inflammation of gastric mucosa by *Helicobacter pylori* after adhesion to sialoglycoproteins of the cell surface (Evans *et al.*, 1988), and meningitis of infants by *Escherichia coli* (Parkkinen *et al.*, 1986; Hanisch *et al.*, 1993). Several bacterial toxins are known that bind to gangliosides in a sialic acid-dependent manner, e.g., cholera or tetanus toxins (Schauer, 1982b; Table V). In addition, it should be mentioned that various antibodies have been described that recognize epitopes containing sialic acids (Schauer, 1988; Zeng and Gabius, 1992b; Varki, 1993).

Sialic acid-dependent receptors have been recognized to play an important role in the adhesion of mammalian cells. This line of research was initiated by the discovery of a family of cell adhesion molecules now generally called selectins (Bevilacqua *et al.*, 1991). Other well-defined sialic acid-dependent adhesion receptors are sialoadhesin (Crocker and Gordon, 1986; Crocker *et al.*, 1991) found on specific subsets of macrophages in bone marrow and lymphatic tissues (e.g., lymph nodes and spleen) as well as CD22 (Stamenkovic *et al.*, 1991, 1992), a B-cell-restricted protein belonging to the immunoglobulin superfamily. In addition, other sialic acid-binding activities from various mammalian tissues have been described and are listed in Table VI.

6.2. Selectins

All three members of this family (Bevilacqua *et al.*, 1991) show a similar primary structure (Bevilacqua *et al.*, 1989; Johnston *et al.*, 1989; Lasky *et al.*, 1989; Larsen *et al.*, 1989; Siegelman *et al.*, 1989; reviewed in Lasky, 1992;

Table VII
Distribution and Binding Specificity of Selectins

Selectin	Cell type	Ligand determinant
E-selectin	Activated endothelia	Sialyl-Le^x and sialyl-Le^a Neu5Acα2,3Galβ1,4(Fucα1,3)GlcNAc and Neu5Acα2,3Galβ1,3(Fucα1,4)GlcNAc
L-selectin	Leukocytes	Sialylated, sulfated, and fucosylated *O*-glycans
P-selectin	Activated platelets and endothelia	Sialyl-Le_x and sialyl-Le_a

McEver, 1992; Varki, 1992b; Bevilacqua, 1993; Bevilacqua and Nelson, 1993; Rosen, 1993). They represent type I transmembrane glycoproteins containing an amino-terminal carbohydrate recognition domain (CRD), a single epidermal growth factor (EGF)-like domain, a variable number of short consensus repeats (SCR) (two for L-selectin, six for E-selectin, and nine for P-selectin), and a relatively short carboxy-terminal cytoplasmic domain. Whereas the homology between the three selectins is 60–70% in the CRD and EGF-like domain, in the SCR only about 40% homology was found. The essential structural elements of the CRD as defined by Drickamer (1988) were deduced from homologies between the hepatic asialoglycoprotein receptor and other sugar-binding proteins. This motif consists of about 120 amino acids with 32 conserved residues—18 identical and 14 conservative. For E-selectin, the crystal structure of a recombinant protein containing the CRD and EGF-like domains has been obtained (Graves *et al.*, 1994).

Besides their structural homologies, the selectins also share common aspects in their function. They all play crucial roles in the initial event of white blood cell adhesion to specific endothelia, the so-called rolling (Lawrence and Springer, 1991). Before firm adhesion, cells flowing in the bloodstream start to slow down by rolling along the endothelial lining of the vessel. This is mediated by selectins interacting with sialic acid-containing ligands. The specificity of this interaction is accomplished by the expression pattern of the receptors and their appropriate ligands (Table VII). At least two of the selectins recognize the same carbohydrate structures sialyl-Lewis^x (sLe^x) or sialyl-Lewis^a (sLe^a), which are expressed at high levels on leukocytes and some tumor cells. Therefore, it has been proposed that selectins are also involved in metastatic events. The cooperation of the selectin family with ICAM-1 and -2, sLe^x on CD11/CD18 during the rolling and adhesion of granulocytes on endothelial cells under the influence of inflammatory cytokines is illustrated in Figure 10 (Kotovuori *et al.*, 1993).

6.2.1. L-Selectin

L-Selectin is found on lymphocytes and other leukocytes and was described under various names (MEL-14 Ag, gp90MEL, LAM-1, Leu8, Ly22, TQ1,

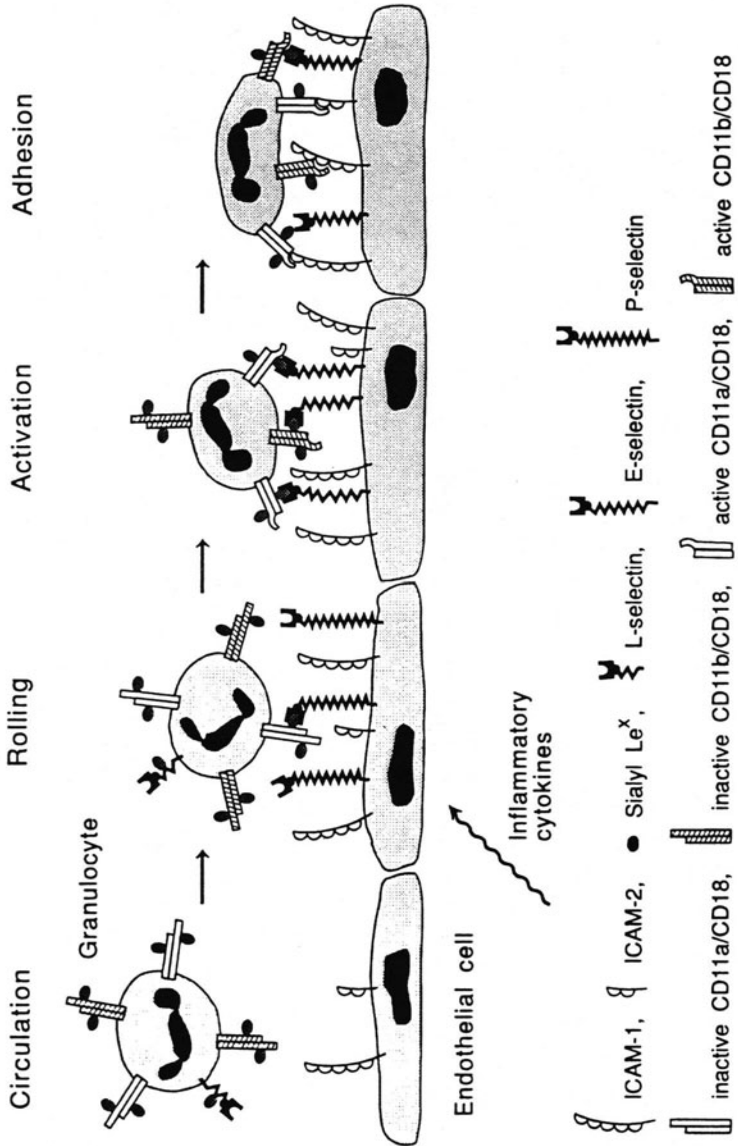


FIGURE 10. Model for granulocyte binding to activated endothelial cells. Circulating granulocytes express L-selctin and inactivated CD11/CD18. Unstimulated endothelial cells express only smalll amounts of ICAM-1 and ICAM-2. After induction by cytokines, ICAM-1 and ICAM-2 expression is upregulated and E- and P-selectin expression is induced. Binding of E- and P-selectin to sLe^x on CD11/cd18 triggers activation of these integrins leading to firm attachment through binding to ICAM molecules. (From Kotovuori *et al.*, 1993.)

DREG56). The functional aspects of L-selectin and recent progress in the characterization of its putative ligands have been reviewed in detail by Rosen (1993). It functions as an adhesion molecule in the homing of lymphocytes to high endothelial venules (HEV) in peripheral lymph nodes, but it is also involved in other leukocyte trafficking events, such as the recruitment of neutrophils to sites of acute inflammation (Lewinsohn *et al.*, 1987; Watson *et al.*, 1991). The requirement of sialic acids in this homing event was first described by Rosen *et al.* (1985). Until now, three mucinlike proteins (Imai *et al.*, 1991), GlyCAM-1 (Lasky *et al.*, 1992), CD34 (Baumhueter *et al.*, 1993), and MAdCAM-1 (Berg *et al.*, 1993) were characterized as potential ligands for L-selectin in HEVs of peripheral lymph nodes. In addition, it was shown that the *O*-linked oligosaccharides from GlyCAM-1 contain essential sialic acid and sulfate residues besides fucose and *N*-acetylglucosamine (Imai and Rosen, 1993; Imai *et al.*, 1993). Possible implications of the characteristic features of these ligands will be discussed in Section 6.6.

6.2.2. E-Selectin

E-Selectin was originally described under the name ELAM-1 as an adhesion molecule expressed on endothelia activated by cytokines (e.g., by interleukin-1 or tumor necrosis factor-α) where it mediates the rolling of leukocytes. Characteristic for the expression of E-selectin is the lag time of about 4 hr between activation of the endothelial cell and occurrence of the receptor on the cell surface due to the requirement of transcription and translation. This is in contrast to the rapid expression of P-selectin (Section 6.2.3). The discovery of a CRD homologous to the other selectins (Bevilacqua *et al.*, 1989) and the knowledge of specific carbohydrate structures found on granulocytes led to the discovery of sLe^x as oligosaccharide recognized by this receptor, in a number of laboratories (Lowe *et al.*, 1990; Phillips *et al.*, 1990; Walz *et al.*, 1990; Tiemeyer *et al.*, 1991). Later, other structures were shown also to bind E-selectin, like the structural analogues of sLe^a or sLe^x containing sulfate instead of sialic acid (Yuen *et al.*, 1992; see Section 6.6 for further discussion). A specific protein ligand for murine E-selectin was purified and partially characterized (Levinovitz *et al.*, 1993).

6.2.3. P-Selectin

P-Selectin also was originally described under different names (GMP-140, PADGEM, CD62), stemming from independent discoveries. It is found on activated platelets and endothelia where it is expressed on the cell surface briefly after activation by cytokines, because of its storage in vesicles under the cell surface. Like E-selectin, it is involved in rolling events of leukocytes on acti-

vated endothelia. The importance of P-selectin in rolling and leukocyte trafficking has been shown convincingly by targeted gene disruption in the mouse (knockout mouse). These mice appear normal and healthy, but their level of circulating leukocytes is elevated and the recruitment of these cells to sites of inflammation is impaired, although they express normal levels of E- and L-selectin and sLe^x (Mayadas *et al.*, 1993). As for E-selectin, shortly after the discovery of its CRD by molecular cloning (Johnston *et al.*, 1989; Larsen *et al.*, 1989), binding of P-selectin to sLe^x and sLe^a as the oligosaccharide structures recognized was demonstrated (Polley *et al.*, 1991; Zhou *et al.*, 1991; Handa *et al.*, 1991; Table VII). For P-selectin a high-affinity ligand protein also was characterized (Moore *et al.*, 1992; Norgard *et al.*, 1993b; Sako *et al.*, 1993; see Section 6.6).

6.3. CD22

This receptor is found on B cells. As a member of the superfamily of immunoglobulin (Ig)-like molecules, it is a type I transmembrane glycoprotein consisting of seven Ig-like domains, a transmembrane domain, and a C-terminal cytoplasmic domain (Stamenkovic and Seed, 1990; Stamenkovic *et al.*, 1991; Wilson *et al.*, 1991). Alternatively spliced transcripts lacking domain 3 and 4 were originally described as CD22α (Stamenkovic and Seed, 1990). However, later studies failed to demonstrate the presence of this protein form on the surface of human B cells (Engel *et al.*, 1993). The murine homologue has also been described (Torres *et al.*, 1992). In contrast to the high homology between murine and human selectins, only 62% of the amino acids are identical between human and murine CD22, with the highest homology in the extracellular domain 7, the transmembrane and the cytoplasmic domain (71, 68, and 67% identity, respectively). The overall genomic organizations of the human (Wilson *et al.*, 1993) and the murine (Law *et al.*, 1993) homologues were resolved, showing the same structure composed of 15 exons. Each Ig-like domain is contained in a separate exon (exon 4 to 10). Interestingly, the gene of CD22 maps to the same chromosome as the most closely related proteins, carcinoembryonic antigen (CEA) and myelin-associated glycoprotein (MAG), suggesting that the genes of these adhesion proteins may have arisen from the same ancestral gene (Wilson *et al.*, 1993; Law *et al.*, 1993).

Recombinant soluble CD22 constructs and CD22-transfected COS cells were used to analyze the specificity of the receptor for different blood cells (Stamenkovic *et al.*, 1992; Torres *et al.*, 1992; Engel *et al.*, 1993; Crocker *et al.*, 1995). These studies demonstrated that CD22 can mediate binding of B cells to B and T cells as well as to neutrophils, monocytes, and erythrocytes. The interaction with T cells is proposed to be involved in early B-cell activation. CD22 has also been implicated to modulate signaling through the surface IgM (sIgM)–cell

receptor complex. Along this line, Leprince *et al.* (1993) showed that CD22 coprecipitates with the complex and that it is phosphorylated rapidly upon sIgM cross-linking. Interestingly, all six tyrosine residues in the cytoplasmic domain are conserved in the human and murine homologues (Torres *et al.*, 1992).

First evidence for sialic acid-dependent binding came from a study (Stamenkovic *et al.*, 1991) reporting that CD22 interacts with CD45RO on T cells and CD75 on B cells, an epitope that depends on the expression of α2,6 sialyltransferase in these cells (Bast *et al.*, 1992; Munro *et al.*, 1992; Stamenkovic *et al.*, 1992). In addition, proteins isolated as ligands for CD22 contain α2,6-linked sialic acids on branched, *N*-linked oligosaccharides which are determinants for binding (Sgroi *et al.*, 1993; Powell *et al.*, 1993; see Section 6.6). Interestingly, in the α2,6 sialyltransferase gene a B-cell-specific promoter leads to cell-type-specific regulation of this enzyme during B-cell development (Wang *et al.*, 1993).

6.4. Sialoadhesin

Sialoadhesin is a receptor found on specific macrophage subpopulations in murine bone marrow, spleen, and lymph nodes (Crocker and Gordon, 1986). High expression of sialoadhesin is restricted to resident bone marrow macrophages in hematopoietic clusters, to marginal zone macrophages in spleen, and to macrophages in the subcapsular sinuses and medullary cord of lymph nodes (Crocker and Gordon, 1989; Crocker *et al.*, 1992). A striking distribution of sialoadhesin was observed in bone marrow on the ultrastructural level, where the receptor is highly enriched at contact sites between macrophages and developing myeloid cells (Crocker *et al.*, 1990). In contrast, no staining was observed at contact sites of the same macrophages to erythroblasts. Whereas most studies on sialoadhesin were done in mouse, the existence of a homologous protein in rat was demonstrated in spleen and lymph node macrophages (van den Berg *et al.*, 1992). In addition, reports on the specificity of a ganglioside binding activity on rat alveolar macrophages (Riedl *et al.*, 1982; Boltz-Nitulescu *et al.*, 1984; Förster *et al.*, 1986) point to the possibility that sialoadhesin may also be present in these macrophages, although murine alveolar macrophages express only low amounts of the receptor (Crocker and Gordon, 1989). Using glycoproteins and glycolipids as well as erythrocytes that, after sialidase treatment, were resialylated with purified sialyltransferases to contain only specific sialylated glycoconjugates, it was shown that sialoadhesin recognizes the sequence Neu5Acα2→3Galβ1→3GalNAc on glycoproteins and glycolipids on cell surfaces (Crocker *et al.*, 1991).

Possible functions for sialoadhesin have been implicated in the development of myeloid cells in the bone marrow and the trafficking of leukocytes in lymphatic organs (Crocker *et al.*, 1991, 1992; van den Berg *et al.*, 1992). Evidence

for this hypothesis comes from the distribution of the receptor in bone marrow (see above) and from cell binding experiments. In experiments with purified sialoadhesin and macrophages expressing the receptor, the receptor bound preferentially to inflammatory and circulating neutrophils (Crocker *et al.*, 1995). Whereas total bone marrow cells were still bound well, lymphocytes and monocytes showed intermediate binding, and binding to murine erythrocytes was barely detectable. In binding assays with frozen sections of spleen and lymph nodes, sialoadhesin can mediate the adhesion of lymphocytes and lymphoma cells (van den Berg *et al.*, 1992). In quantitative binding assays, activated T cells bind better than resting cells, and lowest binding in this cell lineage is to thymocytes. In contrast, activated B cells do not bind sialoadhesin better than resting B cells.

6.5. Other Mammalian Sialic Acid-Dependent Receptors

Besides the receptors described above, other proteins from mammalian sources capable of sialic acid recognition have been described in the literature (Table VI). However, their further characterization as sialic acid-dependent receptors is still needed, since their purification, specificity, or possible function has not as yet been described. Four examples should be mentioned here. (1) Factor H of the alternate complement pathway binds to sialic acid residues and other polyanionic molecules on "nonactivating" cell surfaces and has been inferred to facilitate access of the H protein to C3b on cell surfaces (Meri and Pangburn, 1990). However, to date no study on the specificity of this interaction has been described. (2) A ganglioside binding activity was characterized in membranes from myelin sheets with preferential binding to GD1b, GT1b, and GQ1b (Tiemeyer *et al.*, 1989, 1990), although no protein has yet been purified. (3) From human placenta, a protein was isolated with a specificity toward *O*-acetylated sialic acids (Ahmed and Gabius, 1989), which was shown to be an IgG (Zeng and Gabius, 1992b). (4) Finally, the potential role of a membrane-bound sialidase found in myelin, which binds GM1, should be mentioned here (Saito and Yu, 1993). This phenomenon is discussed in Chapter 8.

6.6. Ligands for Sialic Acid-Dependent Receptors

All receptors discussed above recognize specific carbohydrate structures containing terminal sialic acid residues. Whereas the restrictions toward these structures are more or less stringent depending on the receptor, different glycoconjugates can carry identical oligosaccharides and consequently are potentially bound by the same receptor. In addition, the same glycoprotein can carry different oligosaccharide structures ("microheterogeneity"), depending on the glycosylation machinery of the cell producing it. Since the oligosaccharide determines

whether a glycoconjugate can serve as ligand ("counterreceptor"), we could call the oligosaccharide "ligand determinant." However, the oligosaccharide in question will only be recognized if presented appropriately, emphasizing the importance of the carrier molecule. Another feature of carbohydrate recognition is that only a few functional groups of the oligosaccharide structure are required for binding, as has been shown in many examples of sugar–protein interactions. On some of these functions relatively major changes are tolerated, whereas others cannot be modified at all. Because of the large variability in naturally occurring oligosaccharide structures, sometimes related structures are recognized as well. One example is the similar potential of the isomers sLe^x and sLe^a to serve as ligand determinants for E- and P-selectin (see Sections 6.2.2 and 6.2.3, Table VII). Furthermore, E- and L-selectin bind to glycolipids containing structural analogues of sLe^x or sLe^a containing sulfate instead of sialic acid (Yuen *et al.*, 1992; Green *et al.*, 1992). Considering these aspects, we cannot expect precise receptor–ligand pairs involving simple kinetics in sialic acid-dependent interactions. Studies dealing with the "specificity" of these receptors have to take this into account, especially since experimental approaches often involve artificial presentation of the molecules investigated. One example is the TLC overlay technique, where the oligosaccharides may be presented in an unnatural way including high clustering of single molecules which might not occur on cell surfaces. Also the commonly used expression of the receptor in transfected cells leads to high levels of these molecules on the cell surface not always existing in the natural environment. Although these conditions might lead to the adhesion of cells not found naturally with sufficient expression levels of receptors, the observed specificity reflects the flexibility of protein–carbohydrate interaction, which also allows binding to low-affinity ligands, if the density is high enough. One such example is the binding of soluble P-selectin to HL-60 cells compared to CHO cells transfected with fucosyltransferase expressing sLe^x (Zhou *et al.*, 1991). Whereas on HL-60 cells P-selectin bound to a low number of high-affinity sites, on CHO cells P-selectin bound to a high number of low-affinity sites. Despite the obvious artificial situation in the CHO cells, we cannot rule out that such high number of low-affinity interactions may have biologically relevant roles.

Besides the description of rather diverse molecules that can be bound by selectins, specific glycoproteins binding to these receptors were recently isolated and characterized. Their structural features supply evidence for the importance of the presentation of ligand determinants. Ligands for L-selectin are GlyCAM1 (Lasky *et al.*, 1992), CD34 (Baumhueter *et al.*, 1993), and MAdCAM (Berg *et al.*, 1993), which all are highly glycosylated proteins with mucinlike structures carrying dense clusters of *O*-linked oligosaccharides (Chapter 5). The tissue-specific functionality of MAdCAM is an example of how the cell-specific oligosaccharide structures can function as ligand determinants (Berg *et al.*, 1993).

Although the final structure(s) of the ligand determinant(s) on the native ligand isolated from HEVs of lymph nodes has not been resolved, sialic acid and sulfate are structural components necessary for their biological function (Imai and Rosen, 1993; Imai *et al.*, 1993). The importance of the carrier molecule of ligand determinants was also demonstrated for a P-selectin ligand isolated from neutrophils (Norgard *et al.*, 1993b). Although this glycoprotein carries only a minor fraction of the sLe^x found on these cells, it was the only molecule in cell extracts binding to P-selectin. The primary sequence of a ligand protein cloned from HL-60 cells contains mucinlike elements, like the ligands for L-selectin, since it contains clusters of *O*-linked oligosaccharides required for binding activity (Sako *et al.*, 1993). Also for E-selectin, a specific ligand glycoprotein could be isolated from murine neutrophils and a myeloid cell line (Levinovitz *et al.*, 1993). However, its carbohydrate structure(s) serving as ligand determinant(s) have not been studied. In conclusion, although specific high-affinity ligands for selectins have been described, it is not clear whether only these or also low-affinity ligands are involved in adhesion events.

Considerable progress has been made in the determination of ligands for CD22 (Sgroi *et al.*, 1993; Powell *et al.*, 1993). CD22 requires sialic acids α2,6-bound to branched *N*-linked oligosaccharides as ligands. From B and T cells as well as from lymphoma cell lines, a number of surface glycoproteins could be isolated on CD22 columns, which is in contrast to the very limited number of ligands for selectins. The number and size of these glycoproteins were dependent on the type of cell used as source for CD22 ligands (Sgroi *et al.*, 1993), supplying evidence that for CD22 different cell-type-specific carrier proteins ("counter-receptors") probably with similar ligand determinants can exist. The fraction of *N*-linked oligosaccharides, released from purified ligands, which bound to a CD22 affinity column contained more α2,6-linked sialic acid residues than the oligosaccharides which were only retarded or not bound at all. In addition, a portion of the retarded fraction could be converted to a binding fraction by sialylation with α2,6 sialyltransferase (Powell *et al.*, 1993).

For sialoadhesin, no specific protein or glycolipid ligand has been isolated so far. However, in TLC overlay assays with glycolipid extracts from inflammatory neutrophils or bone marrow cells, sialoadhesin bound to specific ganglioside bands, whereas similar extracts on Western blots did not reveal glycoprotein ligands for sialoadhesin (Kelm *et al.*, 1993), although in principle it is possible to detect glycoprotein ligands for sialoadhesin by Western blotting, as was shown for erythrocyte membrane extracts (Crocker *et al.*, 1991). In addition, treatment of bone marrow cells or inflammatory neutrophils with proteases including *O*-sialoglycoprotease (Abdullah *et al.*, 1992) had little if any effect. Although this is not proof that glycolipids are the ligands on these cells, it supports such a possibility.

6.7. Modifications of Sialic Acids

O-Acetylation and *N*-acetyl-hydroxylation are modifications of sialic acids that are known to have an antirecognition effect on the binding of influenza A and B viruses (Higa *et al.*, 1985; Section 4.1). This is in contrast to the specific recognition of 9-*O*-acetylated sialic acids by influenza C virus (Rogers *et al.*, 1986; see Section 4.1 and Chapter 9).

Only a few studies are known that deal with the effect of sialic acid modifications on mammalian adhesion molecules. Shortening of the glycerol side chain of sialic acids by periodate/borohydrate treatment revealed that this moiety is not important for the binding of E-selectin (Tyrrell *et al.*, 1991), of P-selectin (Norgard *et al.*, 1993b), or of L-selectin (Norgard *et al.*, 1993a). In contrast, for the ligand determinant for CD22, the glycerol side chain seems to be an essential structural element (Sgroi *et al.*, 1993; Powell *et al.*, 1993). However, in none of these studies were naturally occurring sialic acid modifications used.

Experiments with various glycoconjugates and cells demonstrated that sialoadhesin only recognizes Neu5Ac and not Neu5Gc or Neu5,9Ac_2 (Kelm *et al.*, 1994). This leads to the following possible modulatory situation for sialic acid modifications. Cells expressing the ligand determinant for sialoadhesin could mask their ligands by acetylation. However, this mask could be removed by appropriate extracellular esterases thus again allowing interactions of ligands with sialoadhesin. In contrast, Neu5Gc can only be removed by sialidases. However, this would lead to the asialooligosaccharides, which are not ligand determinants for sialoadhesin. A cell expressing Neu5Gc on the surface could only interact with sialoadhesin after consumption of the intracellular pool of CMP-Neu5Gc and exchange of the cell surface sialic acids with Neu5Ac. This model represents an example of how modifications of sialic acid can modulate cellular interactions and how this is affected by their different metabolic pathways.

6.8. Perspectives

Since the characterization of the first sialic acid-dependent receptors at the beginning of this decade, new aspects regarding the role of sialic acids in cell recognition and cell–cell interaction can be investigated. The rather broad specificity toward different ligands (see Section 6.6) potentially allows an almost continuous spectrum of ligands with different affinities to occur on cell surfaces. Theoretically, this adds a new potential in regulating and fine-tuning cellular interactions not possible with highly specific receptor–ligand systems. Therefore, an important aspect of future research will be to examine whether these low-affinity ligands have a biological function (and how this is accomplished), or whether they are just side products of a glycosylation machinery necessary to

produce the high-affinity ligands. In addition, increasing knowledge on mechanisms like clustering of ligand determinants, e.g., on mucinlike molecules, leading to high-affinity ligands, will be helpful in the design of therapeutic drugs specific for certain receptor-mediated interactions. Examples are the increasing efforts to develop carbohydrate-based drugs as inhibitors for selectins for the treatment of undesired inflammatory reactions or for the prevention of cancer metastasis.

The discovery of the selectin family and their close localization on the genome in less than 300 kb raises the intriguing possibility that other gene families of sialic acid-dependent receptors also exist. For example, the gene for CD22 as a member of the Ig superfamily is also localized next to the most homologous genes. Future experiments are needed to clarify whether other members of this family of adhesion receptors recognize sialic acid-containing carbohydrates on their ligands.

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