METABOLISM AND ROLE OF *O*-ACETYLATED SIALIC ACIDS

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In the still growing family of sialic acids with more than 40 different derivatives of neuraminic acid, the interest of the scientific community is increasingly directed towards the *O*-acetylated species. In the past two decades it evolved that *O*-acetylated sialic acids play fundamental roles in the development of organisms, in the regulation of the immune system, in cancer processes and many other biological and pathophysiological events[1-4].

Naturally occurring sialic acids can be *O*-acetylated at all their hydroxyl groups, i.e. at positions C4, C7, C8 and C9 of these carboxylated sugars as it is shown in Figure 1. They mostly are mono-*O*-acetylated, but combinations of acetyl groups at two or more positions generate oligo-*O*-acetylated derivatives. The biological effect is depending on the position relative to the 9-carbon scaffold and whether it is the *N*-acetylneuraminic acid

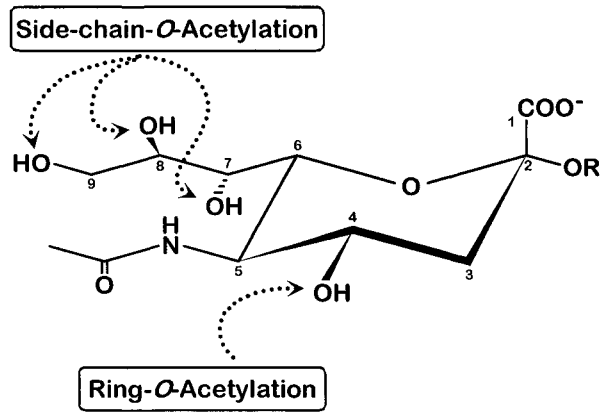

Figure 1. Naturally occurring *O*-acetylated sialic acids
Sialic acid *O*-acetylation can take place at the positions C4, C7, C8 and C9. They can be mono-*O*-acetylated or oligo-*O*-acetylated, e.g. a combination at C7 and C9 leads to the di-*O*-acetylated Neu5,7,9Ac_3.

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(Neu5Ac) or the *N*-glycolylneuraminic acid (Neu5Gc). For example, these modifications prevent a fast degradation of sialylated glycoconjugates by prokaryotic and eukaryotic sialidases, trans-sialidases and sialate lyases[3]. Other biological effects will also be discussed in the paragraphs "Biological functions" of the specific sections.

Although *O*-acetylated sialic acids occur mainly in the animal kingdom, there are some exceptions. Among the prokaryotes, certain sialic acids-expressing bacteria, most of them pathogenic for mammalians, contain *O*-acetylated sialic acids[5], as will be shown later.

It is well known that eukaryotic sialic acid anabolism is a complex enzymatic event taking place in the cytosol and ending up with the activation of free Neu5Ac by CTP in the nucleus. The resulting CMP-Neu5Ac and CMP-Neu5Gc, the latter being synthesised by a cytosolic CMP-Neu5Ac hydroxylase (EC 1.14.99.18)[6], serve as donor substrates in various acceptor-substrate-specific sialyltransferase reactions in the Golgi apparatus. Finally, the

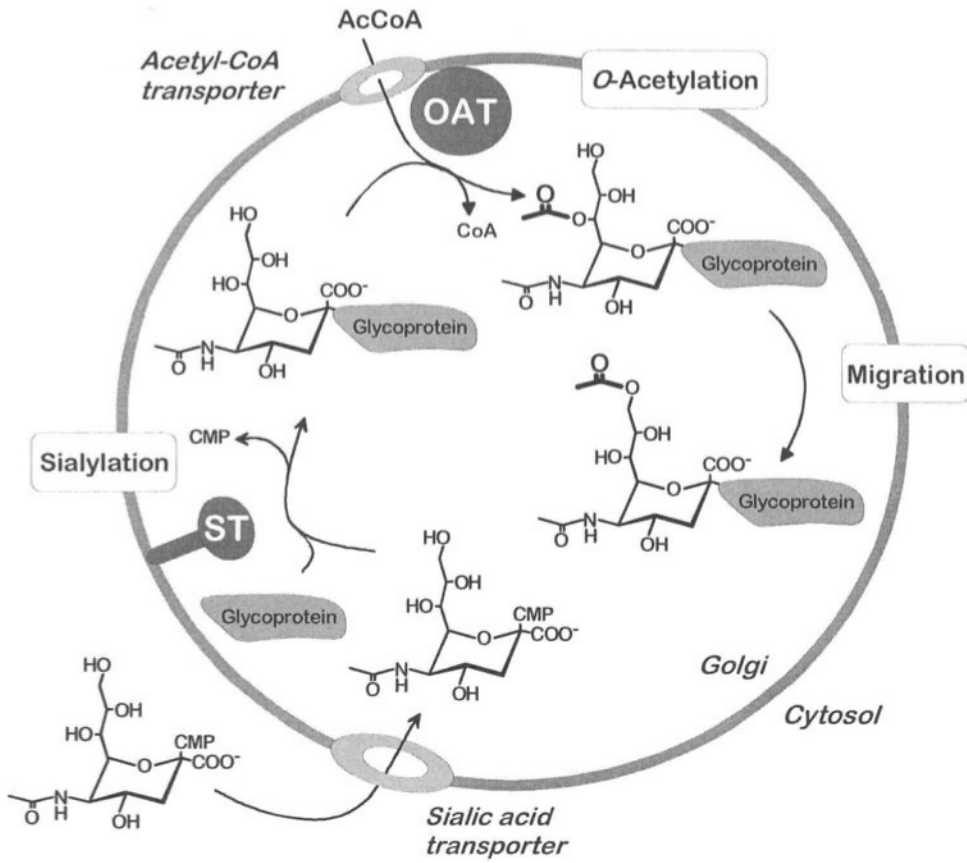

Figure 2: Model of side-chain *O*-acetylation of sialic acids
Sialic acids are transported in their CMP-activated form into the Golgi apparatus, where they serve as substrates for different sialyltransferases (ST). As an example here the transfer onto a glycoprotein is shown, although the same mechanism may function for glycolipid sialylation. AcCoA enters the Golgi via an AcCoA-transporter to provide it for the *O*-acetyltransferase (OAT), which transfers the acetyl moiety to the glycosidically bound sialic acid probably at position C7. After a migration to position C9, which could be enzymatically catalyzed, an additional *O*-acetylation reaction can take place at position C7. This model, which has not yet been firmly established in all details, is based mainly on the references [7] and [8]. From our unpublished analytical data the *O*-acetylation at the stage of CMP-Neu5Ac can not be completely excluded. Sialic acid 4-*O*-acetylation may occur in a similar way.

sialate-*O*-acetylation occurs at the α-glycosidically linked sialic acid[1].

The understanding of the metabolism of *O*-acetylated sialic acids can be divided into two parts. Firstly, the formation of acetyl esters at the different hydroxyl groups of sialic acids by the transfer of activated acetyl groups from acetyl coenzyme A (AcCoA) by diverse *O*-acetyltransferases (Figure 2). Differing in their regioselectivity, basically two groups of sialate-*O*-acetyltransferases exist: the acetyl-CoA:sialate 7(9)-*O*-acetyltransferase (EC 2.3.1.45) is responsible for the frequently found side chain *O*-acetylation which can most impressively be observed in bovine submandibular glands[9], whereas the reaction of the acetyl-CoA:sialate 4-*O*-acetyltransferase (EC 2.3.1.44) leads to the pyranose ring-*O*-acetylation at C4. As shown for the biosynthesis of 9-*O*-acetylated sialic acid in rat liver and bovine submandibular glands, the 4-*O*-acetyltransferase reaction investigated in guinea-pig liver takes place in the Golgi apparatus or trans-Golgi-network. However, up to now no structural information about a purified enzyme or its gene from either tissue is available, although several attempts to isolate the enzyme from different tissues were made by using various techniques including classical enzyme purification or expression cloning[10].

Secondly, several enzymes degrading free or glycosidically bound *O*-acetylated sialic acids are well characterized, like esterases for Neu5,9Ac_2 and Neu4,5Ac_2, respectively, which are found e.g. in influenza C virus and frequently in lysosomes of mammalian cells on the one hand or in mouse hepatitis virus and in horse liver, on the other[3,11-13].

ANALYSIS OF *O*-ACETYLATED SIALIC ACIDS

Before discussing the biochemistry and biology of sialic acid *O*-acetylation in pro- and eukaryotic systems, the very subtle process of the analysis of these sugars will be shortly addressed. A severe problem in the analysis of glycoconjugate-bound *O*-acetylated sialic acids is the initial step of glycosidic bond cleavage, since the mildest hydrolysis, which is possible by sialidases, will lead to an incomplete release of derivatives acetylated at the side chain, whereas 4-*O*-acetylated sialic acids are not released at all[2,3], with the exception of some viral sialidases[14]. Alternatively, hydrolysis with various mild acids is often applied[15], which allows the liberation of all kinds of sialic acids but is a compromise between a maximum release of sialic acids, however, slightly dependent on the type of carbohydrate linkage and the character of the glycoconjugate itself, and a minimal loss of *O*-acetylation. Alkaline treatment, of course, has to be excluded during this kind of analysis. Due to these reasons we have to keep in mind that 20-30% of *O*-acetylated derivatives are lost during careful hydrolysis and the subsequent purification procedure of the liberated monosaccharides by ion-exchange chromatography[16].

Presently, methods for a fast, sensitive and reliable sialic acid analysis, beginning with colorimetric determination, TLC, HPLC[17], Reversed Phase-HPLC of fluorescently labelled compounds[18], also in combination with Electrospray-Ionisation-MS[19], and GC-MS after pertrimethylsilylation[20], to NMR[21] have greatly improved and enable detection of these compounds in the fmol range.

In addition to these physico-chemical techniques, other methods for the analysis of *O*-acetylated sialic acids are known, among them antibody recognition, lectin staining and detection by influenza C viruses. The different methods for the use of influenza C virus as analytical tool[22,23] are based on its receptor-destroying enzyme, the sialate 9(4)-*O*-acetyl esterase (EC 3.1.1.53)[24]. Only few lectins are known to recognize specifically either 9-*O*-acetylated[25,26] or both, 4-*O*-acetylated and 9-*O*-acetylated sialic acids, as found in crustaceans[27,28]. The majority of sialic acid-specific lectins only recognise glycosidically bound Neu5Ac or Neu5Gc[1]. In contrast to the use of lectins, monoclonal antibodies against

sialate side chain *O*-acetylation are well known[29-32], whereas a recognition of 4-*O*-acetyl sialic acids by antibodies has been rarely reported[33].

SIALIC ACID 4-*O*-ACETYLATION

Occurrence

Due to the lack of 4-*O*-acetylated sialic acids in man, where 9-*O*-acetylation is predominantly found, not much interest was focussed to the only possible pyranose ring *O*-acetylation of sialic acids in former sialic acid analyses, though a report of Neu4Ac5Gc-GM3 describes this ganglioside to be a tumor-associated antigen in human colon cancer[33]. However, no additional examples of sialate-4-*O*-acetylation in man have been given.

Another reason for this neglect may have been the previously mentioned poor preparative and analytical tools for sialic acid analysis. In parallel to the development of techniques protecting the *O*-acetylated sialic acids during purification, reports showed these derivatives to occur in a Japanese dace[34], a South American pit-viper[35], the Australian monotreme echidna[36], the horse, the donkey[3], and in the rabbit[37] besides the guinea-pig[38].

Metabolism

As previously mentioned in the introduction, several species- and substrate-specific *O*-acetyltransferases (OAT) occur, none of which has been purified or cloned so far. In this respect the investigation of the 4-OAT from guinea-pig liver, which has been described for the Golgi-membrane bound enzyme[39], seems to be a promising approach.

The enzyme revealed a high affinity to acetyl-CoA and CoA, expressed by an app. K_M and an apparent K_i of 0.6 and 4.2 μM, respectively; CoA inhibited the enzyme in a mixed competitive manner. Therefore, affinity chromatography by either acetyl-CoA or CoA matrices is supposed to be a good strategy to increase the yield of specific enzyme activity and to purify the enzyme to homogeneity. We were able to solubilize the 4-OAT, which is stable even after freeze- and thawing cycles. Among various substrates, including free sialic acids, sialosaccharides and sialoglycoproteins, gangliosides have been shown to be *O*-acetylated most effectively.

The Golgi-membrane-bound 9-OAT in rat liver, extensively investigated by Varki and co-workers[1,7,40], obviously shows parallels with regard to the kinetic and inhibition parameters and thus allows deduction of basic properties for this enzyme family in general. However, the existence of a transmembrane enzyme complex, which was favoured in rat liver, has never been unequivocally confirmed.

Another enzyme system under investigation, which greatly differs from the system described above, is the 4-OAT from horse submandibular glands, first mentioned by Schauer[41]. A soluble enzyme within this tissue, as observed in bovine submandibular glands, could not be verified. Due to the unusual constitution of mucin-secreting cells, an isolation of Golgi-membranes is difficult. However, the endogenous 4-OAT activity was detected in the microsomal fraction of this tissue. Work is in progress to characterize and solubilize the membrane-bound enzyme. It will be very interesting to look at its substrate specificity, since *O*-glycan-bound Neu4,5Ac_2 is the main *O*-acetylated sialic acid in the horse submandibular gland[42], whereas Neu4Ac5Gc-GM3 is the main *O*-acetylated component on horse erythrocyte membranes[43].

The finding of the 4,8-anhydro derivative of Neu5Ac, first described in collocalia mucin from edible birds nest substance by Pozsgay et al.[44], raises the question for its origin.

These authors suggested that this sialic acid modification originates from Neu4,5Ac$_2$ during the release of glycosidically bound sialic acids under acidic conditions. Detailed investigations by Manzi et al.[45], however, revealed a conversion of free, but not glycosidically bound Neu4,5Ac$_2$ to its anhydro compound under alkaline conditions. A small amount of 4,8-anhydro-Neu5Ac during acid hydrolysis of horse serum confirmed the assumption of Pozgay, but does not explain the relatively high amount (10 %) of this anhydro-sialic acid in collocalia mucin. In our laboratory this sialic acid was isolated only from an older preparation of this mucin, whereas two fresh samples obtained from Indonesia and Vietnam, kindly provided by A. Wu, Taipei, contained Neu5Ac exclusively.

Because eukaryotic sialidases are not able to liberate 4-*O*-acetylated sialic acids, and acylneuraminate lyases (EC 4.1.3.3), which split free sialic acids into acylmannosamines and pyruvate, do not accept 4-*O*-acetylated sialic acid as substrates, too, the existence of a further enzyme has been assumed that removes such *O*-acetyl esters prior to further degradation of the sialic acid. This led to the discovery of a sialate 4-*O*-acetylesterase (4-OAE) in horse liver which has been partially purified (Schauer *et al.*, unpublished results). It exists in isoforms and also hydrolyses sialate-9-*O*-acetyl esters, although to a lesser extent[24]. Recently, a viral 4-OAE has been reported to occur in mouse hepatitis virus (MHV)[13], as already mentioned above.

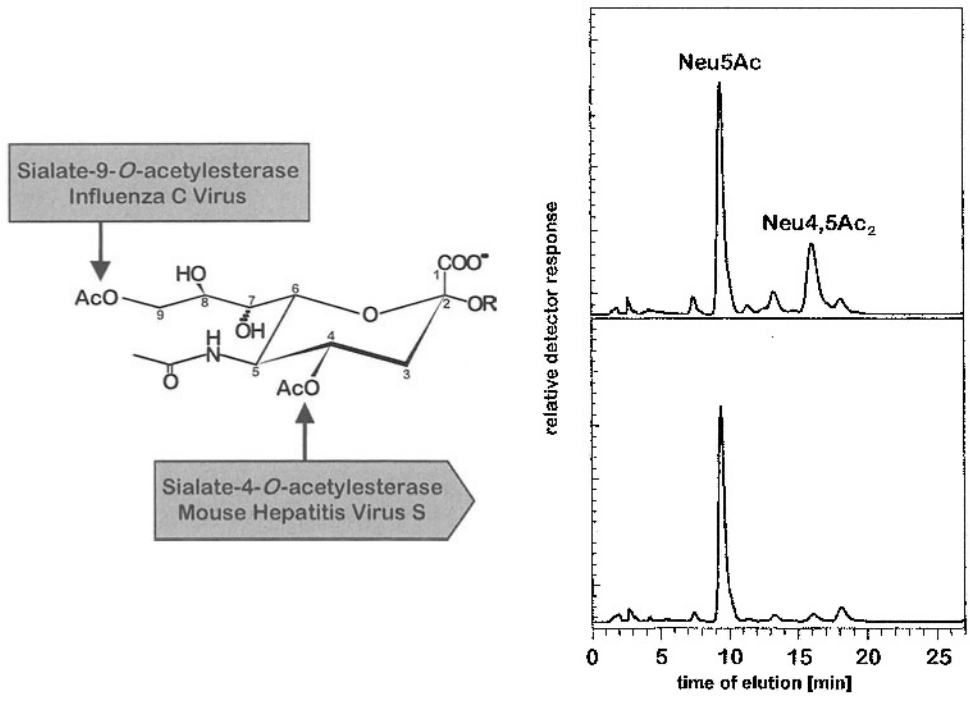

Figure 3: Viral esterases as receptor-destroying enzymes
Mouse hepatitis virus (MHV) is able to de-*O*-acetylate guinea-pig serum glycoprotein, which carry glycosidically bound Neu4,5Ac$_2$, as shown on the right site. The upper chromatogram shows the sialic acid composition of untreated serum glycoproteins, the lower panel displays the situation after treatment of the glycoproteins with MHV containing 4-OAE activity. Sialic acids were analysed after hydrolysis by fluorimetric HPLC. On the left a comparison of the different sites of OAE activities from influenza C virus and MHV are indicated.

Biological function

Still little is known about the role of 4-*O*-acetylated sialic acids compared to the wealth of information on the corresponding side chain modifications. The most obvious effect of the sialic acid 4-*O*-acetyl group is its protective function of the parent molecule towards the degrading enzymes sialidase and acylneuraminate-lyase. As a potential inhibitor of influenza virus infection, Neu4,5Ac_2 does not appear to increase the avidity of interaction with the viruses, since isolated oligosaccharides of equine α_2-macroglobulin are no stronger inhibitors of adsorption than oligosaccharides of human α_2-macroglobulin, which is a relatively poor inhibitor and contains only Neu5Ac. Since Neu4,5Ac_2 is almost resistant to cleavage by viral sialidases, it may protect the inhibitor from inactivation by these enzymes[46,47]. There is no evidence for 4-*O*-acetylated sialic acids serving as ligands for endogenous acceptors as it was investigated with 9-*O*-acetylated species, but the discovery of the viral 4-OAE may give us a hint in this direction.

Thus, the predominant role of 4-*O*-acetylated sialic acids seems to be its influence on the catabolism of respective carbohydrate chains. Therefore, the 4-OAE is involved in the initial step of degradation of such carbohydrate moieties, as was discovered in horse liver[3]. Recent investigations revealed also a 4-OAE in mouse hepatitis viruses (MHV, Figure 3). The function of this esterase is not clear at present, as there is no evidence for any sialate-4-*O*-acetylation in adult murine brain. As MHV infects the juvenile murine brain, a developmentally regulated expression of 4-*O*-acetylated sialic acids in this brain may be expected. Interestingly, lectin-binding investigations in mature male mice point to the existence of sialate-4-*O*-acetylation[48], though the frequent occurrence of sialate-9-*O*-acetylation in this species is well known[49,50].

SIALIC ACID 7(9)-*O*-ACETYLATION

Occurrence

Based on the above mentioned improved analytical techniques increased knowledge of *O*-acetylation of the glycerol side chain of sialic acid and its widespread distribution is now available. The *O*-acetylated derivatives of Neu5Ac and Neu5Gc are present in almost all deuterostomal species[2-4], in a yeast strain[51] and also in a few bacteria, as will be shown later in this article.

The expression is tissue- and development-specific; for example, the human colon mucosa shows high levels of *O*-acetylation of sialic acids, whereas during cancerogenesis the *O*-acetylation decreases already in the first stage of the adenoma-carcinoma sequence[52]. In contrast to this, melanoma[2] as well as basalioma[53] are characterized by a higher expression of *O*-acetylated gangliosides than normal skin; these glycosphingolipids can therefore be considered as tumor-associated antigens. A similar phenomenon was observed during the development of chicken erythrocytes. After hatching, the 9-*O*-acetylation of sialic acids on the surface of erythrocytes is increasing[54].

As another example, side-chain *O*-acetylated sialic acids are also expressed on human leukocytes in a range of 1 to 5 % of the total sialic acid content (G. Kohla, unpublished results,[55]), where they can be used as markers for specific subsets of T-helper cell, known as CDw60, when the 9-*O*-acetylated sialic acid is the terminal sugar of the disialoganglioside GD3[56], which is also expressed on activated B-cells[57]. Flow cytometric analysis showed that each major class of human leukocytes contains a significant fraction of cells expressing 7-*O*-acetyl GD3[30]. Large amounts of various *O*-acetylated sialic acids were also found in

bovine submandibular gland[9,19], rat liver[58], healthy human colon[52] and the erythrocytes from rat, mouse and rabbit[59].

Metabolism

The enzymatic *O*-acetylation of sialic acids was first described in bovine and equine submandibular glands[41,60]. In comparison to the *O*-acetylation of the ring hydroxyl group at C4, described in the previous chapter, here the acetyl esters are formed with the hydroxyl groups of the glycerol side chain of sialic acids. All three hydroxyl groups can be *O*-acetylated, but up to now it is not known whether there are different enzymes responsible for the *O*-acetylation of each hydroxyl group. Following our favoured hypothesis[61], only one enzyme catalyses the acetylation reaction at position C7 and a subsequent migration of the *O*-acetyl groups to C8 and C9 occurs (see Figure 2). Therefore, this transferase is addressed as AcCoA:sialate 7(9)-*O*-acetyltransferase. Data from the bovine submandibular gland system, which represents a biosynthetically highly active tissue, support the latter hypothesis[8].

Only few successful attempts are known for partial purification and characterization of the enzyme. Diaz and co-workers studied the enzyme from rat liver[62]. They found that it is membrane-bound and localized in the Golgi membranes, similar to the 4-*O*-acetyltransferase from guinea pig liver[39]. At least in rat liver, it seems to be co-localized with an acetyl-CoA-transport protein, which can enhance the transferase activity efficiency. The localisation in Golgi membranes supports the momentary hypothesis that glycoconjugates are sialylated in the trans-Golgi or the trans-Golgi network and *O*-acetylation of sialic acids is an event, which modifies glycosidically bound sialo-glycoconjugates at a late step of oligosaccharide maturation.

Another tissue, used for the purification and characterisation of the enzyme, is bovine submandibular gland. As in rat liver, the enzyme activity is membrane-bound and co-localized in microsomes with sialyltransferase activity, typical for Golgi-membranes[8]. In addition, a soluble enzyme activity was found in the preparations of this tissue, and it was partially purified (R. Schauer, unpublished results). Whether this is a product of proteolysis or a splicing variant of the membrane-bound enzyme, is yet unknown.

The primary attachment site of the acetyl residue transferred by this enzyme in bovine submandibular gland seems to be at C7[8]. It is well established that the *O*-acetyl ester of sialic acids can migrate from C7 to a free hydroxyl group at C9[63]. Based on these findings we demonstrated that influenza C viruses exclusively bind to 9-*O*-acetylated ganglioside GD3, but not to the 7-*O*-acetylated derivative (Figure 4). After weak alkaline treatment, which induces migration from position C7 to C9, staining by influenza C virus appeared at the location of previous 7-*O*-acetyl-GD3.

Single *O*-acetyl groups at C8 seem to be very labile, presumably because they migrate quickly to C9. Di- or tri-*O*-acetylated sialic acids may be therefore the result of a combination of such a transfer of acetyl groups, and the additional transfer of a second and later eventually a third acetyl group again to the free hydroxyl group at C7 [3,61].

Since *O*-acetylated sialic acids more or less withstand the attack of sialidases or lyases, sialate-9-*O*-acetylesterases (9-OAE) play an important role in the catabolic pathway of these sialic acid derivatives[64]. Such esterases have been studied in detail in rat liver, bovine brain and influenza C virus[4,11,24,65,66]. So far two different OAE-systems were described in mammalian tissues, a cytosolic enzyme that acts on free sialic acids, whereas another enzyme is a membrane-bound, complex- and high-mannose-type *N*-glycans carrying protein[4], which can act on both, free and glycosidically bound *O*-acetylated sialic acids, although on the latter only to a lesser extent[4,67].

Biological functions

By introducing a more hydrophobic moiety into the sialic acid molecule the *O*-acetyl group changes parameters like size, net charge, hydrogen bonding and conformation of glycoconjugates[68]. One consequence of this is a modulation of their biological functions (see also Table 1).

For example, 7(9)-*O*-acetylated sialic acids influence the haematopoietic development[69] and they prevent the recognition of cell surface glycoconjugates by siglecs, which are sialic acid-dependent adhesion molecules of the immunoglobulin superfamily like sialoadhesin and CD22[70], supposed to function in macrophage and in lymphocyte interactions, respectively, or in the maintenance and integrity of myelinated axons in the case of the myelin-associated glycoprotein (MAG)[71]. A further "anti-adhesion effect" exerted by such *O*-acetyl groups is the hindrance of influenza A and B viruses to sialic acids[72].

For several subsets of T-lymphocytes (e.g. CDw60, 9-*O*-acetylated ganglioside GD3) *O*-acetylation of the glycerol side chain of sialic acids is known to be a differentiation marker [57,73] and is therefore of particular interest from the immuno-cytological point of view. Binding of an antibody, specific for 7-*O*-acetylated GD3, to T-cells stimulates their proliferation, indicating the involvement of this ganglioside in signal transduction processes[30].

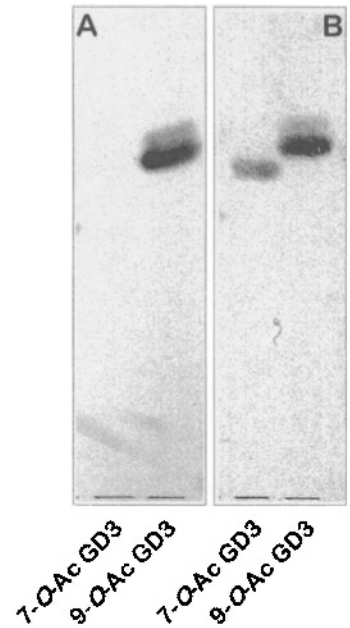

Figure 4: Binding specificity of influenza C virus
The migration of *O*-acetyl groups from position C7 to C9 is demonstrated by staining bands of 7- and 9-*O*-acetylated GD3 using influenza C virus specific for 9-*O*-acetylated sialic acids. In each lane 1µg of the respective ganglioside was applied. Staining was achieved by using the esterase activity of the virus towards the unspecific esterase substrate α-naphtyl acetate. Before alkaline treatment (A), binding only occurs to 9-*O*-AcGD3. After weak alkaline treatment at pH 10 (B) a band corresponding to the former 7-*O*-Ac-GD3 is also stained, indicating a migration to the C9 position of the sialic acid of this ganglioside. *O*-Acetylated gangliosides were a kind gift of Bernhard Kniep (Institut für Immunologie, Technische Universität Dresden, Germany).

Like its increased expression in mammary tumors[74], the presence of 9-*O*-Ac-GD3 on human melanoma[75] and basalioma[53] cells is considered as a tumor-associated antigen. Enhanced expression of melanoma-associated 9-*O*-acetyl-GD3 ganglioside stimulates cellular growth and suppresses certain differentiated phenotypes such as dendrite formation as shown for hamster melanoma cell lines[76]. The expression of 9-*O*-acetyl gangliosides on murine dorsal root ganglions correlates with periods of cell migration and axonal pathfinding[77]. Here the addition of monoclonal antibodies directed towards these gangliosides leads to a collapse of the growth cones. As in other brain regions, the pattern of expression of 9-*O*-acetyl-GD3 in the developing rat hippocampus also correlates with the migration of cells and their processes[78].

The influenza C virus enters the human organism via the respiratory tract, its natural target for binding are the 5-10 % Neu5,9Ac_2 of human nasal mucin[79], although the function of the viral hemagglutinin esterase may be, similar to the function of the sialidases from influenza A and B viruses, the detachment of the budding virus particle from its host cell[80]. By expression of the influenza C virus hemagglutinin esterase in murine neuroblastoma cell lines[81] or in transgenic mice, the significance of *O*-acetylation for the morphogenesis and development of the retina, cerebellum and adrenal gland, and for the organisation and function of neuronal tissue was clearly demonstrated[50].

Table 1: Biological effects of *O*-acetylated sialic acids

Effect	References
Modification of the physicochemical properties of glycoconjugates by increasing the hydrophobicity	82
Inhibition of the activities of sialidase, *trans*-sialidase, endoglycosidase and sialat-pyruvate lyase	2,82,83
Decreased cell degradation, e.g. of erythrocytes	82,84,85
Decreased complement pathway activation	86,87
Decreased binding of influenza A and B viruses, reovirus, *Plasmodium falciparum*, sialoadhesin, CD22 and MAG	72,88,89
Prevention the infection of cattle by *Trypanosoma brucei*	90
Increased binding of influenza C virus, Mouse hepatitis virus, Coronavirus, Encephalomyelitis virus	11,91,92
Decreased antigenicity	82,93,94
Increased bacterial pathogenicity	95
Ligands for binding antibodies and lectins	28,82,96,97
Differentiation- and tumor-associated antigens	50,54,75,82,94,98-105
Stimulation of cell growth	69,74,76,77,81,106

The previously mentioned highly *O*-acetylated sialic acids of human colonic mucins may lead to a better protection of the colon against certain microorganisms[64]. During cancerogenesis, concomitantly with the decrease of sialic acid *O*-acetylation, the expression of sialyl-Lewis(x) antigens on sialo-mucins increases, especially in metastases, because these structures were masked by the ester groups before[107].

BACTERIAL *O*-ACETYLATION

Occurrence

In the bacterial kingdom *O*-acetylated sialic acids or other *O*-acetylated carbohydrates have so far only been found in two surface components of bacteria: in the capsule or in the lipopolysaccharide (LPS). The latter is a surface glycolipid molecule found in the outer leaflet of the outer membrane in Gram-negative bacteria. It consists of a hydrophobic moiety called lipid A, a core oligosaccharide, and the O-specific side chain or O-antigen made of repeating units from different sugars[108]. In contrast to this, the capsular polysaccharides are only carbohydrate polymers comprised of repeating saccharide units.

The first detection of an *O*-acetylated sugar in the O-antigen was in 1969 by Hellerqvist and co-workers, who described an *O*-acetylated abequose residue in the repeating unit of *Salmonella typhimurium*[109]. Later other *O*-acetyl groups on various carbohydrates were found in different enteric bacteria like *Escherichia coli*[110], *Shigella dysenteriae*[111] or *Klebsiella pneumoniae*[112]. In the last years more and more *O*-acetylated sugars of the repeating unit were described in many bacterial strains. Interestingly, nearly all of them are important pathogenic organisms, for example *Proteus penneri*, which is a common cause of urinary tract infections[113], *Acinetobacter calcoaceticus*, a causative agent of various severe nocosomial infections in immuno-compromised patients[114], or the sheep and cattle pathogen *Campylobacter fetus*[115]. *O*-Acetylation was also found in the capsular polysaccharides type 5 and type 8 of the Gram-positive *Staphylococcus aureus*, a major cause of nocosomial infections[116].

O-Acetylated sialic acids were only detected as capsular components of the serotypes A, C, W-135 and Y of *Neisseria meningitidis*[117] and in the capsule or the O-antigen of the LPS in some *E. coli* strains. One of them, the serotype O104:K$^-$:H12 (strain-number: 21701) is an isolate from human faeces. It has no capsule and its O-antigen includes a sialic acid residue. The *O*-acetylation pattern of this sialic acid was discussed controversial. Kogan et al.[118] described an only 9-*O*-acetylated tetrasaccharide and Gamian et al.[119] a 7- and 9-di-*O*-acetylated form as repeating unit. Now it is clear, that the O-antigen of this *E. coli* strain 21701 consists of multiple copies of a tetrasaccharide with an internal sialic acid residue that is always di-*O*-acetylated on C7 and C9, as it is shown in Figure 5.

Metabolism

As outlined above, a sialic acid-specific *O*-acetyltransferase could not yet be purified. Therefore, the strategy to obtain more information about this enzyme had to be changed. In the case of the production of *O*-acetyl-deficient bacterial mutants, it would be possible to get more information about the role of *O*-acetyl groups. But neither with direct mutagenesis using transposons nor with a random mutagenesis system using UV-light it was possible to generate such mutants. A second approach involves the finding of the gene encoding the OAT-protein and the construction of the desired mutants by knocking out this gene. The amino acid sequences of some cloned *O*-acetyltransferases specific for various carbohy-

drates already exist in the database, and they appear to be more or less similar. One of them occurs in *Shigella flexneri*, designated "*oac*", and is responsible for the *O*-acetylation of the O-antigen. Lysogens of these bacteria harbour the temperate bacteriophage Sf6 and the gene for the modification is surprisingly encoded by the phage and not by its host[120]. The integrated prophages can be induced with mitomycin C or UV-light, but both methods had no effect on the *E. coli* strain 21701. Thus, in this strain and in all other bacteria the so far known genes, responsible for *O*-acetylation, are encoded by the bacteria themselves. Slauch *et al.*[121] aligned a series of these proteins that define a family of membrane proteins involved in the acylation of carbohydrate moieties on extracytoplasmic molecules and they showed some homologies between them. These proteins include OfaA from *Salmonella typhimurium* (*O*-acetylation of the O-antigen), GumF from *Xanthomonas campestris pv. campestris* (*O*-acetylation of the O-antigen), Sf6 and several proteins from *Streptomyces spp.* and *Rhizobium spp.* Also the *lag-1* locus responsible for *O*-acetylation of the *Legionella pneumophila* O-antigen that was cloned and characterized by Zou et al.[122] showed homologies to this family.

The ability of this locus to produce *O*-acetylation in former non-*O*-acetylated strains leads to the suggestion that *lag-1* indeed encodes an *O*-acetyltransferase. In order to confirm this, it is necessary to overexpress this or another possible *O*-acetyltransferase and to measure transferase activity, but until now, there is no *in vitro* assay available. To establish an enzyme test, we examined with the *E. coli* strain 21701 various exogenous substrates like de-*O*-acetylated bacteria, their isolated LPS, different gangliosides (GD3, GT1b or GQ1b), sialyllactose or free Neu5Ac in the presence or absence of substances, e.g. different detergents like CHAPS and Triton X-100 as well as DFP as an esterase-inhibitor and AcCoA, but in no case radioactively labelled *O*-acetyl groups were incorporated. So far even the acetyl donor for this reaction is unclear. We assume AcCoA as the donor, although there is no direct evidence for this hypothesis in bacteria. Another open question is where and when the *O*-acetylation reaction takes place. This may occur at the cytoplasmic side of the inner membrane before the O-unit is flipped and polymerized[123], although there exists no direct evidence for this mechanism. Clarification of this situation affords a lot of future work.

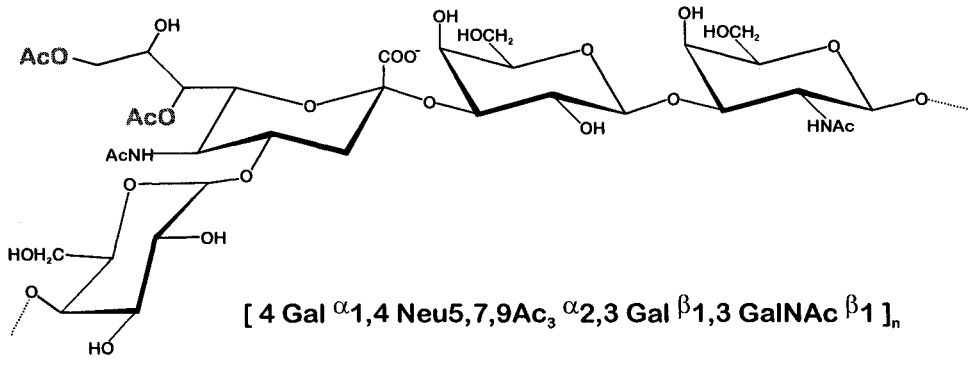

Figure 5: Oligosaccharide repeating unit of the O-antigen in theLPS from *E. coli* strain 21701, Serotype: O104:K⁻:H12

The structure of this unit was analyzed using NMR-Spectroscopy by Ulrich Zähringer (Forschungszentrum Borstel, Zentrum für Medizin und Biowissenschaften, Germany).

Biological functions

In contrast to higher organisms, there exist only little data about the function of this sialic acid modification in microorganisms. As mentioned above, nearly all bacteria with *O*-acetylated sugar residues are important pathogens, indicating that there might be a correlation between *O*-acetylation and pathogenicity. This was shown for *Salmonella typhi*, the causative agent of typhoid fever, with the capsular polysaccharide as a virulence factor that is stabilized by *O*-acetyl groups[124]. Another example for a direct relationship between *O*-acetylation and pathogenicity was demonstrated in *Neisseria meningitidis* group C, which is responsible for approximately 35% of meningococcal diseases in the USA. Most strains of this group produce a homopolymer of α2,9-linked Neu5Ac with *O*-acetyl groups at C7 and C8. About 85% of the strains causing diseases were reported to be *O*-acetyl-positive[125]. This immuno-dominance was also found for *E. coli* K1[126] and was shown for *E. coli* strain 21701, too. Mice immunized with *O*-acetylated bacteria gave a significant higher immune response than mice immunized with de-*O*-acetylated material. Since nothing more is known about the effects of *O*-acetylated sialic acids in bacteria there is still a controversial discussion about the role of these sialic acids in the literature.

CONCLUSIONS AND OUTLOOK

Sialate *O*-acetylation is a crucial cellular event especially in the animal kingdom and in man and causes a vast spectrum of biological effects. Due to their prevailing presence on glycoconjugates of the outermost leaflet on the plasma membrane, sialic acids, *O*-acetylated at their side chains, mainly as Neu5,9Ac_2, mediate or prevent binding of cells to each other and of viruses or bacteria to their host cells. They therefore revealed to be important in cell differentiation, neuronal development, non-immunogenic protection and immuno reactivity, or on the other hand, they are involved in cancer progression and virus invasion. *O*-Acetylated sialic acids thus are great mediators of cellular cross talk.

In contrast, our knowledge about the function of 4-*O*-acetylated sialic acids is limited and restricted to the resistance towards sialic acid degrading enzymes. However, the discovery that mouse hepatitis viruses recognize free and glycosidically bound Neu4,5Ac_2 stimulates the discussion that sialate-4-*O*-acetylation is more than just influencing enzyme reactivity, but may also be important for recognition phenomena similar to the side chain *O*-acetylation.

It is therefore no question that *O*-acetylation is an exciting and promising field of research, which increased rapidly during the past ten years, due to the very sensitive analytical tools that have been developed within this time on the one hand and to the great interest in this sialic acid modification on the other.

Due to the extraordinary interest of the scientific community in sialate-*O*-acetylation, different approaches to purify or clone the sialate-*O*-acetyltransferases from diverse kinds of animals were made. In the tissues and cells investigated, sialate-*O*-acetyltransferases are membrane-bound enzymes situated in the Golgi apparatus. *O*-Acetylation seems to take place preferentially on α2,6- or α2,8-linked sialic acids. The transfection of an α2,6-specific sialyltransferase into CHO-cells provides the substrates for an *O*-acetyltransferase, which was "quiet" before, when the cells expressed α2,3-linked sialic acids only[127]. Remarkably, the richest natural sources of *O*-acetylated sialic acids known are human and bovine mucins as well as 9-*O*-acetylated gangliosides.

All efforts to clone the enzymes failed so far, although various reports seem to get closer to the possibility to identify unequivocally a sialate-*O*-acetyltransferase gene. It was,

however, possible to clone and sequence the gene for one of the components of the hypothetical sialate *O*-acetyltransferase complex, the AcCoA-transporter[128]. In contrast to several attempts made from the molecular biologists point of view, our present approach is a more classical way of enzyme purification to resolve the amino acid sequence. Once the miracle is solved, it will be of great scientific impact to learn more about the regulation of *O*-acetylated sialic acid expression in embryogenesis, immunology and tumor biology. Although the product of the biosynthesis is well characterized, the way it is formed is still waiting to be elucidated.

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