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Review

Structure, function and metabolism of sialic acids

C. Traving and R. Schauer*

Biochemisches Institut, Christian-Albrechts-Universität, Olshausenstr. 40, D-24098 Kiel (Germany),
Fax +49 431 880 2238, e-mail: schauer@biochem.uni-kiel.de

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Abstract. Sialic acids represent a family of sugar molecules with an unusual and highly variable chemical structure that are found mostly in the terminal position of oligosaccharide chains on the surface of cells and molecules. These special features enable them to fulfil several important and even diametrical biological functions. Because of the great importance of sialic acids, it is also worth having a look at their metabolism in order to get an idea of the intimate connection between structure and function of these fascinating molecules and the often serious consequences that result from disturbances in the balance of metabolic reactions. The latter can be due to genetic disorders that result in the absence of certain enzyme activity, leading to severe illness or even to death.

Key words. Acylneuraminate lyase; cell recognition; CMP-*N*-acetylneuraminic acid hydroxylase; sialate-*O*-acetylesterase; sialate-*O*-acetyltransferase; sialic acid (neuraminic acid); sialidase; sialyltransferase.

Structure

Sialic acids comprise a family of about 40 derivatives of the nine-carbon sugar neuraminic acid [1–3]. Among its special structural features are the amino group at position 5 and the carboxyl group at position 1 that confers a negative charge on the molecule under physiological conditions and characterizes it as a strong organic acid (pK 2.2; fig. 1). The unsubstituted form, neuraminic acid, does not exist in nature. The amino group is usually acetylated, leading to *N*-acetylneuraminic acid (Neu5Ac), the most widespread form of sialic acid. Substituting one of the hydrogen atoms in the methyl moiety of the acetyl group by a hydroxyl group results in *N*-glycolylneuraminic acid (Neu5Gc), which is common in many animal species, best investigated in porcine tissues, but has been found in humans only in the case of particular cancer [2]. Another significant variation on the theme is the esterification of the hydroxyl groups mainly at positions 7, 8 and 9 with acetic acid, whereas this so-called *O*-acetylation is more rarely found at position 4, for example in horses [4]. A number of other modifications of the sialic acid molecule are known, including introduction of a lactoyl group at position 9 and of a sulfate or a methyl group at position 8, the latter of which has been found in gangliosides from the starfish *Asterias rubens* [2, 3]. Sialic acid molecules can be substituted in more than one position, for example in 7,8,9-tri-*O*-acetyl-*N*-acetyl- or *N*-glycolylneuraminic acid [1, 3]. Finally, an unusual modification should be mentioned in which there is an additional hydroxyl group present instead of the amino function at position 5 of the sugar, leading to 2-keto-3-deoxy-nonulosonic acid (Kdn). This component has been found in sperm and eggs of teleost fish [3]. There

* Corresponding author.

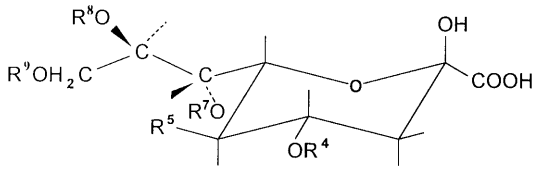
exists a specific pattern of sialic acid derivatives depending on the tissue and the developmental stage of each individual species [3].

The variability of sialic acids is further extended by their location on cells and molecules. They usually represent the terminal sugar residue of a glycan chain [5, 6]; that is, they are linked via C2 to position 3 or 6 of the penultimate sugar or to position 8 of another sialic acid molecule, respectively. In the latter case, which occurs in bacterial capsules and gangliosides of higher animals, the sialic acid molecules are located internally in a glycan chain. However, the most common subterminal sugar residues are galactose and *N*-acetylgalactosamine (GalNAc) [5]. The sialylated glycan chain can itself be part of a glycolipid or glycoprotein molecule or represent an oligo- or polysaccharide.

Occurrence

Sialic acids have been found in the animal kingdom, from the echinoderms upwards to humans [7], whereas there is no hint of their existence in lower animals of the protostomate lineage or in plants. The only known exception is the occurrence of polysialic acid in larvae of the insect *Drosophila* [8]. In addition, there are sialic acids in some protozoa, viruses and bacteria [1, 2, 9–11]. Thus, several strains of *Escherichia coli* contain long saccharide stretches consisting of up to 200 sialic acid molecules, the so-called colominic acid. Sialoglycoconjugates are present on cell surfaces as well as in intracellular membranes (e.g., of the Golgi apparatus). In higher animals they are also important components of the serum and of mucous substances.

The irregular, distribution of sialic acids in nature raises the question, 'Why is there a lack of this sugar in lower eucaryotes and where exactly does it originate in evolution?'



R^5	$R^{4,7,8,9}$	
$-NH-C(=O)-CH_3$	$-H$	(4,7,8,9)
$-NH-C(=O)-CH_2(OH)$	$-C(=O)-CH_3$	(4,7,8,9)
$-NH-C(=O)-CH_2-O-C(=O)-CH_3$	$-C(=O)-CH(OH)-CH_3$	(9)
	$-CH_3$	(8)
$-OH$	$-SO_3H$	(8)
	$-PO_3H_2$	(9)

Figure 1. Chemical structure of the sialic acid molecule and a list of natural substituents, the positions of which are indicated in brackets (modified from [4]). Most sialic acids are derivatives of neuraminic acid, e.g. *N*-acetylneuraminic acid (Neu5Ac) or *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac_2). Desaminoneuraminic acid is 2-keto-3-desoxy-nonulosonic acid (Kdn). For the nomenclature and abbreviations of all natural sialic acids known see [1].

Function

The structural diversity of sialic acid is reflected in the variety of its biological functions [2, 5, 6, 12]. Due to its size and the hydrophilic character, along with its negative charge, this sugar can have a simple physicochemical effect on its environment. Its main function, however, is that of specific phenomena related to cellular and molecular recognition.

Due to their negative charge, sialic acids are involved in binding and transport of positively charged molecules (e.g. Ca^{2+}) as well as in attraction and repulsion phenomena between cells and molecules. Their exposed terminal position in carbohydrate chains, in addition to their size and negative charge enable them to function as a protective shield for the subterminal part of the molecule (preventing e.g. glycoproteins from being degraded by proteases) or cell (as is the case for the mucous layer of the respiratory epithelium). In infectious processes, the colonization of bacteria can be limited by the sialic acid coat covering the host cell surface. Another interesting phenomenon is the spreading effect that is exerted on sialic acid-containing molecules due to the repulsive forces acting between their negative charges [13]. This stabilizes the correct conformation of enzyme or cell membrane (glyco) proteins, for example, and is important for the slimy character and the resulting gliding and protective function of mucous substances, such as on the surface of the eye or on mucous epithelia [14].

Sialic acids take part in a variety of recognition processes between cells and molecules. Thus, the immune system can distinguish between self and nonself structures according to their sialic acid pattern. The sugar represents an antigenic determinant, for example of blood group substances, and is a necessary component of receptors for many endogenous substances such as

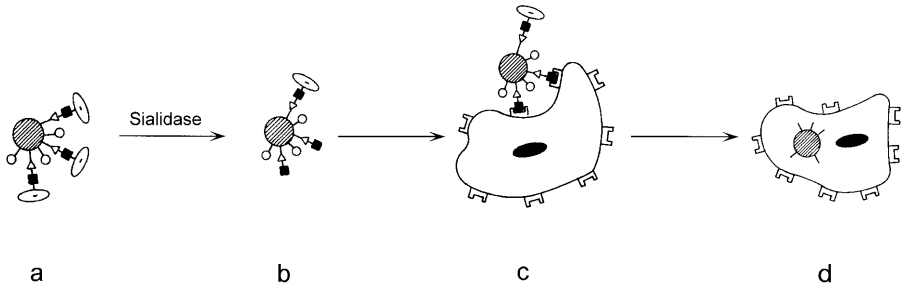


Figure 2. The masking function of sialic acids: mechanism of binding (*b*) and phagocytosis (*c*) of sialidase-treated erythrocytes (*a*) by macrophages. Sialylation enables these cells a long lifetime in circulation [6, 16a, 17]. ▭, negatively charged sialic acid; ■, galactose residue; ○, other membrane components; ⌐¬, galactose-specific lectin on the macrophage surface. Reprinted with permission from: Schauer R., Shukla A. K., Schröder C. and Müller E. (1984) The anti-recognition function of sialic acids: studies with erythrocytes and macrophages. Pure Appl. Chem. **56:** 907–921, © 1998 CRC Press, Boca Raton.

hormones and cytokines. In addition, many pathogenic agents such as toxins (e.g. cholera toxin), viruses (e.g. influenza), bacteria (e.g. *Escherichia coli*, *Helicobacter pylori*) and protozoa (e.g. *Trypanosoma cruzi*) also bind to host cells via sialic acid-containing receptors [1].

Another important group of sialic acid-recognizing molecules belongs to the so-called lectins. They are usually oligomeric glycoproteins from plants, animals and invertebrates that bind to specific sugar residues. Very common sialic acid-binding lectins are wheat germ agglutinin (WGA), *Limulus polyphemus* agglutinin (LPA), *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA), which are specific for α-2,6-linked and α-2,3-linked sialic acids. Plants do not have sialic acids. Therefore, these lectins seem not to have a function for their own metabolism, but might be helpful in the defense against sialic acid-containing microorganisms or plant-eating mammals. There also exist mammalian counterparts of lectins. These include selectins as well as members of the sialoadhesin family ('siglecs' [15]) such as sialoadhesin (Sn), CD22, CD33, myelin-associated glycoprotein (MAG) and Schwann cell myelin protein (SMP) [16]. Selectin and siglec molecules consist of several domains, one of which is responsible for sugar binding. Selectins play an important role in the initial stage of adhesion of white blood cells to endothelia, the so-called rolling of the cells, which may be followed by evasion of the leucocytes into the tissue. They are located on endothelial cells and recognize sialic acids in the sialyl Lewis $(Le)^x$ and sialyl Le^a structure on the surface of leucocytes. Because these molecules are also present on tumor cells, selectins might be involved in tumor metastasis. CD22 is expressed in B cells. Belonging like the selectins to the type I membrane glycoproteins, it is a member of the immunoglobulin superfamily. It mediates binding of B cells to other B or T cells, neutrophiles, monocytes or erythrocytes. The ligand of CD22 is sialic acid bound in α-2,6 linkage to branched N-linked oligosaccharides. Sialoadhesin was found on macrophages from murine bone marrow, spleen and lymph nodes and is thought to be important for the development of myeloid cells in the bone marrow and trafficking of leucocytes in lymphatic tissues.

Binding between these receptors and their ligands depends on the presence of only a few functional groups of the oligosaccharide. Generally, the receptors have a broad specificity with regard to possible ligands. Thus, they bind as well to low-affinity ligands that are presented in high density on a cell surface as to a low number of high-affinity ligands. This is an important feature, as it enables better fine tuning of cell-cell interactions as required for very specific recognition processes.

Another important feature of sialic acids that seems to be in direct contrast to their recognition function is the masking of cells and molecules (fig. 2). Erythrocytes are covered by a dense layer of sialic acid molecules. The normal life span of these red blood cells is around 120 days. During this time, sialic acids are removed stepwise from the surface of the cells by the action of serum sialidase and by spontaneous chemical hydrolysis. The penultimate galactose residues that represent signals for degradation of the respective blood cells then become apparent. Finally, the unmasked erythrocytes are bound to macrophages and phagocytosed [17]. Thus, sialic acids prevent erythrocytes from being degraded, because they mask the subterminal galactose residues. The same mechanism works on other blood cells (thrombocytes and leucocytes) and on various serum glycoproteins, which are bound by hepatocytes after exposure of subterminal galactose residues. However, masking of endogenous structures can also have a detrimental effect,

as can be seen in the case of some tumors that are sialylated to a much higher degree than the corresponding normal tissues. Consequently, the transformed (i.e. foreign) cells are 'invisible' to the immune defense system. The higher degree of sialylation has an immunosuppressive effect and corresponds to higher sialyltransferase activities often seen in tumor tissues. Thus, terminal galactose residues that would otherwise inhibit further cell growth and spreading are masked. This might be one reason for the loss of contact inhibition of cancer cells [18]. The masking effect of sialic acids also helps to hide antigenic sites on parasite cells from the host immune system. This is the case for microbial species like certain *E. coli* strains and gonococci (*Neisseria gonorrhoeae* [19]).

Sialic acid derivatives modulate the function of the respective glycoconjugate they are linked to. This will be discussed in the following sections of this review in the context of the enzymes that catalyze the corresponding modification of the parent sialic acid molecule.

Metabolism

Because of the important biological functions of sialic acid, we should also pay some attention to the metabolism of this sugar, which is rather complex and reveals some interesting insights into the connection between structure and function of a given sialic acid derivative and the narrow borderline between health and severe illness that can depend on the presence or absence of a certain enzyme during sialic acid metabolism.

The reactions of sialic acid synthesis and degradation are distributed among different compartments of the cell (fig. 3). The sugar is synthesized from *N*-acetylmannosamine-6-phosphate and phospoenolpyruvate in the cytosol. After dephosphorylation of the reaction product, Neu5Ac-9-phosphate, the molecule is activated in the nucleus by the transfer of a cytidine monophosphate (CMP) residue from cytidine triphosphate (CTP) through CMP-Neu5Ac synthase. This sugar nucleotide is the only natural case of a β linkage between sialic acid and another compound, because in glycoconjugates it is always α. CMP-Neu5Ac is then translocated into the Golgi apparatus or the endoplasmic reticulum [4, 12]. There, the activated sialic acid can be transferred by a sialyltransferase onto an appropriate acceptor molecule, that is the oligosaccharide chain of a nascent glycoconjugate. The bound sialic acid can then be modified by *O*-acetylation or *O*-methylation before transport of the mature glycoconjugate to the cell surface, whereas the only modification that takes place before the transfer onto the glycoconjugate is the hydroxylation of the *N*-acetyl group of CMP-Neu5Ac, which leads to CMP-Neu5Gc in the cytosol [2].

The key enzyme of sialic acid catabolism is sialidase. Sialic acid residues can be removed from cell surface or serum sialoglycoconjugates by membrane-bound sialidases. Usually, the glycoconjugates that are prone to degradation are taken up by receptor-mediated endocytosis in higher animals. After fusion of the endosome with a lysosome, the terminal sialic acid residues are removed by lysosomal sialidases. In contrast, the corresponding microbial enzymes are mostly secreted to get into contact with their substrate in the environment (see below). A prerequisite for the effective action of sialidases is the removal of *O*-acetyl groups by sialate-*O*-acetyl esterases [1, 2], whereas bound Neu5Gc is a fairly good substrate. Free sialic acid molecules (Neu5Ac or Neu5Gc) are transported through the lysosomal membrane into the cytosol, from where they can be recycled by activation and transfer onto another nascent glycoconjugate molecule in the Golgi. Alternatively, they are degraded to acylmannosamine and pyruvate with the aid of a cytosolic acylneuraminate lyase. The latter enzyme is also produced by many bacterial species.

In the following sections more information is given about some of the enzymes that are involved in sialic acid metabolism.

Sialyltransferase (EC 2.4.99.1)

This enzyme catalyzes the transfer of CMP-activated sialic acid molecules onto an acceptor substrate in the trans-Golgi network after their transport through the Golgi membrane. Meanwhile, the sialyltransferase primary structures of many mammalian tissues and from some bacterial species are known, and today there exists a family of at least 15 members creating $\alpha 2 \rightarrow 3$, $\alpha 2 \rightarrow 6$, $\alpha 2 \rightarrow 8$ and $\alpha 2 \rightarrow 9$ linkages between Neu5Ac and the accepting sugar [1, 20]. A stretch of about 50 amino acids is conserved in sialyltransferases, the 'sialyl motif' that is thought to be involved in substrate binding. Generally, these enzyme proteins consist of an N-terminal cytoplasmic domain, a signal membrane anchor domain, a stem region and the large C-terminal catalytic domain. The acceptor specificity of these enzymes is very high. Thus, in rat liver, OH6 of β-Gal and the *N*-acetyl group of GlcNAc are required for activity of the $\alpha 2 \rightarrow 6$ sialyltransferase. The $\alpha 2 \rightarrow 3$ sialyltransferase needs HO3, HO4 and HO6 of β-Gal, and even subterminal sugars as well as the polypeptide or lipid part of an acceptor molecule have an influence. The donor specificity is less pronounced, for not only Neu5Ac but also several derivatives can be transferred. The enzyme activity might be regulated by Ca^{2+}/calmodulin and phosphate, and is much increased and varied in their linkage specificity in many tumors, leading to a higher degree and different mode of sialylation. The design of

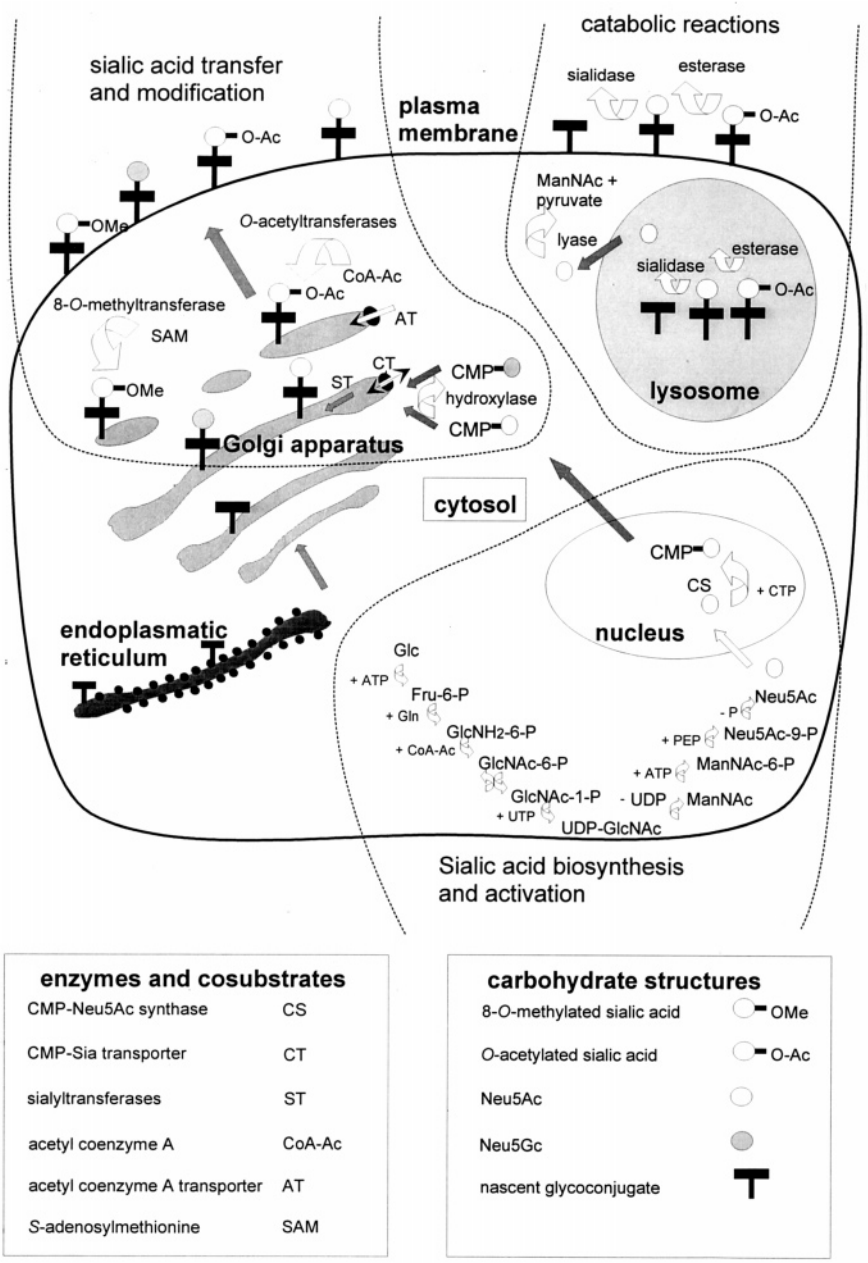


Figure 3. Metabolism of sialic acids (modified from [12]). The enzymatic reactions involved in sialic acid biosynthesis, activation, transfer, modification and catabolism are shown with their intracellular localization.

sialyltransferase inhibitors is of great interest for the study of the modulation of sialylation, for example in tumors, and much effort has been made in finding synthetic inhibitory substances derived from donor and acceptor molecules in sialyltransferase reactions. Natural inhibitors have also been found that are probably involved in the regulation of sialoglycoconjugate synthesis, for example heparine and special proteins from rat and calf brain (reviewed in [1]). The most promising strategy for the inhibition of sialylation, which may also have an impact in cancer treatment, is the synthesis of CMP-Neu5Ac analogs [21, 22].

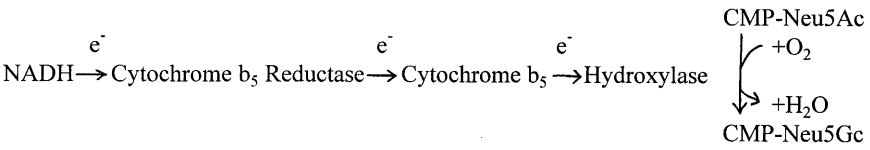


Figure 4. The biosynthesis of Neu5Gc. Electron-transferring components involved in the CMP-Neu5Ac hydroxylase reaction.

CMP-*N*-acetylneuraminic acid hydroxylase (EC 1.14.99.18)

This enzyme catalyzes the reaction by which Neu5Gc is formed from CMP-Neu5Ac. This sialic acid derivative is common in higher animals from the echinoderms to mammals [1, 2, 23]. In these species, a higher Neu5Gc level is found in adult animals than in juvenile ones. So the sugar occurs in many adult murine tissues [24]. It is, however, absent from humans, except in certain tumors [4]. Thus the expression of this modification seems to be dependent on the physiological differentiation and malignant transformation of the tissue [4]. The presence of Neu5Gc in human tumors can be traced by specific antibodies. The corresponding Neu5Gc-presenting antigens on malignant cells are called Hanganutziu-Deicher antigens. The amount of Neu5Gc in tumors is very low. It is conceivable that the otherwise dormant or repressed CMP-Neu5Ac hydroxylase gene is reexpressed in this state [2].

Neu5Gc is cleaved by sialidases and acylneuraminate lyases at a slower rate than Neu5Ac [2]. Therefore, this modification extends the life span of the corresponding glycoconjugates. On the other hand, the damaging effect that is exerted by sialidases from pathogenic microorganisms on the host tissue is also reduced by the presence of Neu5Gc on the host cell surface [2]. Furthermore, the interaction of sialic acid-specific receptors with Neu5Gc is often weaker than with Neu5Ac [12]. However, there also exist specific receptors for Neu5Gc. The only known pathogen that is able to bind to Neu5Gc-containing ligands is *E. coli* K99 [2]. Because Neu5Ac and Neu5Gc are transferred onto glycoconjugates with equal efficiency by sialyltransferases, changing the activity of the hydroxylase represents the only possibility for regulating the proportion of Neu5Gc in the sialic acid pool of the cell [2].

The CMP-Neu5Ac hydroxylase is a multicomponent system (fig. 4; [2]). At first, there are reducing equivalents required, which are provided by nicotinamide adenine dinucleotide (phosphate) (reduced form) (NAD(P)H). From there, the electrons are transferred onto cytochrome b_5 by NAD(P)H cytochrome b_5 reductase. The hydroxyl-ase protein itself has binding sites for cytochrome b_5 and for the substrate CMP-Neu5Ac. Another stretch of amino acids has also been found that are characteristic for Rieske iron-sulphur proteins [25]. These conserved motifs have been identified in the primary structures of this enzyme known to date from pig submandibular gland and mouse liver [25, 26]. Corresponding areas have been shown to be conserved in several other proteins from chloroplasts, mitochondria and microorganisms, whereas one oxygen of the O_2 molecule is introduced into CMP-Neu5Ac, yielding CMP-Neu5Gc, the second oxygen is reduced to water. Thus the hydroxylase is in fact a monooxygenase. The two mammalian CMP-Neu5Ac hydroxylases studied are soluble enzymes, which corresponds to the cytosolic location of the hydroxylase substrate [24] and is interesting because many related enzyme systems like the cytochrome P450-dependent hydroxylases are membrane-bound. It is believed that cytochrome b_5 and NAD(P)H cytochrome b_5 reductase are bound to the membrane of the endoplasmic reticulum in mammals, whereas the hydroxylase itself is located in the cytosol [27]. In starfish, there seems to exist a tighter association of the system components since the CMP-Neu5Ac hydroxylase is found bound to subcellular membranes [28]. The significance of this intimate contact between them can be deduced from the inhibitory effect of increasing ionic strength or dilution on the hydroxylase activity, which might inhibit ionic interactions between the components. The hydroxylase enzyme proteins from mouse liver and pig submandibular gland have been extensively investigated. They are monomers with a molecular weight of 65 kDa [25] and reveal a very high substrate affinity and also a pronounced substrate specificity [2]. The CMP-Neu5Ac hydroxylase has a broad pH optimum between pH 6.4 and 7.4 [24].

Sialate-8-*O*-methyltransferase (proposed EC 2.1.1.78)

This enzyme transfers a methyl group from *S*-adenosylmethionine onto position 8 of sialic acids, either free or bound to glycoproteins and gangliosides. These sialic acid derivatives have so far only been found in echinoderms [3]. In *Asterias rubens*, the 8-*O*-methyltransferase is membrane-associated. It has been solubilized and

purified 22,000-fold [29]. The molecular weight is 58–62 kDa, and the temperature optimum is 37 °C. The enzyme is optimally active between pH 7.4 and 8.3 with no obvious maximum. 8-*O*-Methylated sialic acids might represent a stop signal for oligosaccharide chain elongation in echinodermal glycoconjugates or may guide the branching of oligosaccharide chains frequently found in echinoderms [2, 23].

Sialate-4-*O*-acetyltransferase (EC 2.3.1.44) and sialate-7(9)-*O*-acetyltransferase (EC 2.3.1.45)

These enzymes are involved in creating some of the most important sialic acid derivatives, in which one or more of the hydroxyl groups in positions 4, 7, 8 and 9 are substituted by acetyl groups. These acetylesters are alkali labile, which needs to be considered during isolation of these derivatives. Procedures with the least saponification are performed between pH 4 and 6. As already mentioned for Neu5Gc, the occurrence of *O*-acetylated sialic acids is tissue-specific and developmentally regulated [3]. Thus, expression in human and rat colon strongly increases after birth, but is on the other hand quite variable, depending on bacterial products and environmental stimuli [30].

A broad spectrum of *O*-acetylated sialic acids has been found in bovine submandibular gland with most of the sialic acid being *O*-acetylated [31], as well as in human colon mucosa. *O*-Acetylated sialic acids are also present in gangliosides. The level of such sialic acids in colon cancer is lower than in normal tissue [32, 33], which is in contrast to melanoma cells [2] and in basalioma [34], which exhibit more *O*-acetylated sialic acids than normal skin. There is also a large amount of them present on red blood cells from rat, mouse and rabbit as well as in the blood plasma from donkeys [35]. The occurrence of 4-*O*-acetylated compounds, however, has been reported for horse, donkey, guinea pig and the Australian monotreme *Echidna*, for example [23].

O-Acetylation influences many properties of the sialic acid molecule (for reviews see [1, 3, 5, 6, 23]), such as its size and net charge, which change the conformation of gangliosides, among others [36]. Furthermore, the shape of the molecule is altered, and hydrogen bonds are formed in another way, resulting in a less hydrophilic character. The consequence is the modulation of the biological function of the respective sialic acid derivative. *O*-Acetylation influences the adaptation of molecules or cells at their environment, for example the antigenicity of bacterial polysaccharides and complement activation by human erythrocytes. *O*-Acetylated derivatives can either inhibit or enable the recognition of epitopes by antibodies and represent differentiation or tumor antigens. The main sialoglycosphingolipid of melanoma cells is disialoganglioside (GD3) [3], which is not restricted to this type of cancer. This is, however, the case for the 9-*O*-acetylated derivative of this glycoconjugate, which therefore represents a tumor-associated antigen in melanoma as well as in basalioma [34]. Furthermore, *O*-acetylated sialic acids are important for the morphogenesis and development of the retina, cerebellum and adrenal gland, and for the organization and function of neural tissues. The expression of sialate-*O*-acetylesterase from influenza C virus in some tissues of transgenic mice revealed the significance of *O*-acetylation for cell contact and organization of developing tissues in the embryo [37]. Esterified sialic acids protect the colon against certain microorganisms [38] and prolong the life span of cells and molecules, because they cannot be degraded by sialidase as fast and as easily as nonacetylated sialic acids. *O*-Acetylation of murine red blood cells confers resistance to malaria parasites. Also, the binding of several pathogenic viruses, such as influenza A and B, is inhibited by *O*-acetylation of the host cell surface. On the other hand, binding of influenza C and other viruses is in fact dependent on the presence of 5-*N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac_2) [12, 39]. Due to the more pronounced masking effect of *O*-acetylated sialic acids, they support the biological function of the corresponding structures. These assumptions are based upon experiments by Varki et al. [37], who observed that mutations concerning *O*-acetylated sialic acids are rare in animals.

At least two enzymes introduce *O*-acetyl groups into sialic acids: sialate-4-*O*-acetyltransferase is being investigated in equine submandibular gland and in guinea pig liver, whereas sialate-7(9)-*O*-acetyltransferase was studied in rat liver Golgi apparatus and enriched from bovine submandibular gland, as summarized in [1–4, 40, 41]. All these *O*-acetyltransferases are Golgi membrane-bound enzymes which modify glycosidically bound sialic acids.

4-*O*-Acetyltransferase was solubilized from this subcellular site of guinea-pig liver and found to *O*-acetylate, with the aid of acetyl coenzyme A, a great variety of substrates, both free and glycoconjugate-bound sialic acid (M. Iwersen et al., unpublished). The enzyme, however, is absolutely specific with regard to the insertion site of the *O*-acetyl group at C-4. In contrast, the exact specificity of the bovine and rat 7(9)-*O*-acetyltransferase or the possible existence of various enzymes is not yet known. Although it can incorporate ester groups only into the glycerol side chain of sialic acids, the hypothesis that this can happen only and primarily at C-7 has not yet unequivocally been confirmed. Evidence for this mechanism at least in bovine submandibular gland, however, is accumulating [1, 3, 42], as after short-term incubation of Golgi-enriched membranes from this tissue only 7-*O*-acetylated Neu5Ac was found. From this

Table 1. Properties of the sialate-9(4)-*O*-acetylesterases from horse liver, bovine brain and influenza C virus [adapted from ref. 48].

Properties	Horse liver	Bovine brain	Influenza C virus
Size (kDa)	56.5	56.5	88
Subunits	–	–	2, à 30 and 65 kDa
pH optimum	8.5	7.4	7.8
K_m (mM)			
4-MU-acetate	n.d.	0.17	0.13
Neu5,9Ac_2	1.1	18	0.8
V_{max} (U × mg protein^{-1})			
4-MU-acetate	n.d.	0.55	11
Neu5,9Ac_2	2	6.9	7.4

n.d., not determined; MU, methylmbelliferyl. Reprinted with permission from: Schauer R., Reuter G. and Stoll S. (1988) Sialate-*O*-acetylesterases: key enzymes in sialic acid catabolism. Biochimie **70:** 1511–1519, © 1998 Elsevier, Paris.

position the *O*-acetyl group migrates to C-9, probably via C-8, where as a primary ester group it is more stable. Then, according to a hypothesis by Schauer [40], further *O*-acetyl residues may be transferred to the free OH at C-7 by the 7-*O*-acetyltransferase, until the whole side chain is *O*-acetylated. Such oligo-*O*-acetylated sialic acids are frequently found in bovine submandibular gland and human colon mucins. If this theory is correct, a second *O*-acetyltransferase, which may be specific for the hydroxyl at C-9, for example, would not be necessary. The isomerization from Neu5,7Ac_2 to Neu5,9Ac_2 is possible by a spontaneous, nonenzymatic migration of the *O*-acetyl groups as shown by Kamerling et al. [43]. However, according to recent experiments [42] it is likely that this isomerization is an enzymatic process, since in Golgi membranes it occurs more rapidly than a mere physical mechanism would under the experimental conditions, and it can be much delayed by the application of enzyme inhibitors. It was shown by [44] in rat liver that sialic acid *O*-acetylation is not a simple process but is linked to an acetyl coenzyme A transporter that transfers the coenzyme from the cytosol to the Golgi compartment. Many attempts to clone *O*-acetyltransferases have so far failed, although transfection of various transcripts increased *O*-acetylation of sialic acids in cultured cells [45, 46]. Thus, efforts should be continued to purify these enzymes as a promising strategy to reach this goal. In contrast to the enzyme itself, the cloning of the acetyl coenzyme A transporter has been reported [47].

Sialate-*O*-acetylesterases (EC 3.1.1.53)

When it became known that the glycosidic linkage of sialic acids can be better hydrolyzed by sialidases after the removal of *O*-acetyl groups, the existence of an enzyme was postulated as a 'missing link' in sialic acid catabolism, which hydrolyzes the ester linkage between the acetyl and the sialic acid hydroxyl groups. The enzymes able to catalyze this reaction were identified – the so-called sialate-*O*-acetylesterases [48]. They occur in viruses, bacteria, and in humans and other vertebrates [1–3]. Generally, they remove 9-*O*-acetyl groups from free or bound sialic acid, whereas the equine enzyme additionally acts on 4-*O*-acetyl residues. Because 7-*O*-acetyl groups are able to migrate nonenzymatically to position 9, they could be also cleaved by these enzymes. A common feature of sialate-*O*-acetylesterases is a serine residue in the active center. With respect to the substrate, the carboxyl group seems to be essential for the reaction. Furthermore, an arginine appears to be present, which is a common feature of enzymes that act upon anionic substrates [49].

Sialate-*O*-acetylesterases have been studied in detail in horse liver [48] and rat liver [50], in bovine brain and in influenza C virus [3, 39, 48, 51, 52]. The enzymatic properties of three of these enzymes are presented in table 1, whereas the substrate specificity is described in detail in [48]. The best substrates are 9-*O*-acetylated sialic acids, but acetyl groups in position 4 of the sialic acid can also be hydrolyzed to different degrees. There exist multiple forms of this enzyme in horse liver that differ in their pI and substrate specificity [48, 49]. Furthermore, the ring conformation of the substrate is more critical for the rate of cleavage than the position of acetyl groups in the side chain [48]. In mammalian systems two kinds of sialate-*O*-acetylesterases have been studied, a cytosolic enzyme that acts on free sialic acid molecules, whereas another enzyme is a water-soluble glycoprotein with complex and high-mannose-type N-linked sugar chains [3]. Another interesting example is the enzyme from influenza C virus, which is part of the heterotrimeric HEF glycoprotein of the virus capsid [53]. This glycoprotein consists of a hemagglutinin specific for the virus receptor Neu5,9Ac_2, the 9-*O*-acetylesterase removing the 9-*O*-acetyl groups and thereby destroying the receptor, and a fusion protein that enables the uptake of the virus particle into the cell. The

primary structures of several HEFs are known. Corresponding enzymes have also been described for other viruses [54, 55]. The viral esterases are important for the release of the viral progeny, because as they exit the cell, the virus particles would become stuck immediately to 9-*O*-acetylated sialic acids on the cell surface via their hemagglutinin were the receptor not destroyed before by the esterase.

The specificity of the esterase from influenza C virus was used to develop a very sensitive test for the detection of as little as 65 fmol of 9-*O*-acetylated sialic acids in glycoconjugates [56]. At first, the biological sample is incubated with the virus at 4 °C. At this temperature, the viral hemagglutinin binds to putative substrate molecules, while the esterase is still inactive. In a second step, the temperature is raised to 37 °C for enzyme activation, and the synthetic substrate α-naphthyl acetate is added, which is cleaved to a significantly higher degree by the esterase than natural substrates. Further steps result in the production of a colored product. This virus test can be used for sialic acid detection on thin-layer chromatograms, nitrocellulose membranes, gels and microtiter plates. The bound enzyme can also be visualized by antiviral antibodies. This technique was used for histochemical staining of O-acetylated sialic acids by Harms et al. [57]. Alternatively, Klein et al. [58] developed an assay using chimeras from the influenza C virus HE portion and the F_c portion of human immunoglobulin (Ig)G, which enables the detection of esterified sialic acids in tissue sections and also on living cells in a very sensitive manner.

Sialidases (EC 3.2.1.18)

Sialidases have been called 'neuraminidases' or RDEs (receptor-destroying enzymes, because they were first described for viruses). They are the key enzymes of sialic acid catabolism, hydrolyzing the glycosidic linkage between sialic acid molecules and the penultimate sugar of the carbohydrate chains of oligosaccharides and glycoconjugates. The free sialic acid molecules can then be further degraded by acylneuraminate lyases (see below) or retransferred onto glycoconjugates by sialyltransferases after activation to CMP-Neu5Ac (see fig. 3 and the text above). The enzymes occur in higher animals of the deuterostomate lineage that possess the corresponding sialic acid-containing substrates as components of an autonomous sialic acid metabolism. However, they have also been found in a variety of microorganisms, such as viruses, bacteria and protozoa [13, 59]. Some of the latter organisms do not contain or produce sialic acid themselves. This raises the question, 'What is the biological function of sialidases in these microbial species?' Interestingly, they often live in close contact with higher animals as hosts, for example as parasites. Here, they may have a nutritional function, enabling their owners to scavenge host sialic acids for use as a carbon and energy source (see below for comparison with trans-sialidases). For some microbial pathogens, sialidases are believed to act as virulence factors, allowing successful competition with the host by alleviating their spread in host tissue [60]. The Gram-positive anaerobic species *Clostridium perfringens* causes gas gangrene [61]. This severe or even fatal disease develops when deep wounds are becoming increasingly anoxious due to damage to the surrounding blood vessels and are contaminated with these bacteria or their spores. In the absence of oxygen, the bacteria begin to divide rapidly. During propagation they excrete a cocktail of enzymes with different substrate specificities, that is proteases, hyaluronidases, collagenases, phospholipases, lecithinases and also a sialidase, which degrade a variety of host structures. Sialidases play a crucial role in this scenario, as they remove the first line of host defense by cleaving the terminal sialic acid residues. Thus, subterminal molecules including proteins become easily accessible. Multiplication and spread of the bacteria in the tissues can be very rapid (10 cm per hour). A possible role for sialidases is that of spreading factors which facilitate the propagation of bacteria and their invasion of host tissue [38, 62]. Another effect of sialidase overflow is anemia: red blood cells are covered by a dense coat of sialic acid molecules that is removed by this dysregulated sialidase action. As a consequence, the galactose residues are demasked on the blood cell surface after removal of sialic acids, presenting a signal for degradation by liver hepatocytes ([6]; see fig. 2). Due to the resulting lack of negative charge on the erythrocyte surface, the cells tend to aggregate, which leads to thrombosis [13, 63]. Additionally, enzymes, hormones and serum glycoproteins are inactivated by high sialidase levels [13, 64]. Another possible role of sialidases from parasites is the demasking of subterminal host cell structures, which then serve as receptors for the parasites and toxins, as in the case of cholera [13, 65]. The pathophysiological significance of sialidases can also be seen from infection of humans with influenza A or B. Here, the sialidase function is analogous to that of *O*-acetylesterase in influenza C virus (see above) and thus represents another 'receptor-destroying enzyme'. The sialidase enables release of the viral progeny, and by cleavage of host sialic acids leads to impairment of the viscosity of the protective mucous layer of the upper respiratory apparatus.

Nevertheless, the role of sialidases as factors in pathogenesis is controversial. On the one hand, they confirm the impact of pathogenic microbial species like *Clostridium perfringens*. On the other hand, these enzymes are factors common in the carbohydrate catabolism of

many nonpathogenic species, including higher animals. They do not, however, exert a direct toxic effect. Instead, their detrimental effect depends on the massive amount of enzyme that is released [63] into the host together with other toxic factors upon induction [13] by host sialic acids under nonphysiological conditions, which leads to a variety of cell damage. In summary, sialidases are common enzymes in sugar catabolism that become dangerous only when they improve the parasitic or pathogenic potential of a microorganism with respect to its environment. Also for these species, however, the enzyme has an additional nutritional function.

A special sialidase subgroup is represented by the trans-sialidases, which combine the features of sialidases and sialyltransferases. These enzymes preferably transfer the sialic acid molecule directly from one glycosidic linkage onto another sugar residue instead of a water molecule. The most common 'alternative' acceptor molecule is β-galactose, as a terminal component of oligosaccharides and glycoproteins. The involvement of a sialidase-like enzyme in sialylation has so far only been found in some species of the genus *Trypanosoma* [66–68]. The causative agents of several very important infectious diseases belong to these flagellated protozoa. Trypanosomes are divided into African and Central South American species and reveal a complex life cycle, during which they are transmitted from mammalian to insect hosts and back again. The different stages in this life cycle are accompanied by significant changes in the morphology and physiology of the parasites. The American species *Trypanosoma cruzi* causes Chagas disease in humans [69], for which vectors are insects of the genus *Rhodnius* and *Triatoma*, respectively. In this case, the parasites penetrate the host cell membranes, for example of the heart muscle, thus evading the immune system, before intracellular propagation starts. In the African species *T. brucei*, infection of humans and domestic animals occurs via a sting of the Tse-Tse fly *Glossina* sp. *T. brucei*, which is the infectious agent of sleeping sickness in humans and Nagana disease in cattle, respectively, uses another strategy to escape host immune defense by successive expression of a series of variable surface glycoproteins (VSGs). Consequently, the immune response of the host is always one step behind. According to these different strategies for maintaining an advantage over the host, trans-sialidase expression is restricted only to certain stages in the life cycle. In *T. cruzi*, the enzyme is present in the mammalian bloodstream form and may be involved in the invasion of host cells. *T. brucei*, on the other hand, reveals transsialidase activity only in the procyclic form, colonizing the gut of the fly [70]. Its function in this species seems to be completely different, consisting in the transfer of sialic acids from the surface of sucked mammalian blood cells or from serum glycoproteins onto the parasite's own surface glycoproteins [71]. Thereby, the trypanosome would be protected from the proteases and glycosidases of the fly's digestive tract as well as from possible immunological attack [69].

The trans-sialidase from *T. cruzi* has been found to consist of several domains, among them an N-terminal domain responsible for sialidase activity and two further domains that are involved in sialyltransferase activity [72]. Interestingly, trypanosomal species possessing trans-sialidases do not contain enzymes for sialic acid anabolism [66].

Sialidases have been extensively investigated, especially in microbial species. Why do we work on sialidases? First, we want to obtain information about the relationship and evolution of these enzymes, and here primary structure data enable us to draw some conclusions. When did sialidases appear in evolution? Why is there a gap in the occurrence of sialidases in lower animals? Were these enzymes invented twice in bacteria and in animals? Are they evolutionarily old, distributed universally in those early days, and the genetic information for sialidase production lost later in some groups of organisms? Or did they only emerge in higher animals, followed by horizontal transfer of the corresponding genetic information to some microorganisms? For such a pathway, close contact between the partners exchanging genetic information would be necessary, as is the case in host-parasite interactions. Other possible vehicles for horizontal gene transfer are viruses. Finally, free DNA molecules can be taken up by several naturally transformable bacterial species by the process of transfection. Gas gangrene represents a proper scenario for horizontal gene transfer with bacterial cells being in intimate contact with damaged host cells. It is also conceivable that the genetic information might have been transferred from bacteria to eucaryotes. Schauer and Vliegenthart [73], however, propose an exchange of information in the reverse direction. For some strains of *Salmonella typhimurium* it has been shown that sialidase genes are spread between strains of this species by horizontal gene transfer [74].

To learn something about the evolution of sialidase genes, sialidase primary structures had to be elucidated. Table 2 presents a list of species for which the corresponding primary structures are known. The alignment of sialidase primary structures [92] reveals that – apart from some conserved regions – the overall homology between the sequences is low. The conserved areas point to a single evolutionary origin of sialidases. The percentage of identical amino acids between each pair of sequences can be calculated and the values used to construct a dendrogram of sialidase relationships. This is shown for known clostridial primary structures in figure 5. It can be seen that clostridial sialidases form a group of closely related enzymes. The sialidase relation-

Table 2. List of known sialidase primary structures.

Organism	Type of sialidase	Reference
Influenza B virus	membrane-associated	[75]
Homo sapiens	endoplasmic reticulum	[76]
	lysosomes	[77]
Cricetulus griseus	cytosolic	[78]
Rattus norvegicus	cytosolic	[79]
Macrobdella decora	secreted	[80]
Trypanosoma cruzi	cell surface, trans-sialidase	[81]
	cell surface, sialidase	[82]
Trypanosoma rangeli	secreted	[83, 84]
Actinomyces viscosus	cell surface	[85]
Bacteroides fragilis	secreted	[86]
Clostridium perfringens	secreted 'large' isoenzyme	[87]
	cytosolic 'small' isoenzyme	[88]
Clostridium septicum	secreted	[89]
Clostridium sordellii	secreted	[90]
Clostridium tertium	secreted	[90a]
Micromonospora viridifaciens	secreted	[91]
Salmonella typhimurium	cytosolic	[74]
Vibrio cholerae	secreted	[65]

ships within this group, however, contradict the data of 16S ribosomal RNA (rRNA) analysis: according to the latter, *C. perfringens* is most highly related to *C. septicum* [93]. In contrast, the highest degree of similarity was found between the 'small' sialidase isoenzyme from *C. perfringens* [88] and the enzyme from *C. sordellii* [90], whereas the close relationship between the 'large' isoenzyme from *C. perfringens* [87] and the one from *C. septicum* [89] is expected. This is another clue to the above-mentioned horizontal gene transfer. Interestingly,

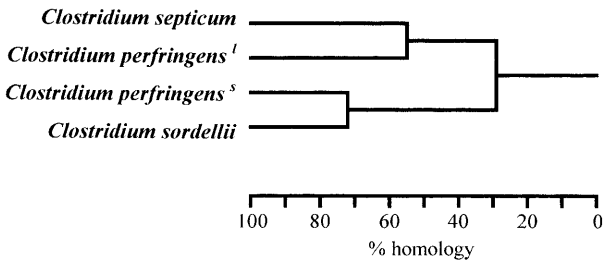


Figure 5. Dendrogram of similarities among sialidase primary structures from various *Clostridium* species based on identical amino acids (modified from [87]); index 'l', large isoenzyme; index 's', small isoenzyme. Reprinted with permission from: Traving C., Schauer R. and Roggentin P. (1994) Gene structure of the 'large' sialidase isoenzyme from *Clostridium perfringens* and its relationship with other clostridial *nanH* proteins. Glycoconj. J. **11:** 141–151.

the 'small' sialidase isoenzyme from *C. perfringens* is located near a phage attachment site on the chromosome of this species [94, 95]. According to one hypothesis [96], the genetic information for sialidases was spread by horizontal gene transfer at two time points in evolution: genes that were acquired by microbial species at an earlier time are well adapted to their environment, which can be seen from their broad substrate specificity and the fact that they are secreted into the medium to make contact with their substrates (e.g. the 'large' isoenzyme from *C. perfringens*). Sialidase genes that have been transferred during a more recent event reveal a limited substrate specificity and have not been fully adapted to their new 'host' because they still lack a signal for excretion of the enzyme proteins (e.g. the 'small' sialidase isoenzyme from *C. perfringens*).

Another aim of the work is to elucidate the catalytic mechanism of these enzymes. This is especially important, as sialidases are involved in the pathogenesis of several infectious diseases (see above). Therefore, they are of great importance in medicine and the pharmaceutical industry for the analysis of oligosaccharides and the development of sialidase inhibitors, for example as potential drugs against influenza infections [97]. Some insight into the catalytic mechanism of these enzymes can be gained from the analysis of the corresponding primary structures. Concerning the alignment of sialidase primary structures, we already mentioned the existence of conserved regions [98]. The most prominent conserved motif is the so-called Asp box, which is a stretch of amino acids of the general formula -**S**-X-**D**-X-**G**-X-**T**-**W**- where X represents variable residues [92, 98]. The name was chosen because of the aspartic acid residue. The five different residues are conserved to different degrees. This motif is found four to five times throughout all microbial sequences with the exception of viral sialidases, where it is only found once or twice or is even absent. The third Asp box is more strongly conserved than are Asp boxes 2 and 4. Interestingly, the space between two sequential Asp boxes is also conserved between different primary structures. The Asp boxes were believed to participate in catalytic action. However, increasing insight into the three-dimensional structure of sialidases contradicts this idea, attributing to them simply a structural role [99].

In contrast to the Asp boxes, the 'FRIP' motif is located in the N-terminal part of the amino acid sequences. It encompasses the amino acids -X-**R**-X-**P**- with the arginine and proline residues being absolutely conserved [2]. The arginine is directly involved in catalysis by binding of the substrate molecule [100]. Also important for the catalytic reaction is a glutamic acid-rich region, which is located between Asp boxes 3 and 4 as well as two further arginine residues. There are also several single, conserved residues dispersed throughout the sequence.

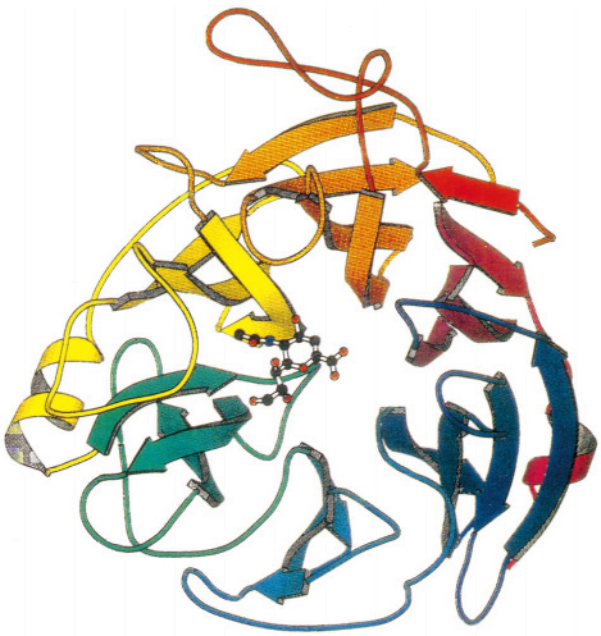


Figure 6. Secondary structure model of the sialidase protein from *Salmonella typhimurium* with the bound inhibitor Neu2en5Ac; view from above on the active center (from [99]). Reprinted with permission from: Crennell S., Garman E., Laver G., Vimr E. and Taylor G. (1993) Crystal structure of a bacterial sialidase (from *Salmonella typhimurium* LT2) show the same fold as an influenza virus neuraminidase. Proc. Natl. Acad. Sci. USA **90:** 9852–9856, © National Academy of Sciences, USA.

Meanwhile, X-ray analysis has been performed with complexes between the sialidase inhibitor 5-*N*-acetyl-2-deoxy-2,3-didehydroneura minic acid (Neu2en5Ac), and both the monomeric enzyme from *S. typhimurium* and the tetrameric enzyme from influenza virus, respectively [99]. Despite of the low overall homology, the three-dimensional structure of viral and bacterial sialidase monomers is nearly identical (fig. 6): the secondary structure consists of six groups each comprising four antiparallel β-pleated sheets in addition to two small α-helical segments. The 64 antiparallel β sheets are positioned like propeller blades around a central axis through the active site [99]. The viral tetramer is stabilized by C-terminal extensions interacting with the first and second sheets of adjacent monomers. The Asp boxes are located at equivalent positions on the surface of the protein monomers from the different species, namely at the turn between the third and fourth strands of the first four sheets. Their aromatic residues form a hydrophobic core stabilizing the turn, whereas the aspartic acid residues point into the solvent. Thus, they might represent contact sites between subunits of the protein. On the other hand, there are only one or two Asp boxes in tetrameric influenza virus sialidases. Another possible role for these motifs might be involvement in the process of enzyme secretion. This is in agreement with the location of these regions on the surface of the enzyme molecule. The carboxylate group of the substrate is stabilized by three arginine residues in the active center [101]. One of them descends from the FRIP region.

These data together with mutagenesis experiments [102] provide enough information to develop a model of the catalytic process [99, 100]: The sialoside substrate is activated by a protonated arginine residue planarizing the ring of Neu5Ac in the region of the oxygen and carbon atoms 1–3. A tetrahedral transition state is formed with a positive charge at carbon atom 2. For hydrolysis, proton donation from the solvent is necessary, which is facilitated by the presence of an aspartic acid residue.

While this overall reaction mechanism seems to be generally valid for sialidases, there are also some differences [101]. Thus, there is a strong interaction between the oxygen of carbon atoms 8 and 9 of the glycerol side chain and the glutamic acid residue in influenza virus sialidase, which leads to a slow turnover of the substrate by the viral sialidase. In *Salmonella*, there exist two strong and one weak hydrogen bonds between the enzyme and O4, whereas in influenza virus there is only one weak bond. This is an important point, for most sialidases are absolutely unable to cleave 4-*O*-acetylated sialic acids. However, this is the case for the viral enzyme, although at a slow rate. Therefore, analogs of the sialidase inhibitor Neu2en5Ac have been developed, containing an amino or guanidino group at C-4. These substances are strong inhibitors of enzyme activity [97]. Especially the guanidino derivative represents a potent drug against influenza infections that is in stage III of clinical trials [103].

Certain bacterial sialidases reveal a more complex structure consisting of several domains with special functions. Thus, the enzyme from *Vibrio cholerae*, which has also been crystallized [104], proved to contain two lectin-like domains flanking the central catalytic domain. These domains reveal a sandwichlike structure composed of seven and six antiparallel β sheets, respectively, and probably serve in binding of carbohydrates. The actual ligands, however, are so far unknown. A galactose-binding pocket was identified as a part of a jelly roll domain in the sialidase from *Micromonospora viridifaciens* [105]. It is linked via an immunoglobulin-like domain to the catalytic part of the molecule. The galactose-binding pocket is only 30 Å away from the sialic acid binding site. So each of the two domains seems to interact with a different part of the sialoside substrate.

Table 3 presents a list of microbial sialidases that have been purified and characterized. Although these proteins reveal a high degree of variability with respect to their

properties, they can be classified into two groups according to their size (see above; [96, 106]): 'small' proteins of around 42 kDa and 'large' ones of 60 to 70 kDa. The primary structures of the 'large' sialidases contain extra stretches of amino acids between the N-terminus and the second Asp box as well as between the fifth Asp box and the C-terminus. It is believed that they contribute to the broader substrate specificity of the 'large' sialidases.

On the other hand, it is tempting to assume that the sequence areas that are additionally present in some primary structures encode extra domains such as the galactose binding domain of the *M. viridifaciens* enzyme [105]. The 'small' sialidases are not Ca^{2+}-dependent in contrast to some of the 'large' enzymes. The only common feature of all sialidase proteins is a slightly acidic pH optimum. Remarkably, trans-sialidases operate best under neutral conditions. Usually sialidases are specific for Neu5Ac that is $\alpha 2 \rightarrow 3$, $\alpha 2 \rightarrow 6$ or $\alpha 2 \rightarrow 8$ linked to the subterminal sugar chain of poly- and oligosaccharides, glycoproteins and gangliosides. Neu5Ac-bound $\alpha 2 \rightarrow 3$ in most cases is hydrolyzed at the highest rate [1]. The endosialidase of bacteriophage E acts exclusively upon $\alpha 2 \rightarrow 8$ bound Neu5Ac and Neu5Gc. Ganglioside GM1 is a substrate only for the enzyme from *Arthrobacter ureafaciens*. With the exception of *S. typhimurium*, the first reaction product of the sialidase reaction is the α form of Neu5Ac, which then mutarotates to the more stable β form. Finally, an enzyme has been identified that splits Kdn instead of Neu5Ac, a so-called Kdnase, in the loach *Misgurnus fossils*, in the ovary of rainbow trout and in *Sphingobacterium multivorum* [107–109].

Less knowledge exists so far about sialidases from higher animals, although this is a very interesting and important field. The first phylum in the animal kingdom to contain sialidases is the echinoderms. The enzyme from *Asterina pectinifera* ovary has a pH optimum of 3 to 4 and hydrolyzes a broad spectrum of substrates, including sialyloligosaccharides, glycoproteins and gangliosides, as was also found in human placenta [110]. Mammalian sialidases with different substrate specificities were found in lysosomes (specific for sialyloligosaccharides, glycoproteins, gangliosides), the Golgi, the plasma membrane and the cytosol (sialyloligosaccharides and glycopeptides).

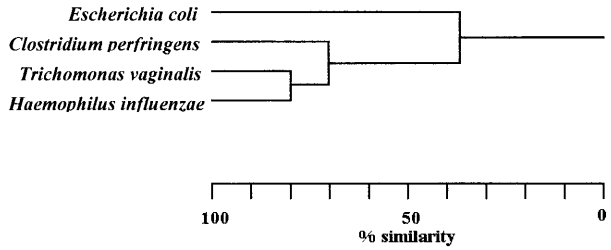


Figure 7. Dendrogram of the relationship between lyase primary structures calculated from homologous amino acids (modified from [129]). Reprinted with permission from: Traving C., Roggentin P. and Schauer R. (1997) Cloning, sequencing and expression of the acylneuraminate lyase gene from *Clostridium perfringens* A99. Glycoconj. J. **14:** 821–830.

The significance of sialidases for mammals can be seen from the severe consequences of genetic diseases concerning sialidases [111]. Deficiency of the human lysosomal sialidase leads to sialidosis, an autosomal recessive genetic disorder that can be classified into type I (late onset) and type II (infantile onset) cases [77]. Frame-shift and missense mutations have been found to be responsible for sialidosis. The lysosomal sialidase gene was localized on chromosome 6 in proximity to the human lymphocyte antigen (HLA) locus, which corresponds to the localization of this gene in the major histocompatibility complex (MHC) of rat and mouse. The lysosomal enzyme is most similar to the sialidase from *S. typhimurium* but has three glycosylation sites. The lysosomal as well as the cytosolic mammalian sialidases share the presence of Asp boxes, the FRIP region and even the same fold with their microbial counterparts, pointing to a single evolutionary origin of all sialidases [77].

Another inherited lysosomal storage disease is galactosialidosis with sialidase and β-galactosidase being deficient secondary to the lack of cathepsin A/protective protein [112]. All these components are part of a 1.27-MDa complex. Normally, sialidase is synthesized as a 45.5-kDa precursor, including a signal peptide of 47 amino acids. Removal of the latter and glycosylation leads to the mature protein of 48.3 kDa that is found in lysosomal and plasma membranes. In addition, the enzyme is only active in association with cathepsin A, which is important for the correct conformation of the sialidase and protects against intralysosomal proteolysis. The same is true for β-galactosidase. In galactosialidosis, missing interaction with cathepsin A leads to sialidase and galactosidase deficiency due to abnormal proteolytic cleavage and rapid degradation of the two proteins.

A sialidase with unique features has been described for *Macrobdella decora* [80]. It releases 2,7-anhydro-Neu5Ac and is absolutely specific for Neu5Ac$\alpha 2 \rightarrow 3$ Gal. The calculated mass is 83 kDa. Like the enzyme from *A. pectinifera*, it is similar to the enzyme from *S. typhimurium*, and shares the presence of Asp boxes and the FRIP region with microbial sialidases, but the similarity with other, especially mammalian sialidases, is only slight.

Table 3. Properties of different microbial sialidase proteins [adapted from ref. 113].

Species	Size (kDa)	Subunits	Location	T_{opt} (°C)	pH_{opt}	Substrate spectrum	pI	Ca^{2+}-dependent
Clostridium perfringens 'small' isoenzyme	43	1	cell-bound	37	6.1	limited	5.1	no
Clostridium sordellii	45	1	medium	36	6.0	'small' substrates, serum glycoproteins	4.4	no
Micromonospora viridifaciens	41	1	medium	58	5.0	broad	5.6	no
Salmonella typhimurium	41.3	1	intracellular	n.d.	n.d.	n.d.	n.d.	no
Trypanosoma cruzi transmembrane	85	1	membrane-bound	n.d.	7	n.d.	n.d.	no
Clostridium perfringens 'large' isoenzyme	73	1	medium	55	5.0	broad	5.2	no
Vibro cholerae	90, 82	1	medium	n.d.	n.d.	broad	n.d.	yes
Clostridium chauvoei	300	2	medium	37	5.5	gangliosides, glycoproteins	3.4	yes
Clostridium septicum	111	3	medium	37	5.3	glycoproteins	acid	no
Actinomyces viscosus	150[a] 180[a]	1	cell-bound	37	5.0	gangliosides	4.8	no
Bacteroides fragilis	165	3	n.d.	n.d.	6.1	α(2,8)-linked sialic acid, sialyllactose	n.d.	yes
Trypanosoma brucei trans-sialidase	67	n.d.	membrane-bound	35	6.9	limited	n.d.	no

n.d., not determined; [a]determined using different methods. The respective references can be obtained from the authors. Reprinted with permission from: Traving C. and Schauer R. (1996) Sialinsäuren – ein Schutzschild auf Zellen. Futura **3:** 168–178, © 1998 Boehringer Ingelheim Fonds, Stuttgart.

Acylneuraminate lyases (EC 4.1.3.3)

The last but not least group of enzymes in sialic acid metabolism splits acylneuraminic acids into acylman nosamines and pyruvate. Their distribution corresponds to that of sialidases: in higher animals they provide an alternative to the recycling of sialic acid molecules [1, 2, 113]. Furthermore, they have been found in microorganisms that either possess sialic acids or sialidases for access to foreign sialic acids. In these microbial species, they not only have a nutritional function but are important for the regulation of intracellular sialic acid concentration. For example, in certain *E. coli* strains that metabolize both biosynthetically derived and exogenous sialic acids and which are unable to degrade sialic acids due to mutations in their lyase genes, this sugar can reach toxic levels inside the cell [114]. Finally, acylneuraminate lyases are used in industry for the synthesis of sialic acids and their analogs [115]. This application is especially important, as sialic acid isolation is a difficult and expensive procedure. There even exist reports about the physiological, in vivo synthesis of sialic acids in *E. coli* by means of acylneuraminate lyase [116, 117]. Furthermore, lyases are tools in sialic acid analysis: the degree and rate of cleavage that is obtained with these enzymes for different substrate analogs enables the characterization of peaks in high-pressure liquid chromatography (HPLC) analysis [41, 118].

What are the topics we are especially interested in concerning acylneuraminate lyases? Because the distribution of sialic acid aldolases is as unusual as already described for sialidases, we would like to learn something about the relationship and evolution of these enzymes in addition to how they function. Based on data about the *E. coli* enzyme [114, 119, 120], it is also interesting to obtain more knowledge about the regulation of lyase expression and activity. And most important, the possibilities for synthesizing unusual sialic acid analogs by letting lyase act in the reverse direction should be evaluated.

The most extensively studied lyase is the enzyme from *E. coli*, for which the primary structure is known [121, 122] and, following purification and crystallization [123, 124], the three-dimensional structure has been resolved [125]. It is produced on industrial scale by biotechnological methods [126]. Meanwhile, several further microbial primary structures have become known [127–129]. In contrast, so far the only well-studied mammalian lyase has been isolated from pig kidney [130]. However, there exist expressed sequence tags (ESTs) [complementary DNA (cDNA) deduced from messenger RNA (mRNA), thus representing only the genetic information of proteins expressed in the respective tissue] from human fetal heart and mouse in the GenBank database (fetal heart: W79930 [131]; mouse: AA162738 [132]), which show a distinctive degree of homology with procaryotic

lyase sequences. From the alignment of microbial lyase primary structures (fig. 7; [129]) it can be seen that acylneuraminate lyase proteins form a more homogenous group when compared with sialidases. Thus, the length of the complete microbial sequences is almost identical, corresponding to molecular weights of about 33 to 37 kDa that were calculated from the primary structures or determined for the denatured proteins, respectively.

Earlier investigations pointed to a dimeric [133] or trimeric [123, 124] structure of the native enzyme. However, the crystal structure of *E. coli* lyase proved it actually to be a tetramer [125]. The same was found after cross-linking experiments with porcine kidney lyase (U. Sommer et al., unpublished). The comparatively high number of 86 (!) amino acids has been found to be completely conserved among four of the microbial sequences. Nine of them were identified to be present in the active site of *E. coli* lyase [125]. The highest degree of homology exists between the lyases from the Gram-negative bacterial species *Haemophilus influenzae* [127] and the eucaryotic protozoon *Trichomonas foetus* [128], whereas the degree of homology between *H. influenzae* and *E. coli* is low. The enzyme from *C. perfringens* [129] stands somewhere in between these two groups. This finding is surprising and in contrast to the phylogenetic relationship of the species. The relationship between the known parts of the two putative mammalian primary structures is even closer, as they are almost identical (86%) on the amino acid level (U. Sommer et al., unpublished). Accordingly, 23 of the 24 amino acid residues of the four porcine lyase peptides obtained so far (U. Sommer et al., unpublished) match the corresponding residues in the EST sequences. From the partial sequences of the mammalian enzyme it can clearly be seen that microbial acylneuraminate lyase sequences and those from higher animals have a common origin in evolution.

Table 4. Properties of acylneuraminate lyases from *Clostridium perfringens* [133, U. Sommer et al., in preparation], *Escherichia coli* [123–125], and pig kidney [130, 134, U. Sommer et al., unpublished]; slightly modified from ref. 113.

Properties	*Escherichia coli*	*Clostridium perfringens*	Pig kidney
Size (kDa)	98[a], 105[b]	100	58
Subunits	4	2	1
pI	4.5	4.7	5.6
Temperature optimum (°C)	75[a], 80[b]	65–70	75
Thermostability	yes	yes	yes
pH optimum	7.7	7.2	7.2
K_m Neu5Ac (mM)	3.6[a], 3.3[b]	2.8	3.7
Metal ions required	no	no	no

[a]Based on the data of [123]; [b]based on the data of [124]. Reprinted with permission from: Traving C. and Schauer R. (1996) Sialinsäuren – ein Schutzschild auf Zellen. Futura **3:** 168–178, © 1998 Boehringer Ingelheim Fonds, Stuttgart.

The homogeneity of acylneuraminate lyases concerning the primary structure continues on the level of the proteins: as can be seen from table 4, the enzymes from *E. coli*, *C. perfringens* and pig kidney are not only very similar in size, but have almost identical pH optima and kinetic properties [113]. Of special interest is the high temperature optimum and thermostability that has been found to be a common feature of these enzymes. So far nothing is known about the structural basis for this thermostability. However, in this context it is notable that the lyases from *E. coli* and pig kidney seem to be tetramers [125, 134]. A tetrameric structure has been found for many thermostable enzymes. In addition, the stability of such proteins is often increased by salt bridges (e.g. between glutamic acid and Ca^{2+}; G. Taylor, personal communication).

Concerning substrate specificity, acylneuraminate lyases cleave not only Neu5Ac but also Neu5Gc at a reduced rate [1, 130, 135]. Neu5Gc accumulates in the cytosol after lysosomal degradation of corresponding sialoglycoconjugates. In sialic acid catabolism, however, there exists no further reaction for changing Neu5Gc into Neu5Ac, which would lead to a further decrease in the relative amount of Neu5Gc. Only via acylneuraminate lyase can it be slowly degraded to ManNGc and pyruvate. Even *O*-acetyl groups are tolerated in certain positions, like in the sialic acid side chain, but not at C-4, as with most sialidases. Generally, modifications at positions 2, 3, 4, 7 and 8 of the sialic acid substrate diminish enzyme activity more than variation at position 5 or 9, respectively [130, 136].

From earlier investigations about the catalytic mechanism of lyases by means of amino acid-modifying reagents [137] and with new data about the crystal structure of *E. coli* [125], we know that a Schiff base forms between the ε-amino group of a lysine residue and the carbonyl group of the substrate. Accordingly, acylneuraminate lyases belong to the class I aldolases (Schiff base formation) in contrast to class II aldolases, which are characterized by the dependence of their activity on metal ions. Furthermore, a histidine or tyrosine residue seems to be involved in the catalytic process as a proton donator for the hydroxyl group at C-4 of the substrate. Finally, inhibition experiments showed one or more SH groups to be important for the reaction [130, 137, U. Sommer et al., unpublished]. These observations fit well with the conclusions that can be drawn from the alignment. Thus, lysine 165 and tyrosine 137 of the *E. coli* sequence are conserved in all known structures, whereas no histidine has been found in an appropriate position [129]. Most of the amino acids that have been described as flanking the active site pocket in the *E. coli* enzyme [125] are also conserved.

Interestingly, a subfamily of enzymes could be defined due to the overall structural and functional relationship of its members to acylneuraminate lyases [138]. Apart from the 'genuine' sialic acid aldolases, this group of

proteins encompasses several enzymes that catalyze different reactions, for example the synthesis of dihydrodipicolinate. However, all of them share a similar three-dimensional structure.

Nees and Schauer postulated the existence of an operon in *C. perfringens* containing the genes of sialic acid catabolism [139], which encode an extracellular sialidase and a cytosolic acylneuraminate lyase. The expression of both enzyme activities has been observed in various microbial species [64, 140, 141]. Recently, a partial open reading frame (ORF) respresenting a putative lyase gene was identified downstream from the sialidase gene in *C. tertium* [90a]. Because the sialic acid molecules have to enter the cell to become available for the lyase, the existence of a sialic acid permease was also postulated for *C. perfringens* on the basis of experimental observations [139]. While this protein is so far hypothetical in *C. perfringens*, there already exist extensive investigations concerning the corresponding component of *E. coli* [142]. Meanwhile, even the genes for the transporter of this species [143] and a putative transporter of *H. influenzae* [127] have been cloned. In *E. coli*, the operon for sialic acid catabolism encompasses the genes for lyase and permease, whereas a sialidase gene is absent [114]. Nevertheless, *E. coli* cells successfully compete for free extracellular sialic acid molecules that have been produced by other sialidase-excreting microorganisms because they can induce their permease very quickly [114]. In addition, there exist separate gene clusters for sialic acid biosynthesis and polymerization in *E. coli* strains forming a sialic acid-containing capsule. Here, the regulation of the sialic acid metabolism is very complex because the use of exogenous sialic acids has to be coordinated with the biosynthetic steps. The lyase functions as a valve to dissipate excess sialic acid [114, 119].

Concluding remarks

We hope that this survey provides some insight into the fascinating world of sialoglycobiology. Although sialic acids have been the aim of research for 6 decades, this field of science has become a matter of great interest only in the last 10–15 years, when the high structural diversity of this molecule became more and more obvious and also knowledge increased regarding the significant role of this sugar in many biological processes of animals and humans, both in the state of health (e.g. recognition and contact between cells and molecules, neuronal transmission, ion transport, reproduction, differentiation, masking, protection), and in many important diseases (infections and inflammations; cancer; neurological, cardiovascular, endocrinological and autoimmune diseases; genetic disorders and so on). Although much has been learned about sialic acids and their metabolism, much remains to be discovered and every question answered raises new ones. This is all the more important since many findings might lead some day to useful medical applications. To take just a few examples, there are especially extensive studies currently in progress concerning the enzymatic and genetic basis, and the biological role, of *O*-acetylation, *N*-acetyl hydroxylation, the mechanism of selectin action and its potential for curing diseases as well as the role of sialic acids in receptor physiology, transmembrane signalling, hormonal control, tumor metastasis, alcohol abuse and the mechanisms by which trans-sialidases participate in the pathogenesis of tropical diseases caused by trypanosomes. As mentioned throughout the text, many questions remain with regard to the evolutionary roots of sialic acid enzymes, whether these are mono- or oligophylogenetic, whether and in which direction horizontal gene transfer took place, and even in which animal phylum sialic acids have their origin. Although echinoderms were originally considered to be the 'inventors' of sialic acids and many of their anabolic and catabolic enzymes [73], the genetic and chemical origin of this monosaccharide family may have occurred much earlier in evolution. To this possibility points the finding of sialic acids in *Drosophila* embryos [8] and in cicada larvae (Y. Malykh, unpublished results), but also the existence of nucleic acid sequences with high homology to sialic acid-metabolizing enzymes (sialic acid synthetase and CMP-Neu5Ac-synthase) in the archaebacterium *Methanococcus janashii* (E. Vimr, personal communication).

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