Glycoconjugate Journal 17, 485–499, 2000
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Achievements and challenges of sialic acid research

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Sialic acids are one of the most important molecules of life, since they occupy the terminal position on macromolecules and cell membranes and are involved in many biological and pathological phenomena. The structures of sialic acids, comprising a family of over 40 neuraminic acid derivatives, have been elucidated. However, many aspects of the regulation of their metabolism at the enzyme and gene levels, as well as of their functions remain mysterious. Sialic acids play a dual role, not only are they indispensable for the protection to and adaptation of life, but are also utilised by life-threatening infectious microorganisms. In this article the present state of knowledge in sialobiology, with an emphasis on my personal experience in this research area, is outlined including a discussion of necessary future work in this fascinating field of cell biology.

Keywords: **sialic acid diversity, sialic acid functions, sialic acid future aspects, sialic acid metabolism, sialic acid occurrence**

Introduction

Alfred Gottschalk, who wrote the first book on sialic acids (Sia) [1] and investigated the carbohydrate composition of sialic acid-rich mammalian mucins, introduced me to this field in the 1960s and pointed to the novelty of carbohydrate research in biological systems by saying "we are just at the shores of an unexplored continent". He not only had coined, together with Gunnar Blix and Ernst Klenk, the term "sialic acid" [2], but had also obtained early insight into the great biological significance of this monosaccharide family, for example, the observation of an antiproteolytic effect in developing frog eggs (reviewed in [3]). Earlier, Ernst Klenk introduced the name "neuraminic acid" to a substance, which he had isolated after methanolysis of gangliosides and which we now know as neuraminic acid-β-methyl glycoside [4]. The main achievement of Gunnar Blix was to crystallize, for the first time in 1936, this incidentally being the year of my birth, several Sia species hydrolytically released from bovine submandibular gland mucin [5]. Stimulated by this promising field of research I gladly accepted a post-doctoral position at the newly founded Ruhr-University in Bochum in the laboratory of Hans Faillard and ventured into this "sticky" research area, which has kept me attracted since 1967. Faillard's attention was focused mainly on the synthetic aspects of Sia science and his results are summarized in Refs. [3,6–8]. In the following I will summarize the results of my research groups, first at the university in Bochum, and since 1976 in Kiel, on sialic acids, and will discuss the future mainstreams and challenges of this special field in glycosciences.

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Occurrence, isolation and analysis of sialic acids

When I entered the sialic acid field, the main area of interest was still in the chemistry of this exciting monosaccharide, but also in its distribution in nature and metabolism. The main pathway of the biosynthesis, transfer and degradation of Sia were already known, mainly by the work of Saul Roseman and coworkers (summarized in Refs. [1,6–9]). I started with the isolation and structural analysis of the various Sia found in different animal species, as well as with the study of their behaviour towards the action of sialidases and sialate-pyruvate-lyases. The availability of an anion-exchange technique in combination with adsorption chromatography on cellulose powder with butanol/propanol/water (1:2:1) of Sia liberated from their glycosidic linkage by sialidase or mild hydrochloric acid enabled the isolation and purification of larger quantities of individual Sia [10]. This was a prerequisite for the structural analysis of, for example, the number and position of *O*-acetyl groups, which was tentatively possible by quantitative periodate oxidation followed by a dropping mercury electrode [10], as well as by several colorimetric and thin-layer chromatographic methods, summarized in [11]. However, exact Sia analysis, especially of minute amounts, became only possible after the development of a combination

of gas chromatography and mass spectrometry (GC/MS) [12]. This was the beginning of a long and fruitful collaboration with the group of J.F.G. Vliegenthart and J.P. Kamerling at Utrecht University and led to the discovery of many new neuraminic acid derivatives in a variety of natural sources. Thus, the number of Sia species increased to over fourty, including 2-keto-3-deoxy-nonulosonic acid (Kdn) and its derivatives [8]. These comprise those with *N*-acetyl- or *N*-glycolyl groups, combined with *O*-acetyl residues or the less frequent *O*-lactyl, *O*-methyl or *O*-sulfate groups (Figure 1). Furthermore, Sia were discovered with a double bond between C-2 and C-3 (5-*N*-acetyl-2-deoxy-2,3-didehydro-neuraminic acid, Neu2en5Ac) found in the free form in animal fluids, first in human urine [13], or with an internal anhydro linkage (5-*N*-acetyl-2,7-anhydroneuraminic acid, Neu2,7an5Ac) in animals and in, for example, human cerumen (summarized in [8]). For the identification of trace amounts of all these Sia in biological materials the technique described in Ref. [12], based on pertrimethylsilylether-, methyl- or trimethylsilylester derivatives, is still of great value due to its specificity and sensitivity. Previous purification of Sia, by ion-exchange chromatography for instance, in order to get a better resolution of sialic acid mixtures, is recommended. Another GC/MS method has been described by Zanetta [14] using heptafluorobutyric anhydride derivatives, which also allows the identification of different Sia in mixtures, even from impure samples, in minute quantities.

Although these techniques, including NMR-spectroscopy of free Sia [7,8], require sophisticated equipment and specialist knowledge, other known tools [11] can be routinely used in normal laboratories. With fluorimetric HPLC [15], now in wide use, individual Sia can easily be analysed in fmole quantities. However, also in this case purification of Sia is recommended and care should be taken that the reagent used for Sia derivatization (1,2-diamino-4,5-methylene-dioxybenzene, DMB) does not contain impurities [11]. The first separation of Sia by HPLC was carried out in our laboratory by Shukla *et al.* [16] in 1982.

With these tools available, it is possible, and necessary, to study in the future the wider occurrence of different Sia in biological materials. So far, Sia analyses have been carried out only sporadically, these have, however, revealed that these monosaccharides do not occur randomly. They are expressed both quantitatively and qualitatively depending on the microorganism or animal species, as well as on the kind of tissue or cell and on the function or developmental stage. Large differences in diseased cells were observed, which will be discussed later. Thus, in the last 20 years, Sia have more often been found in microorganisms, for example in lipopolysaccharides [8,17] first observed in the core region of the LPS of purple bacteria of the genus *Rhodobacter* [18], or in trypanosomes like in *Trypanosoma cruzi* [19]. Unexpectedly, Sia were also identified in developing insects, in *Drosophila melanogaster* [20] and in the cicada *Philaenus spumarius* [21], in both cases in the form of polysialic acid, of the nerve ganglia and Malpighian tubules, respectively. Neuraminic acid derivatives were also described in *Galleria mellonella* [22]. The presence of Sia has also been reported in cultured insect cells [23]. To my knowledge, there are no further reports on the occurrence of Sia in other lower animals. Only from the echinoderms onwards, Sia seem to exist regularly, although, of course, not yet all species have been investigated [8]. Sporadic, earlier reports of this monosaccharide in plants could not be confirmed by modern analysis. We have studied several plant sources, including the mucin from *Drosera binata*, but did not find Sia, although we obtained for the first time structural data from this biologically important acid polysaccharide [24]. The systematic analysis of Sia in the animal kingdom or under different physiological or pathological states will show the real distribution of Sia in the living systems and will help to better understand the roles of the various Sia species and to get more insight into their regulation

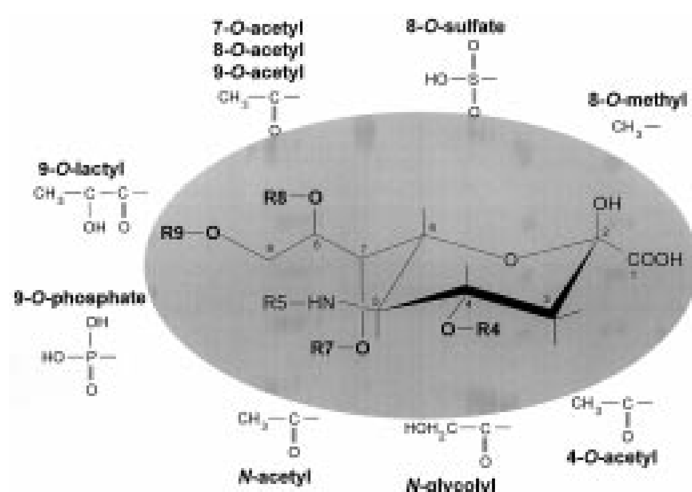

Figure 1. The family of naturally occurring sialic acids. The nomenclature including abbreviations is described in Ref. [8]. For the distribution in nature of these neuraminic acid derivatives, as well as of Kdn, not shown here, see Refs. [6–9,50].

at the gene level. For instance, why does 4-*O*-acetylated Sia occur less frequently than the 9-*O*-acetylated Sia? Similarly, why is Neu5Gc lacking in some species while it is highly expressed in others? What is the evolutionary significance of these phenomena? Why are there such pronounced changes in tumours in the type and amount of Sia?

NMR- and MS-techniques have been well adapted to the structural analysis of bound sialic acids as components of oligosaccharides or glycoconjugates. Improved separation methods, as well as looking to lower animals will reveal new and exotic structures. In the starfish *Asterias rubens* for example, new gangliosides [25] and a new type of oligosialic acid was detected in which *N*-glycolylneuraminic acid (Neu5Gc) is linked to another Neu5Gc residue by the hydroxyl of the glycolyl residue [-Neu5Gc(α2-O5)Neu5Gc(α2-O5)Neu5Gc(α2-] [26,27]. The sialyltransferase responsible for this linkage is unknown. Furthermore, in echinoderms there exist gangliosides and glycoproteins with sialic acids involved in the formation of branches of the glycan chains (summarized in [8]). The elucidation of such structures combined with various sialic acids including *O*-methylated and *O*-sulfated ones will increase our knowledge and understanding of the diversity of complex carbohydrates.

More attention should also be focused on the histochemistry of Sia, to get more insight into the location, the amount and the type of Sia in individual cells. For this purpose antibodies, lectins, including the hemagglutinin-esterase of influenza C virus, and chemical techniques using periodate and *p*-aminobenzaldehyde (Schiff's reagent; PAS) are available (reviewed in [8,28]). For example, with anti-Neu5Gc antibodies an accumulation of Neu5Gc-containing glycoconjugates on the surface of pig lymphocytes was found [29,30].

Biosynthesis of sialic acids

An overview of Sia metabolism and the localization of the individual steps in cell compartments is shown in Figure 2. While the biosynthetic route of the maternal Sia Neu5Ac is well known, as reviewed in [6–9], the enzymatic reactions modifying this monosaccharide are at present still being intensively studied. Ever since the discovery, in porcine submandibular gland mucin, of large quantities of Neu5Gc, and high amounts of *O*-acetylated Sia in bovine submandibular glands [5–9], we have taken an interest in the metabolism of these Sia. The oxidative conversion of the *N*-acetyl group of Neu5Ac to the *N*-glycolyl residue of Neu5Gc had already been observed in 1968 in surviving slices of pig submandibular glands [31]. However, it took until 1988 to determine the substrate for this reaction, that being CMP-Neu5Ac [32]. This finding led to a rapid sequence of publications from various laboratories, mainly of Akemi Suzuki in Tokyo, Ajit Varki in San Diego and our group, summarized in [29]. The enzyme requires oxygen and NADH, which obtains electrons *via* the ubiquitous electron transport system composed of cytochrome b_5 reductase and cytochrome b_5. In mammals, this hydroxylase is soluble, in contrast to starfish, where it is membrane-bound [33]. However, using hydroxylase antibodies and electron microscopy, in pig lymphocytes the enzyme was found to be attached to the surface of the nucleus and to some neighbouring microsomes [30]. From the cytosol, the CMP-Neu5Gc produced is transported into the Golgi lumen together with the corresponding Neu5Ac glycoside [34].

Interestingly, most animals of the deuterostome lineage, from echinoderms to the Great Apes, express this enzyme and Neu5Gc, respectively. However, in healthy human tissues only traces (0.1% and less) of this Sia exist, although this amount in some tumours has been reported to increase up to a few percent [29]. However, enzyme activity has never been reported in normal or malignant tissues. The reason for this deficiency in man is the lack of a 92 bp region of the cDNA coding for the human CMP-Neu5Ac hydroxylase [35,36]. The resulting mRNA codes for a truncated protein lacking a considerable position of the *N*-terminus, including the binding for the Rieske iron-sulphur centre [37], which is probably essential for catalytic activity. Other biosynthetic routes or dietary sources must therefore be considered to be responsible for the minute amounts of human Neu5Gc. Some experimental basis for the latter possibility was provided by Nöhle *et al.* [38,39]. When feeding mice and rats with free sialic acids, sialyllactose and porcine submandibular gland mucin radioactively labelled in the Sia moiety, it was found that free Sia were rapidly adsorbed from the intestine and excreted by the urine. This process was, however, much slower from the glycosidically bound Sia, with a small proportion, including Neu5Gc, of this radioactivity later being found in liver glycoproteins. This shows that Neu5Gc from a dietary source can be reused, at least in these animals. However, evidence that this may also happen in human tissues is being provided by immunological experiments. These show that Neu5Gc-reactivity occurs in endothelial cells of blood vessels from fetal tissues and carcinoma rather than in stromal cells [40].

Another frequent modification of Sia, from echinoderms to man, is *O*-acetylation. This occurs either at the pyranose ring or at the different hydroxyls of the side-chain ([6–9,41] for review). At least two enzymes catalyse these esterifications, either an acetyl coenzyme A:sialate-4-*O*-acetyltransferase or a corresponding 7(9)-*O*-acetyltransferase. First insight into the biosynthesis of these Sia was obtained in 1970 [42] by the incubation of radioactively labelled acetate into surviving slices of bovine and equine submandibular glands. It took a long time until the enzyme reactions could be demonstrated in *in vitro* systems. These enzymes are localized in the subcellular membrane fractions of guinea pig liver [43] and equine submandibular gland (Tiralongo *et al.*, unpublished) in the case of 4-*O*-acetylation, as well as in rat liver [44] and bovine submandibular gland [45] in the case of 9-*O*-acetylation. Solubilization of an active enzyme from these membranes by detergent has proved to be difficult, but enrichment from guinea pig liver and bovine submandibular gland was possible (unpublished). There is evidence that the

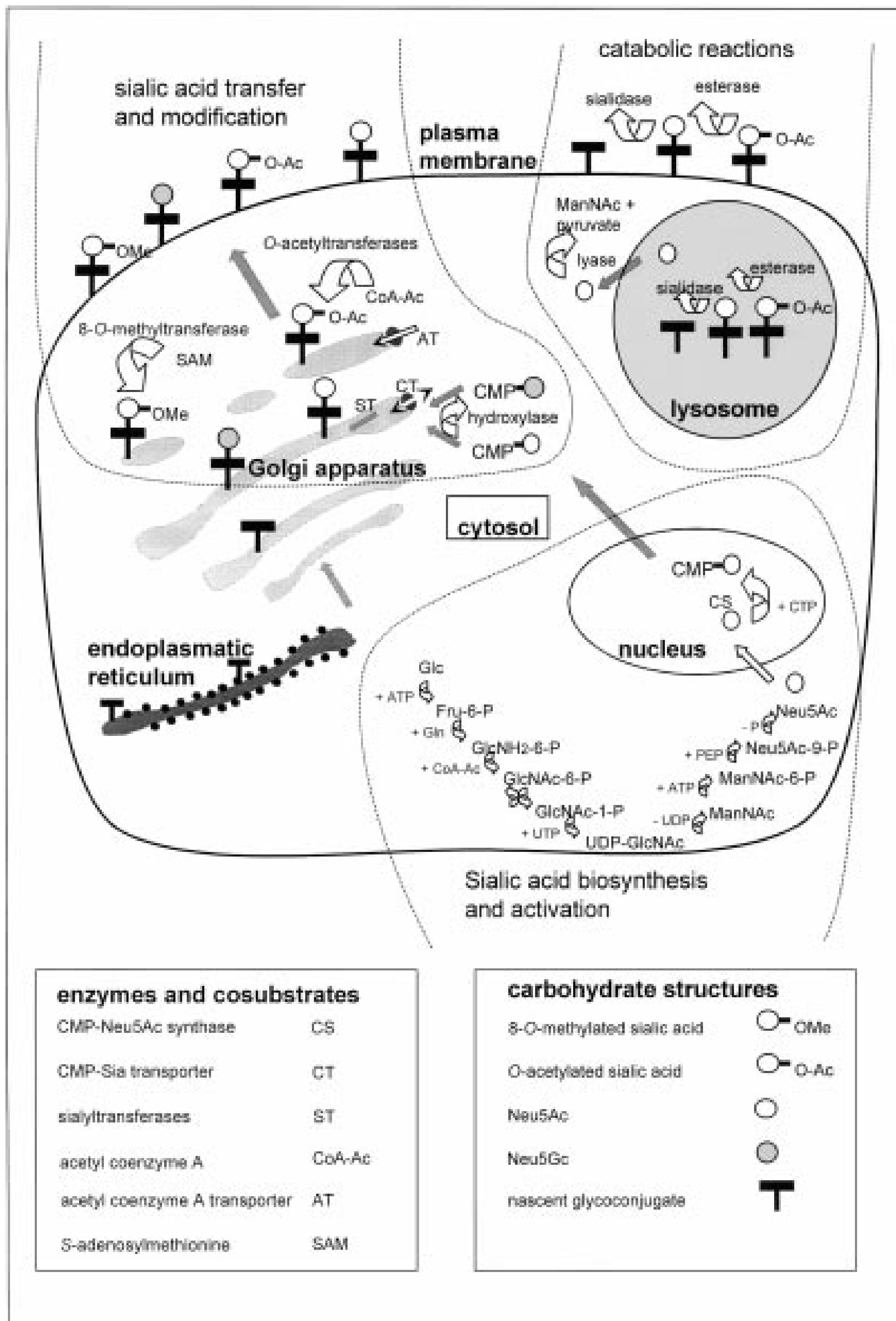

Figure 2. Metabolism of sialic acids. The enzymatic reactions involved in sialic acid biosynthesis, activation, transfer, modification and catabolism are shown with their intracellular localization. (Reproduced from Ref. [55] with permission of the publishers, Birkhäuser Verlag, Basel.)

primary site of the bovine *O*-acetyltransferase is at C-7 forming *N*-acetyl-7-*O*-acetylneuraminic acid (Neu5,7Ac_2), from where the *O*-acetyl group migrates to C-9, probably accelerated by an enzyme, a "migrase" [45]. A non-enzymatic but slow isomerization of Neu5,7Ac_2 to Neu5,9Ac_2 was earlier shown to occur under physiological conditions by NMR spectroscopy [46]. No primary structure or gene encoding the sialate *O*-acetyltransferase is yet available. Several approaches utilising expression cloning led to an increased production of *O*-acetylated Sia, but failed to obtain the gene coding for the enzyme [41,47]. We are trying to reach this goal *via* enzyme purification in order to obtain peptide sequences of the *O*-acetyltransferases, however, this approach has also proved to be rather tedious.

The availability of the primary structure and more knowledge on enzyme properties, as well as on the regulation of gene expression, especially of the 7(9)-*O*-acetyltransferase is urgently required, since this enzyme not only seems to be involved in differentiation and molecular contacts, but also in human tumour biology (see below).

Kdn may also be *O*-acetylated, for example in amphibia (Zanetta *et al.*, unpublished), but nothing is known with regard to the biosynthesis or function of this modification.

Another modification, found in some echinoderm species but only in minute amounts in higher animals (Zanetta *et al.*, unpublished), is the methylation of Sia at position 8 [8]. The enzyme responsible for this reaction is *S*-adenosyl-L-methionine:sialate-8-*O*-methyltransferase which was isolated from the gonades of the starfish *Asterias rubens* [48]. In the presence of Mn^{2+} ions it *O*-methylates both free and glycosidically linked Neu5Ac and Neu5Gc. With regard to the function of this substitution it can only be assumed, on the basis of some observations, that it influences the action of sialidases and the biosynthesis of specific glycan structures. Why this modification, as far as we know, has largely been lost during evolution is unknown. The resolution of this question may be one of the challenges for future research. This is in contrast to *N*-acetyl hydroxylation and *O*-acetylation which have been conserved from echinoderms to mammals.

Sulfation at the same position is also restricted mainly to echinoderms [8]. 8-*O*-Sulfated Neu5Gc was first isolated from sea urchin glycoproteins [49]. It is also considered to influence glycan growth, for example by terminating it by Sia capping [50]. Nothing is known about its biosynthesis and it will be a task of the future to identify a corresponding sialate-8-*O*-sulfotransferase, probably using 3′-phosphoadenosine-5′-phosphosulfate (PAPS) as coenzyme. This will increase our understanding of the astonishingly pronounced Sia diversity, as well as the biology of lower deuterostomes.

Evidence is accumulating that 9-*O*-lactylated Sia is not a rare modification and is increasingly being found in higher animals in significant proportions [8,51]. The biosynthesis of such Sia also awaits further elucidation. In a study with equine liver homogenates we observed the formation of Neu5Ac9Lt in the $105\,000 \times g$ supernatant after the addition of glycoprotein-bound Neu5Ac [52]. The lactyl donor substrate is unknown, however, lactate itself was excluded.

In the future it will also be necessary to investigate the de-/re-*N*-acetylation process of Sia in glycoconjugates, first observed in gangliosides of melanoma cells by A. Varki's group [50,53]. Nothing is known about the enzymes and genes involved, although this reaction is assumed to have great potency in the regulation of cellular events. Furthermore, whether Sia lactones (54, Zanetta *et al.*, unpublished) are formed spontaneously or can also be the result of enzyme action, is another challenging question.

These anabolic enzymes, either already established or still hypothetical, which are involved in creating the Sia diversity, are summarized in Figure 3. They contribute to the enormous variety of complex carbohydrates and are involved in manifold biological and pathological processes. We seem to understand only a few of these regulatory functions and solving these problems will be beneficial for cell biology.

Catabolism of sialic acids

Since sialic acids, being in exposed positions on both macromolecules and on cell surfaces, are potent regulators of cellular functions, their turnover or vulnerability towards degrading enzymes, often from a microbial origin, is of great interest, also with regard to genetic disorders [8]. Early on we became attracted to sialidases, first as a tool to isolate Sia [10] and then in their distribution in nature, as well as in their properties, inhibitors, and molecular genetics (summarized in [8,55]). In early studies it was most striking that Neu5Gc and *O*-acetylated Sia were released from their glycosidic linkage by all sialidases at a much slower rate, with 4-*O*-acetylated derivatives being totally resistant. This is not only of theoretical interest but also of practical significance, especially when studying the biological role of Sia in biological materials where different Sia species occur.

Work in several laboratories revealed two motifs in the sialidase primary structures, one a 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 [56]. This motif is found four to five times in all microbial sequences studied with the exception of viral sialidases, where it is found only once or twice or is even absent [57]. It also occurs in the sialidases from eukaryotic origin, that is, from mammals, including man, and from trypanosomes (summarized in [55]). Likewise, trypanosomal trans-sialidases (see below) have this motif. In contrast to the Asp boxes, which are found throughout the sequence, another motif, FRIP, is located in the *N*-terminal part. It encompasses the amino acids –X-**R**-X-**P** with the arginine and proline residues absolutely conserved. While this region, *via* arginine, is directly involved in catalysis by binding of the substrate molecule, the Asp boxes are believed, based on X-ray analysis, to play a role in maintaining the enzyme structure.

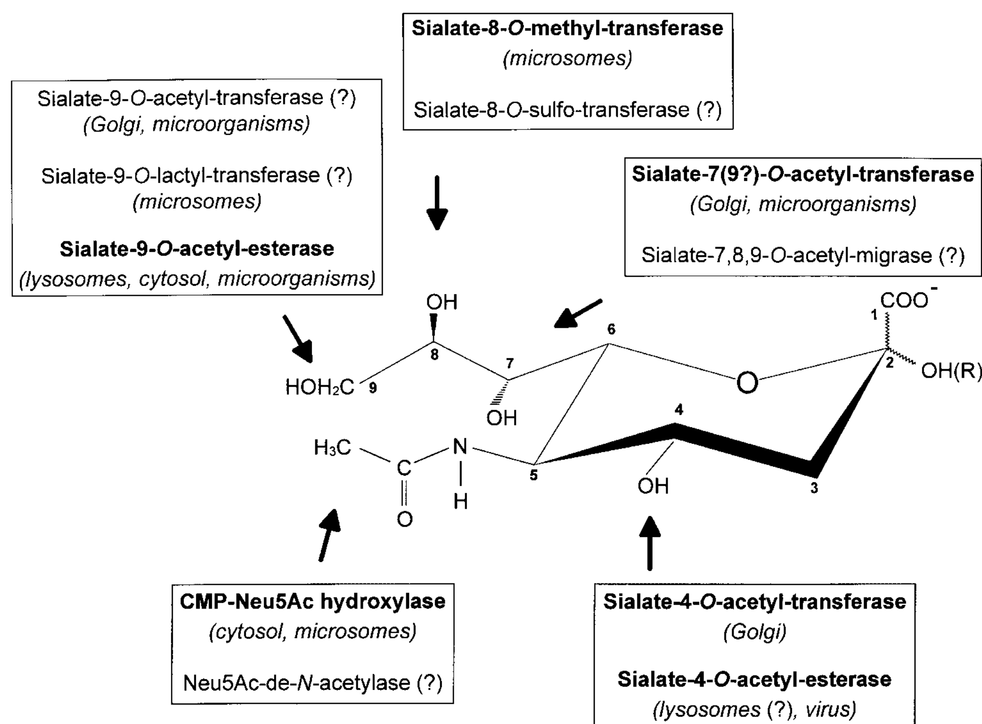

Figure 3. Neu5Ac-modifying enzymes responsible for sialic acid diversity. The well-characterized enzymes are shown in bold print, whereas the existence of those with a question mark is likely but has not yet unequivocally been demonstrated. (Taken from Ref. [50] with permission of the publishers, Wiley-VCH, Weinheim.)

The relationship of pro- and eukaryotic sialidase genes, which is also mirrored in similar catalytic properties and substrate specificities, although exceptions exist, suggests a common origin of the members of this superfamily [55]. There may have occurred an exchange of sialidase genes between animals and bacteria or other microorganisms, possibly with the aid of phages. Further studies will show in which direction this transfer took place, although it is conceivable that microorganisms acquired the sialidase gene from animals they had infected and that this may have contributed to their pathogenicity. Microbial and viral sialidases are involved in many inflammatory processes, which may result in chronic autoimmune diseases, due to demasking of membrane components by Sia loss (see below) [8,55,58]. Research in this field is therefore rapidly proceeding and potent inhibitors are in demand. For instance, relatively high sialidase activities are observed in the wounds and even in the blood serum of gas oedema patients caused by the anaerobic bacterium *Clostridium perfringens*. This can be detected by a fluorimetric enzyme assay and by specific sialidase antibodies which enable a fast diagnosis and consequently specific therapy of this aggressive disease [59]. Unfortunately, an inhibitor with a high inhibitory constant is not yet available, as is also the case for other sialidases from virulent bacteria.

In contrast to bacterial, and also trypanosomal trans-sialidases, potent inhibitors are now available against influenza virus sialidase. This enzyme, together with the hemagglutinin component, is essentially involved in the binding and release of viruses from infected cells [8,55]. Based on the knowledge that Neu2en5Ac is a natural inhibitor of most sialidases, including viral ones, as well as the shape and distribution of charges in the catalytic pocket of viral sialidases which allows hydrolysis of Sia even with substituents at C-4, in contrast to non-viral sialidases [60], the 4-amino- and 4-guanidino derivatives of Neu2en5Ac were synthesized [61]. The latter compound especially was found to be a very strong inhibitor of influenza A and B virus sialidases. This rationally designed inhibitor, and derivatives thereof, are now in medical use and hinder or weaken virus propagation and correspondingly the pathological symptoms of influenza.

A special form of sialidase is the trans-sialidase, which combines the features of sialidases and sialyltransferases. This enzyme preferably transfers the sialic acid molecule from one glycosidic linkage onto another sugar (galactose) molecule instead of water and forms exclusively α2,3 linkages (for reviews see [8,55]). A sugar nucleotide donor, as for the classical sialyltransferases, is not involved. First hints for the possible existence of such an enzyme were obtained during a cooperation with Miercio Pereira, when we identified sialic acids (Neu5Ac and Neu5Gc) in the American trypanosomal species *Trypanosoma cruzi* and found that they were not synthesized by the parasites themselves but were derived from the incubation medium. The Sia had not just been adsorbed to the cells but seemed to be covalently incorporated into the cell

surface molecules. Since these trypanosomes also expressed a sialidase, we speculated that this enzyme may be responsible for the acquisition of the sialic acids [19]. It is known, and utilised for synthetic purposes, that the sialidase reaction under certain conditions is slightly reversible. In 1992 Schenkman's group discovered the trans-sialidase reaction in *T. cruzi* [62], after Previato *et al.* [63] had obtained more evidence for a new route of Sia acquisition. One year later Markus Engstler described this enzyme in the African species *T. brucei* [64]. Strikingly, the catalytic properties of these enzymes were rather similar, pointing to conserved structures and important functions. This is astonishing because the African and American continents began to drift apart many million years ago.

So far, only the trans-sialidase from *T. cruzi* has been purified and the primary structure elucidated, however the first trypanosomal sialidase isolated and recently crystallized, which did not express trans-sialidase activity, was from *T. rangeli* [65,66].

T. cruzi trans-sialidase consists of several domains, one being responsible for its sialidase activity, which also contains Asp boxes, and the other being responsible for the sialyl transfer [67]. Also a galactose binding, lectin-like area exists. With regard to African trans-sialidases [68], neither an enzyme has been isolated, although work in our laboratory on *T. brucei* and *T. congolense* trans-sialidase is ongoing [64,68], nor have the primary structures of the active enzyme proteins been unequivocally elucidated. However, *via* expression cloning in several laboratories promising results are evolving. Gene analysis and enzyme expression of the real trans-sialidase is hampered by the existence of several gene copies in the trypanosomes under study.

Since sialylation of the trypanosomal surface by Sia from host glycoconjugates is considered to protect these parasites from the mammalian host's or the insect vector's defense systems and thus much contributes to the virulence of trypanosomes [69] or of Leishmania after transfection of this enzyme [70], search for effective inhibitors or purified enzyme for vaccination is of great medical importance. On the African and American continents many millions of people and cattle are suffering from trypanosomiasis (Chagas and sleeping diseases). Much work has also to be done to investigate in detail the enzyme properties and catalytic mechanism of trans-sialidases, as well as their molecular genetics and occurrence in other trypanosomal species. In contrast to many other sialidases, trans-sialidases have not yet been crystallized. The relationship of these enzymes to animal sialidases gives raise to the assumption that they may have been derived from genes of higher eucaryotes or even acquired from that source. Indeed, a hint for the existence of a trans-sialidase in human serum was recently described [71].

Another unique sialidase is that from the leech *Macrobdella decora*, which represents an internal trans-sialidase, releasing 2,7-anhydro-Neu5Ac instead of Neu5Ac specifically from α2,3-linkages [72]. It is quite possible that such sialidases forming an internal glycosidic bond are more frequent, since such anhydro sialic acids were found in several animals at relatively large amounts (unpublished data; it has to be noted that this Sia with a blocked glycosidic hydroxyl escapes fluorimetric HPLC analysis).

More research is required in future on the distribution of these different kinds of sialidases/trans-sialidases in micro-organisms and eukaryotic cells; their location within the cell, besides lysosomes; their substrate specificities, i.e. do they prefer gangliosides [73], glycoproteins or oligosaccharides; the influence of their primary structure on properties studied by site-directed mutagenesis [74]; their occurrence as complexes with other enzymes like β-galactosidase and protease; the regulation of their gene expression; the involvement in genetic diseases like galacto-sialidosis [8]; and the pathological consequences of sialidases from microbial infections. The role of these enzymes in inflammation is a largely unknown field deserving more attention.

The enzyme succeeding sialidase in the catabolic sequence is the sialate-pyruvate-lyase (aldolase, EC 4.1.3.3) (for reviews see [8,55,75]). It is localized in the cytosol of mammalian cells or secreted by bacteria into the medium. It does not exist in viruses and has never been reported in trypanosomes. The role of this enzyme is the degradation of Sia in mammalian cells and thus the regulation of recycling of these monosaccharides. In bacteria, the Sia released by sialidases are mainly used for the production of energy and as a carbon source. This requires permeases for the transport of free Sia from lysosomes to the cytosol in mammalian cells [8] (Figure 2) and for uptake into bacteria [55].

The bacterial and mammalian lyases studied, most recently from pig kidney, are similar in their catalytic properties and primary structures. Most strikingly, Neu4,5Ac$_2$ is, as is the case with sialidases, resistant to the action of lyases. The enzymes are rather thermostable and are optimally active between 65 and 80°C. They belong to the class I aldolases, that is, a Schiff's base is formed between the keto group of Neu5Ac at C-2 and a lysine residue of the enzyme protein, prior to the aldol cleavage reaction of Sia between C-3 and C-4. Correspondingly, absolutely conserved lysine and tyrosine residues, the latter probably being involved in stabilization of the Sia side-chain, have been found in the primary structures of a growing lyase family by gene cloning. These amino acids are localized in the catalytic centre of *Escherichia coli* lyase, as shown by X-ray analysis of the crystallized enzyme [76]. Based on the unpublished observation (Traving *et al.*) that the primary structures of the kidney and the microbial lyase are similar, the question is once again raised, like with sialidases, about the evolutionary origin of these enzymes. More knowledge about these phenomena as well as the catalytic mechanism, the regulatory function and, for example, the exact location in the cytosol of mammalian cells by immuno-histochemical methods of these Sia-specific lyases should be obtained by future work. This also includes the use of lyases for the

enzymatic synthesis [8] of natural and modified Sia for biological and pharmacological studies.

Since sialic acids are often *O*-acetylated and, in the case of this esterification at C-4, completely resistant to the action of mammalian and bacterial sialidases, esterases were assumed to exist as initiators of Sia catabolism. Even horse liver sialidase was unable to release Neu4,5Ac$_2$ from glycoproteins [77], although these animals seem to metabolise Sia normally. This pointed to a missing link in Sia metabolism, namely sialic acid-specific esterases. Correspondingly, two enzymes were first detected in horse liver, hydrolysing *O*-acetyl groups from Sia either at C-9 or at C-4 [78]. In the years following these findings, esterases specific for free and/or glycosidically linked Neu5,9Ac$_2$ were discovered in various mammalian and human cells, as well as in tissues such as erythrocytes, liver and brain. These enzymes are localized in the cytosol or in lysosomes (for reviews see [8,9,55]). Various bacterial strains also produce this enzyme, probably as a spreading factor by making *O*-acetylated Sia more vulnerable towards microbial sialidase, as observed on human colon epithelia [58].

When Rudolf Rott in the early 1980s observed that influenza C virus lacking sialidase activity agglutinates erythrocytes only from some mammals, such as chicken, in a Sia-dependent manner, it seemed logical to investigate the Sia nature of these cells. This led to the discovery of Neu5,9Ac$_2$ as a specific "receptor" for these viruses [79]. Following this discovery we identified an esterase which represented a second receptor-destroying enzyme (RDE) known, the first being sialidase from, for instance, influenza A and B viruses. This sialate-9-*O*-acetylesterase is combined with the hemagglutinin and a fusion protein (HEF) of the virus and may be involved in the infection mechanism. The tertiary structure of this complex was recently published [80]. Such an esterase has also been found in corona viruses. Human nasal and bronchial mucin contain a low percentage of Neu5,9Ac$_2$, which may enable attachment of the viruses and infection of the underlining epithelium. Influenza C virus is a specific and very sensitive detector of Neu5,9Ac$_2$ in tissue sections and isolated glycoproteins or gangliosides on thin-layers or in gels. This can be applied to tumour diagnosis, in for example melanoma [81] and basalioma [81a].

A third RDE was recently discovered in mouse hepatitis virus strain S, binding as a hemagglutinin-esterase (HE) complex exclusively to 4-*O*-acetylated Neu5Ac [82]. The three RDE's now known are summarized in Figure 4. Sia-specific hemagglutinin-esterase complexes seem to occur in other virus species, including those infecting fish (R. Vlasak, personal communication). These examples show that different Sia are involved in distinct inflammatory processes and that elucidation of the mechanisms will provide rewarding future projects, also giving chemists the opportunity to synthesize inhibitors of the adhesion or esterase activity as therapeutical tools.

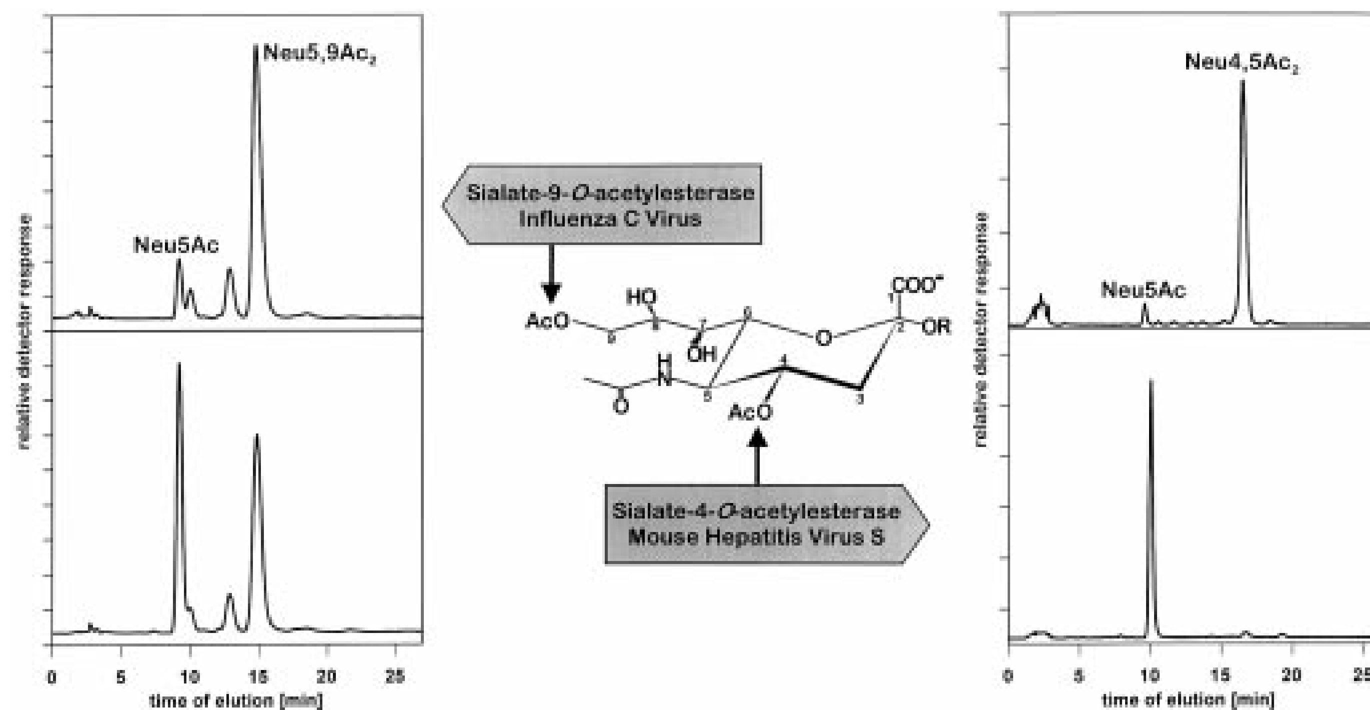

Figure 4. Viral sialate-*O*-acetylesterases as two new "Receptor-Destroying Enzymes". The chromatograms (HPLC, fluorescent Sia derivatives show the conversion of Neu4,5Ac$_2$ and Neu5,9Ac$_2$ into Neu5Ac under the influence of mouse hepatitis and influenza C viruses, respectively. The third and historically first RDE known is sialidase of influenza A and B viruses [8]. (Taken from Ref. [82] with permission of the American Society for Microbiology.)

A hypothetical hydrolase which seems to be widely distributed and awaits investigation, as mentioned above, is the de-*N*-acetylase of bound Sia [8,9]. This enzyme is believed to be involved in cell regulation and, for instance, in the synthesis of cyclic sialyl 6-sulfo Le^x on human leucocytes, which is biologically inactive but can be reactived by hydrolysis of the intramolecular amide bond [83].

Physiological and pathophysiological roles of sialic acids

The external position of Sia on glycoproteins and gangliosides, as well as on the outer cell membranes implies a strong influence in cell biology. Since it is not possible to describe all functions, the reader is referred to various reviews [6–9,28,55]. Here only the main principles of biological effects will be discussed and future perspectives of sialobiology will be outlined.

Firstly, the roles of Sia may be divided into more general ones, irrespective of the variable structures, and those exerted by chemical modifications. Secondly, the biology of Sia may be viewed from its dual role, either masking recognition sites [84] or, in contrast, representing a biological target, that is, allowing recognition by a receptor protein, thus representing a ligand or counter-receptor [28]. The latter role may be again modulated or even abolished by Sia substituents, most effectively by *O*-acetyl groups (see below). These manifold possibilities make understanding of Sia difficult, as the environment of these monosaccharides and the nature of the molecule to which they are bound may influence the biological effects. However, it provides a basis for predicting biological events, if the amount of Sia on cells increases or decreases or if the Sia nature typical for a given cell or tissue changes. In the following, drawing on our own experience, some light will be shed on this situation.

Due to their negative charge Sia are involved in the binding and transport of positively charged molecules as well as in the attraction and repulsion of cells and molecules [3,6–9,50]. In this way, as components of glycoproteins, they contribute to the high viscosity of mucins lining and protecting endothelia, for instance in the intestine or on the surface of frog eggs. Similarly, they influence the conformation of, for example, gangliosides [85] and contribute to the supramolecular structures in cell membranes, thus influencing their functions. These physico-chemical properties of Sia may be influenced by hydrophobic substituents such as *O*-acetyl or *O*-methyl groups or by hydroxylation of the *N*-acetyl group, providing more hydrophilic properties and even a further site for glycosidic linkage (see above). By sulfation, as observed in echinoderms, a very acidic Sia is produced. The negative charge of Sia also contributes to the anti-proteolytic effect of Sia in glycoproteins, as well as hindering the action of some endoglycosidases [3,8].

The latter observations can be grouped into the anti-recognition effects of Sia, exerted by the negative charge in combination with the bulky, hydrophilic molecule. This is a very large and important field comprising the masking of the penultimate sugars which nature has designated to be recognized by receptors, like galactose, or the less specific shielding of antigenic sites in macromolecules or in cell membranes [28,84]. Desialylation, in the first case, leads to recognition by galactose-specific lectins and, in the second case, to better recognition of macromolecules and cells by the complement or the immune system. The purpose of the first effect is to target molecules and cells to specific sites, which, however, often leads to degradation. Therefore, this may be of physiological or pathological importance.

The first reported example for this phenomenon was the uptake of desialylated serum glycoproteins by hepatocytes [86]. A similar function was observed between liver and spleen macrophages and erythrocytes, first observed with human red blood cells [87], which have lost 10–20% of their surface Sia (1–2×10^7 molecules/cell) either by ageing or under the influence of sialidase from blood serum or microorganisms. The red cells bind to the phagocytes *via* their demasked galactose residues to a galactose-specific receptor and ultimately are taken up and degraded [88]. This mechanism can work without the involvement of immunoglobulin or complement and represents the main sequestration process of aged cells [89]. Sialidase-treated lymphocytes also attach to macrophages by this mechanism (Figure 5), however, are not engulfed but are released after about one day incubation due to resialylation of the cell surface [90]. Also malignant cells can be eliminated in this way, and it is understandable that oversialylation, often observed in such cells, protects them from humoral and cellular defense systems and thus increases their malignancy. Sialylation of microorganisms follows the same strategy, allowing better survival in the host organisms. This can be achieved in different ways, like total

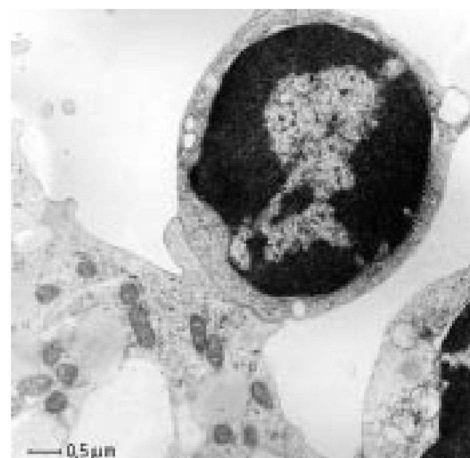

Figure 5. Binding of a sialidase-treated rat lymphocyte to a peritoneal macrophage, a part of which is visible on the lower left side, by the galactose-recognizing receptor after 8 h of incubation. Transmission electron microscopy. (C. Fischer, Diploma thesis, University of Kiel, 1988; unpublished.)

synthesis of sialic acids and possibly polysialic acids (colominic acid in *E. coli* strains, [8]), the acquisition of Sia from the host with the aid of trans-sialidases in some trypanosomal strains (as described above), or the transfer of Sia from CMP-glycoside of the host by a sialyltransferase expressed by the pathogenic bacterium (for example gonococci, studied in detail by the group of Smith, [17]).

A second example is the production of auto-antibodies, which may lead to chronic diseases like glomerulonephritis [58], however, it should be mentioned that this can only occur after cell membrane desialylation, possibly by bacterial or viral sialidases. Carbohydrates, including Sia, may be antigenic determinants, but they often shield antigenic sites and thus weaken the immuno-reactivity. Sia render cells as "self", not allowing recognization by the immune system. The loss of Sia makes these cells more "non-self" and, therefore vulnerable.

In contrast, sialic acid molecules take part in a variety of recognition processes. This may be the most important role of these monosaccharides, especially since their involvement in the interaction of mammalian cells became known only in the last ten years. Until then Sia-recognizing receptors were known only from some viruses, bacteria, low animals and plants. For reviews see Ref. [28,91]. When studying the galactose-receptor from rat peritoneal macrophages, we observed a modulating effect of Sia on affinity [92], thus initiating considerations that Sia may not only have an anti-recognition function. Shortly thereafter a fruitful cooperation began between Paul Crocker, Jim Paulson and Soerge Kelm, resulting in the discovery of a Sia-binding lectin (Sn, sialoadhesin) in macrophages from murine bone marrow cells [93]. This receptor belongs to the immunoglobulin super-family (IgSF), of which, until now, at least ten members have been discovered in several laboratories, including CD22 on B-cells, and MAG, the myelin-associated glycoprotein on oligodendrocytes and Schwann cells as the earliest and best characterized species [28]. All these receptors have been denominated "Siglecs" [94].

Another group of mammalian Sia-recognizing lectins are the selectins found on endothelial cells, which also possess repeating domains [28]. They participate in the initial stage of adhesion of white blood cells to endothelia, along which they may begin to role and eventually evade into the underlaying tissues, which are damaged by the lack of oxygen, in for example transplants or infarcts, or which are inflamed. Cytokines play here an essential role. Selectins preferably recognize sialylated Lewis structures ($SiaLe^x$). Since these can also occur on tumour cells, selectins can be involved in the formation of metastases.

These principal roles of Sia, as either a masking agent or a receptor, can be influenced by chemical modification of Sia [8,28,29,41,50], as summarized in Figure 6. The masking function is increased and prolonged, by modifications retarding the release by sialidases of the corresponding Sia, Neu5Gc and *O*-acetylated derivatives, from glycoconjugates and thus

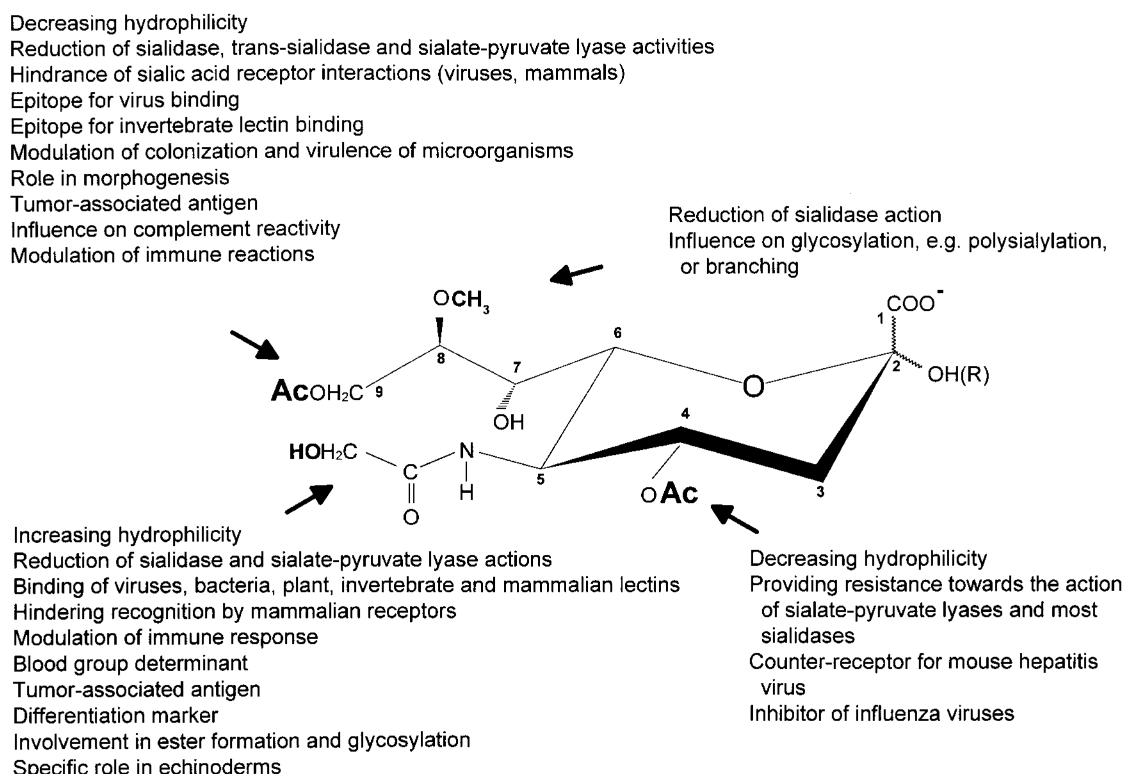

Figure 6. Survey of the biological significance of the sialic acid substituents. (Taken from Ref. [50] with permission of the publishers, Wiley-VCH, Weinheim.)

extending the life-time of molecules and cells. Dramatic changes may occur in the counter-receptor function of Sia, either by abolishing or modifying it. As an example, hydroxylation of Neu5Ac leads to better binding to the murine and human CD22, but sialoadhesin and MAG do not tolerate an *N*-glycolyl residue [95]. Also other Siglecs including those from man and Great Apes bind to Neu5Ac and Neu5Gc with different affinities, from which it may be concluded that loss of Neu5Gc in humans influenced the evolutionary process [96]. The attachment of some virus species, however, was found to be improved by hydroxylation [29].

With regard to *O*-acetylation, the necessity of such ester groups for influenza C and mouse hepatitis virus binding was described above. In contrast, 9-*O*-acetylation prevents the attachment of influenza A and B viruses, as well as of malaria parasites [41]. Also the interaction with Siglecs (CD22, MAG, Sn) is much hampered by this modification [28,97], as well as the binding of sLe^x to selectins [98]. In the latter case, loss of *O*-acetylation of sLe^x in human colon cancer facilitates metastasis by the mechanism outlined previously in this review. It is also thought that in this area we are beginning to understand the role of Sia in the communication between cells and its physiological and pathological consequences. More knowledge will accumulate also in the interaction of host Sia with viruses and other microorganisms, which have "misused" these monosaccharides for infection (see above). Attention was recently focused on *Helicobacter pylori*, often the cause of gastric diseases, of which several strains bind to the epithelia *via* Sia [99]. The receptor–ligand interaction involved in colonization can be inhibited with soluble ligands, sialylated oligosaccharides and glycoproteins, such as from milk, which gives these substances a great "glycopharmacological" potential. This will gain significance as alternative therapies to treatment with antibiotics.

Reversible sialylation is believed, together with other glycosylated structures, to regulate cell adherence and mobility during embryogenesis and also malignant growth [8,28]. A model of reversible sialylation of galactose residues was designed [28], which implies reversible interaction of cells by the galactose-recognizing receptor and may be significant especially in developmental biology. Evidence is also increasing that glycosylation and sialylation, respectively, influence the function of receptors and, for example, of ion-channel proteins [100]. The recent, rather striking report [101] that in *Drosophila* "Fringe" is a β1,3-*N*-acetylglucosaminyltransferase initiating elongation of *O*-linked fucose residues on "Notch" thus altering the ability of Notch to bind to "Delta" ligands involved in the regulation of signalling pathways during the development of this insect, points also in this direction.

Different types of Sia may be involved in such processes, which may explain the variation of not only the total Sia content when compared to the adult, but also the proportion of, for instance Neu5Gc, as observed in fetal calf [102] and rat [103] tissues. Remarkably, there was almost no *O*-acetylation in fetal calf, similar to chicken erythrocytes, which express $Neu5,9Ac_2$ in erythrocytes only after hatching [104].

Further research is required to understand the fine mechanism of action of such differentiation markers. This is also valid for corresponding changes on tumour growth. There are reports on the expression of small amounts of Neu5Gc, for example on gangliosides, in human tumours [8,9,29,40]. Since the hydroxylase activity has never been detected in such tissues and Neu5Gc can be derived from food (see above), a nutritional origin of Neu5Gc is presently suggested [29]. Gangliosides, especially GD3, with $Neu5,9Ac_2$ as terminal sugar are also considered as tumour-associated antigens. Increased amounts are found in tumours of neuroectodermal origin such as brain, mammary and skin tumours, but also in rapidly growing normal tissues (summarized in [41,81,82]). It can only be speculated that this transmembrane signalling events stimulate angiogenesis, thus accelerating tumour growth.

Conclusion and outlook

It may have become obvious that Sia are involved in many biological processes, and future directions have been indicated throughout the text. The main roles seem to be structural and protecting effects, especially on cell membranes, and to regulate molecular interactions and thus the cross-talk between cells and infectious agents. Since these are crucial events in cell biology, the regulation of the "right" amount of Sia is necessary. This is also true for the molecules to which Sia are bound. Thus, imbalances can emerge not only from alterations in the biosynthesis and transfer of the different Sia, but also of the supporting molecules. This may be due to changes in gene expression or to the influence of hormones or toxins and can result in genetically inherent or acquired "glycoprotein deficiency syndromes". If hereditary, these diseases are now named "Congenital Disorders of Glycosylation" (CDG) [105]. Not much is known on hormonal effects, but insulin for example influences the sialylation of cells [106]. Alcohol is an example of a toxin that decreases sialylation, also of gangliosides, and explains the neurological effects of alcohol abuse [107,108]. Sialic acids will have a great future in medical sciences and one of the most recent examples are encephalopathies caused by prions, the glycosylation residues of which are sialylated [108a]

These observations require further studies on the regulation of the biosynthesis of sialylated glycoconjugates and on the rate-limiting metabolic events. Fascinating insight has recently been obtained into the molecular genetics and properties of the key enzyme of Sia metabolism, the UDP-GlcNAc epimerase [109], as one step in this direction. The dietary aspect of glycobiology is becoming more and more recognized, not only with regard to the influence on bacterial and viral colonization in mucous epithelia but also with regard to supply of the organism with special carbohydrates, including Sia [110,111].

The high Sia content of milk, especially of colostrum, as well as the relatively slow Sia biosynthesis rate observed in rat fetuses at birth [112] points to the latter aspect.

Sialyltransferase inhibitors are becoming more important, since "over-sialylation" is a widely studied phenomenon of various tumour cells [8,113] and selectins recognizing Sia have been shown to be involved in metastasis [114]. One of the first studies on the inhibition of sialyltransferases by CMP-Neu5Ac derivatives was carried out in our laboratory [115], but stronger inhibitors are required for therapeutic application [116].

Carbohydrates also play an eminent role in immunology [117]. The effect of Sia on cells being "self" and the role of Siglecs, for instance enabling the communication between B and T lymphocytes has been mentioned. Here, the problem of xenotransplantation of pig tissues is addressed, since these animals express, apart from the immogenic epitope α-galactose [118], relatively large amounts of Neu5Gc [8,29]. The antigenicity of this Sia species for man may create a further problem in the transplantation of tissues from pig.

Polysialic acids represent a further large and fast developing area of sialobiology, which has not been discussed in this review [119]. Their great impact in the regulation of cell adhesion and growth, especially in developmental, neurological and tumour biology [120,121] and also in insect larvae [18,19], render research in this area rewarding.

This article has hopefully shown that future research on sialic acids and the molecules to which they are bound is worthwhile and will lead to better understanding of their function.

Acknowledgements

I take this opportunity to thank my former and present associates at the universities of Bochum and Kiel for their cooperation over so many years and I am happy to express my gratitude to many colleagues, all over the world, for their fruitful cooperation, friendship and stimulating discussions. Joe Tiralongo's important support with this article is greatfully acknowledged.

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