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J. Montreuil, J.F.G. Vliegenthart and H. Schachter (Eds.), *Glycoproteins II*

CHAPTER 11

Chemistry, biochemistry and biology of sialic acids

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List of Abbreviations

Abbreviations used for sialic acids (Sia) are included in Tables 1 and 13.

Alt	altrose
Ara	L-arabinose
Asn	L-asparagine
Asp	L-aspartate
CDP	cytidine diphosphate
Cer	ceramide
CI	chemical ionization
CMP	cytidine monophosphate
CoA	coenzyme A
CTP	cytidine triphosphate
C7-Neu5Ac	Neu5Ac missing the C8-C9 part
C8-Neu5Ac	Neu5Ac missing the C9 part
Da	Dalton
DFP	diisopropylfluorophosphate
DIG	digoxigenin
DMB	1,2-diamino-4,5-methylenedioxybenzene
DNA	desoxyribonucleic acid
EI	electron impact
EPR	electron paramagnetic resonance
FAB	fast atom bombardment
Fuc	L-fucose
FucN	L-fucosamine
Gal	D-galactose
GalNAc-ol	*N*-acetylgalactosaminitol
GalANGro	*N*-galacturonyl-2-aminoglycerol
GalNAc	*N*-acetyl-D-galactosamine
GDP	guanosine diphosphate
Glc	D-glucose
GLC	gas–liquid chromatography
GlcA	D-glucuronic acid
GlcNAc	*N*-acetyl-D-glucosamine
GlcN	D-glucosamine
GD3	disialoganglioside
GM1	monosialoganglioside
Gul	gulose
Hep	heptose
HPLC	high-performance liquid chromatography
HPTLC	high-performance thin-layer chromatography
IgG	immunoglobulin G
IgM	immunoglobulin M
Leu	L-leucine
Man	D-mannose
ManNAc	*N*-acetyl-D-mannosamine
MS	mass spectrometry
MU	4-methylumbelliferyl
NMK	nucleoside monophosphate kinase
NMR	nuclear magnetic resonance
NOESY	nuclear Overhauser enhancement spectroscopy
P, Pi	phosphate
PAD	pulsed amperometric detection
PEP	phosphoenolpyruvate
PK	pyruvate kinase
PPase	inorganic phosphatase
PPi	pyrophosphate
PYR	pyruvate
ROESY	rotating-frame nuclear Overhauser enhancement spectroscopy
QuiNAc	*N*-acetyl-D-quinovose

S	sulfate	Thr	L-threonine
SDS/PAGE	sodium dodecyl sulfate/polyacrylamide gel electrophoresis	TOCSY	total correlation spectroscopy
		UDP	uridine diphosphate
Ser	L-serine	UV	ultraviolet
TADH	*Thermoanaerobium brockii* alcohol dehydrogenase	Xyl	D-xylose
		2D	two-dimensional
TFA	trifluoroacetic acid		
TLC	thin-layer chromatography		

1. Introduction

Since the discovery of *N*-acetylneuraminic acid, the most universal sialic acid, at the end of the 1930s [1,2] as well as the structural and stereochemical elucidation of its free and bound forms at the end of the 1960s (reviewed in ref. [3]), there has been a continual increase in the number of sialic acid types (1994: more than 40) recognized to occur in a variety of living organisms. It is now generally accepted that naturally occurring sialic acids are monosaccharides which influence many important biological and pathological phenomena.

In previous articles [3–5], the former literature has been extensively reviewed with respect to a number of chemical, biological, metabolic, functional as well as historical aspects. Since 1982 [4,5], the proliferation of the literature on the chemistry, biochemistry and (molecular) biology of sialic acids has accelerated dramatically, and several short reviews dealing with specific aspects of sialic acids have appeared [6–12].

The aim of this chapter is to collate a mixture of, what is in our opinion, relevant information published before 1982 and new data appeared since then. Because of the profusion of biochemical and biological data published in the last few years, it was necessary to select those reports which, we believe, reflect potential trends in the future development of sialobiology.

2. General characteristics of sialic acid

In Table 1 a survey of the 43 naturally occurring members of the sialic acid family [13–51], together with their abbreviations and typical biological sources, is presented. The general name "sialic acid" is derived from the Greek "sialos", meaning saliva. Taking into account the Rules for Carbohydrate Nomenclature, as recommended by the IUPAC-IUBMB Joint Commission on Biochemical Nomenclature (1995), the mother-molecule neuraminic acid, which does not occur in free form in nature, due to its immediate cyclization to form an internal Schiff base, is systematically named 5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid (Fig. 1), and abbreviated as Neu, whereby the D-notation is implied in the trivial name. Chemically, this nine-carbon-containing monosaccharide is a 2-keto-carboxylic acid, a deoxysugar, and an aminosugar. The amino group is generally *N*-acetylated (5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid;

Table 1
Survey of established structures of naturally occurring members of the sialic acid family[a]

Name	Abbreviation	Typical biological sources	Reference(s)
Neuraminic acid	Neu	does not occur in free form; only found in gangliosides	[13,14]
N-Acetylneuraminic acid	Neu5Ac	oligosaccharides, polysaccharides and glycoconjugates from man, higher animals, and microorganisms; free form in body fluids	[13,15–18]
5-*N*-Acetyl-4-*O*-acetyl-neuraminic acid	$Neu4,5Ac_2$	ungulate, monotreme and guinea pig oligosaccharides and glycoconjugates	[15,16,18–21]
5-*N*-Acetyl-7-*O*-acetyl-neuraminic acid	$Neu5,7Ac_2$	animal glycoconjugates; bacterial polysaccharides	[15,16,18,22–24]
5-*N*-Acetyl-8-*O*-acetyl-neuraminic acid	$Neu5,8Ac_2$	bovine glycoproteins; bacterial polysaccharides	[15,22–24]
5-*N*-Acetyl-9-*O*-acetyl-neuraminic acid	$Neu5,9Ac_2$	human and animal glycoconjugates; bacterial (lipo)polysaccharides	[15,16,18,22–26]
5-*N*-Acetyl-4,9-di-*O*-acetyl-neuraminic acid	$Neu4,5,9Ac_3$	equine glycoproteins	[15]
5-*N*-Acetyl-7,9-di-*O*-acetyl-neuraminic acid	$Neu5,7,9Ac_3$	bovine glycoproteins and bacterial lipopolysaccharides	[15,16,18,22,23, 26,27]
5-*N*-Acetyl-8,9-di-*O*-acetyl-neuraminic acid	$Neu5,8,9Ac_3$	bovine glycoproteins	[15,16,18,22,23]
5-*N*-Acetyl-7,8,9-tri-*O*-acetyl-neuraminic acid	$Neu5,7,8,9Ac_4$	bovine glycoproteins	[15,22]
5-*N*-Acetyl-9-*O*-L-lactyl-neuraminic acid	Neu5Ac9Lt	human and animal glycoproteins; free form in body fluids	[15,28,29]
5-*N*-Acetyl-4-*O*-acetyl-9-*O*-lactyl-neuraminic acid	$Neu4,5Ac_2$9Lt	equine glycoproteins	[15,30]

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Table 1, *continued*

Name	Abbreviation	Typical biological sources	Reference(s)
5-*N*-Acetyl-8-*O*-methyl-neuraminic acid	Neu5Ac8Me	starfish glycoconjugates	[15,31]
5-*N*-Acetyl-9-*O*-acetyl-8-*O*-methyl-neuraminic acid	Neu5,9Ac$_2$8Me	starfish glycoconjugates	[31]
5-*N*-Acetyl-8-*O*-sulpho-neuraminic acid	Neu5Ac8S	sea urchin glycolipids	[32]
5-*N*-Acetyl-9-*O*-phosphoro-neuraminic acid	Neu5Ac9P	biosynthetic intermediate to Neu5Ac	[33]
5-*N*-Acetyl-2-deoxy-2,3-didehydro-neuraminic acid	Neu2en5Ac	body fluids and tissues	[15–17,34]
5-*N*-Acetyl-9-*O*-acetyl-2-deoxy-2,3-didehydro-neuraminic acid	Neu2en5,9Ac$_2$	urine and tissues	[35]
5-*N*-Acetyl-2-deoxy-2,3-didehydro-9-*O*-lactyl-neuraminic acid	Neu2en5Ac9Lt	urine and tissues	[35]
5-*N*-Acetyl-2,7-anhydro-neuraminic acid	Neu2,7an5Ac	urine, wet cerumen, leech	[36–39]
N-Glycolylneuraminic acid	Neu5Gc	glycoconjugates from most higher animals	[13,15,16,18,25, 40]
4-*O*-Acetyl-5-*N*-glycolyl-neuraminic acid	Neu4Ac5Gc	ungulate glycoconjugates	[15,16,18]
7-*O*-Acetyl-5-*N*-glycolyl-neuraminic acid	Neu7Ac5Gc	glycoconjugates from most higher animals	[15,22]
8-*O*-Acetyl-5-*N*-glycolyl-neuraminic acid	Neu8Ac5Gc	bovine glycoproteins	[36]
9-*O*-Acetyl-5-*N*-glycolyl-neuraminic acid	Neu9Ac5Gc	glycoconjugates from most higher animals	[15,16,18,22]
7,9-Di-*O*-acetyl-5-*N*-glycolyl-neuraminic acid	Neu7,9Ac$_2$5Gc	bovine glycoproteins	[15,22]
8,9-Di-*O*-acetyl-5-*N*-glycolyl-neuraminic acid	Neu8,9Ac$_2$5Gc	bovine glycoproteins	[15,22]
7,8,9-Tri-*O*-acetyl-5-*N*-glycolyl-neuraminic acid	Neu7,8,9Ac$_3$5Gc	bovine glycoproteins	[15,22]
5-*N*-glycolyl-9-*O*-lactyl-neuraminic acid	Neu5Gc9Lt	porcine glycoproteins	[36]
5-*N*-glycolyl-8-*O*-methyl-neuraminic acid	Neu5Gc8Me	starfish glycoconjugates	[15,31]
9-*O*-Acetyl-5-*N*-glycolyl-8-*O*-methyl-neuraminic acid	Neu9Ac5Gc8Me	starfish glycoconjugates	[31]
7,9-Di-*O*-acetyl-5-*N*-glycolyl-8-*O*-methyl-neuraminic acid	Neu7,9Ac$_2$5Gc8Me	starfish glycoconjugates	[41]

continued on next page

Table 1, *continued*

Name	Abbreviation	Typical biological sources	Reference(s)
5-*N*-Glycolyl-8-*O*-sulpho-neuraminic acid	Neu5Gc8S	sea urchin glycolipids	[42]
N-(*O*-Acetyl)glycolylneuraminic acid	Neu5GcAc	rat thrombocytes	[43]
N-(*O*-Methyl)glycolylneuraminic acid	Neu5GcMe	starfish glycolipids	[44]
2-Deoxy-2,3-didehydro-5-*N*-glycolyl-neuraminic acid	Neu2en5Gc	body fluids and tissues	[45]
9-*O*-Acetyl-2-deoxy-2,3-didehydro-5-*N*-glycolyl-neuraminic acid	Neu2en9Ac5Gc	urine	[35]
2-Deoxy-2,3-didehydro-5-*N*-glycolyl-9-*O*-lactyl-neuraminic acid	Neu2en5Gc9Lt	urine and tissues	[35]
2-Deoxy-2,3-didehydro-5-*N*-glycolyl-8-*O*-methyl-neuraminic acid	Neu2en5Gc8Me	starfish	[35]
2,7-Anhydro-5-*N*-glycolyl-neuraminic acid	Neu2,7an5Gc	rat urine	[36,38]
2,7-Anhydro-5-*N*-glycolyl-8-*O*-methyl-neuraminic acid	Neu2,7an5Gc8Me	starfish	[36]
2-Keto-3-deoxynononic acid	Kdn	fish, amphibian and mammalian glycoconjugates; bacterial polysaccharides	[46–50]
9-*O*-Acetyl-2-keto-3-deoxynononic acid	Kdn9Ac	fish glycoconjugates	[51]

[a] If possible, typical biological sources have been indicated. In the case of a sialic acid structure being proven by mass spectrometry and/or NMR spectroscopy, reference numbers refer in general to such studies or to review articles including this sialic acid.

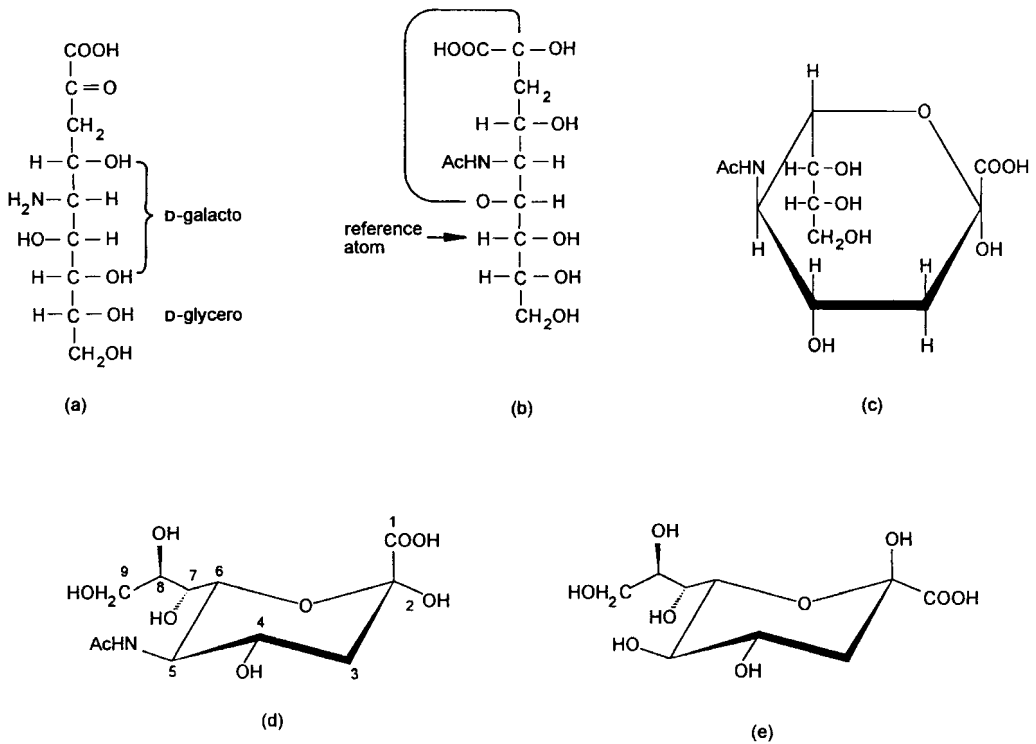

Fig. 1. Chemical structures for simple sialic acids in different views. (a) 5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid (Neu, open chain, Fischer projection formula); (b) 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosonic acid (α-Neu5Ac, Fischer projection formula, note that C7 is the anomeric reference atom); (c) α-Neu5Ac (Haworth projection formula); (d) α-Neu5Ac ($^2C_5$ chair conformation); (e) 3-deoxy-D-*glycero*-β-D-*galacto*-non-2-ulopyranosonic acid (β-Kdn, $^2C_5$ chair conformation). Note that the D-notation is part of the trivial name.

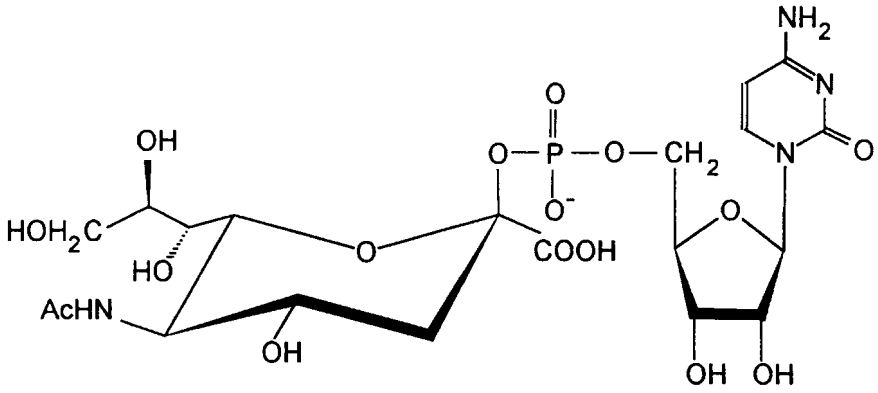

Fig. 2. Chemical structure of cytidine 5′-(5-acetamido-3,5-dideoxy-D-*glycero*-β-D-*galacto*-non-2-ulopyranosyl-onate monophosphate (CMP-β-Neu5Ac).

N-acetylneuraminic acid; Neu5Ac) (Fig. 1) or *N*-glycolylated (5-hydroxyacetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid; *N*-glycolylneuraminic acid; Neu5Gc). As is evident from Table 1, the hydroxyl groups may be free, esterified (acetylated, lactylated, sulfated, phosphorylated) or etherified (methylated). In the case where a sialic acid bears a hydroxy group instead of an amino group at C5, so-called deaminated neuraminic acid, it is systematically named 3-deoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid (Fig. 1), and is abbreviated as Kdn (2-keto-3-deoxynononic acid; also in this case the D-notation is part of the trivial name). Sialic acids are relatively strong acids, e.g. Neu5Ac has a pK_a value found in the range of 2.2–3.0 in various studies with an average of 2.6. This strong acidity is responsible for processes such as autohydrolysis of sialic-acid-containing carbohydrate chains. Conformationally, sialic acids adopt the $^2C_5$ chair conformation, having the glycerol side chain in an equatorial orientation [52]. Due to hydrogen bonding of HO7 and HO8, leading to a *trans*-orientation of these groups, the glycerol side chain is not as flexible as one might expect. This has consequences for the course of the mild periodate oxidative degradation of this moiety in the case of substitution at C9 [53]. Free sialic acids have mainly the β-anomeric ring structure (>93%), whereas glycoconjugate-bound sialic acids occur specifically in the α-anomeric form [54]. In nucleotide-bound sialic acids, i.e. CMP-Neu5Ac (Fig. 2), a β-configuration for the glycosidic bond is present [55]. Crystalline Neu5Ac occurs specifically in the β-anomeric form [56].

With respect to *O*-acetylation patterns in sialic acids, it is worthwhile to mention that spontaneous migration of *O*-acetyl groups can occur between C7, C8 and C9. At pH values at which no significant de-*O*-acetylation is observed (e.g. physiological pH values), Neu5,7Ac_2 can readily transform into Neu5,9Ac_2, whereas Neu5,7,9Ac_3 yields an equilibrium of Neu5,7,9Ac_3 and Neu5,8,9Ac_3 in a molar ratio of approximately 1:1; Neu4,5Ac_2 does not give rise to *O*-acetyl migrations [23]. Also starting from α-Neu5,8,9Ac_3 4-aminophenylthio-glycoside a 1:1 equilibrium between the 8,9- and 7,9-di-*O*-acetylated derivatives is established [57]. 9-*O*-Acetylated *N*-acylneuraminic acids have been found in both glycoconjugates and oligo/polysaccharides of different

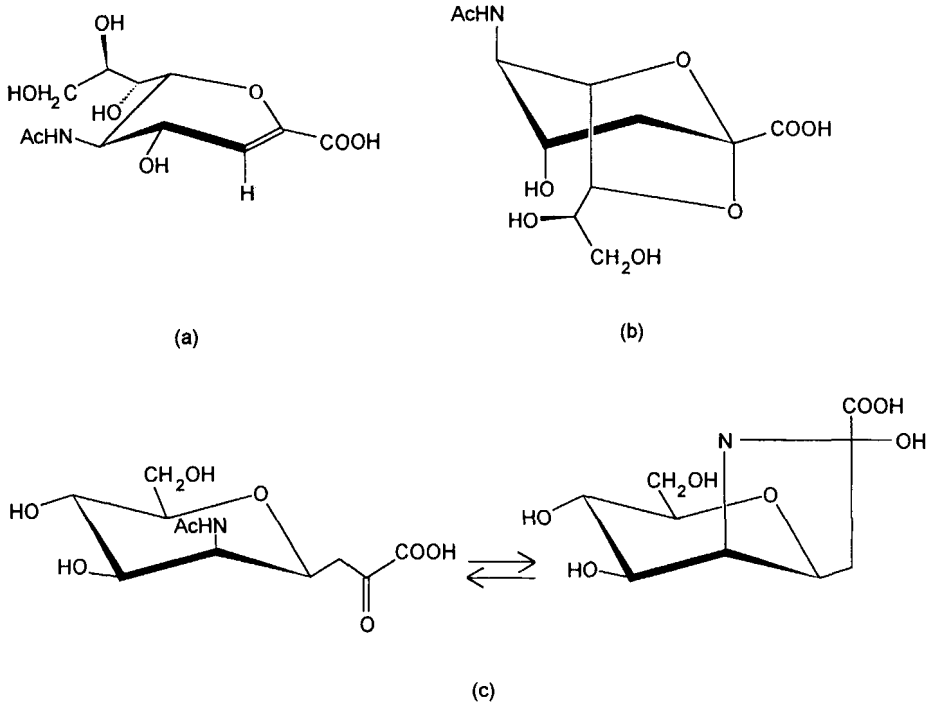

Fig. 3. Chemical structures of (a) 5-*N*-acetyl-2-deoxy-2,3-didehydro-neuraminic acid (Neu2en5Ac); (b) 5-*N*-acetyl-2,7-anhydro-neuraminic acid (Neu2,7an5Ac); and (c) the tautomers of 5-*N*-acetyl-4,8-anhydro-neuraminic acid (Neu4,8an5Ac).

biological origin (Table 1). The same is true for 7-*O*-acetylated *N*-acylneuraminic acids. It should be emphasized that Neu5,9Ac$_2$ and other side-chain-*O*-acylated sialic acids occur in man [58] (see also sections 8.4.2 and 8.4.3), e.g. in human colon. Ungulates form the major source for 4-*O*-acetylated *N*-acylneuraminic acids [13], whereas minor sources are monotremes [19], guinea pigs [21] and humans [59]. In an evaluation of the naturally occurring *O*-acylation patterns, it is evident that *O*-acyl groups are most frequently found at C9 of both Neu5Ac and Neu5Gc. *O*-Methylated and *O*-sulfated *N*-acylneuraminic acids have only been found in lower animals, e.g. in echinoderms [13,31,42].

5-*N*-Acyl-2-deoxy-2,3-didehydro-neuraminic acids like Neu2en5Ac (5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid, Fig. 3) occur in free form in nature. Moreover, they can be generated from corresponding CMP-*N*-acylneuraminic acids in a non-enzymatic elimination reaction, occurring under physiological and, much faster, under alkaline conditions [34,35]. Small amounts of Neu2en5Ac are formed by a water elimination side reaction from Neu5Ac during influenza-B-virus-sialidase-catalyzed desialylations of sialoglycoconjugates [60].

5-*N*-Acetyl-2,7-anhydro-neuraminic acid (Neu2,7an5Ac, Fig. 3), which occurs in free form in nature [36,37], can be generated from Neu5Ac(α2–3)Gal(β1– containing glycoconjugates using a sialidase isolated from the leech *Macrobdella decora* [38,61].

It is clear that such a sialidase has a highly unusual specificity (see sections 5.1, 8.4.6 and 9.2). The methyl ester of Neu2,7an5Ac is formed as a very minor by-product during methanolysis [62].

5-*N*-Acetyl-4,8-anhydro-neuraminic acid (Neu4,8an5Ac, Fig. 3), having CH_2–CO–COOH as a side chain of a pyranose ring with the $^7C_4$ conformation, does not occur as such in nature. Initially, it has been isolated from an acid hydrolysate of edible bird's nest substance [63]. Furthermore, Neu4,8an5Ac was detected in a sialic acid mixture obtained after acid hydrolysis of equine serum, whereas incubation of Neu4,5Ac_2 under alkaline conditions showed, in addition to de-*O*-acetylation, a partial conversion into Neu4,8an5Ac [41]. Heating of solid sodium Neu5Ac (3 h, 140°C) or a solution of Neu5Ac at pH > 11 or at pH 2.0 (30 min, 80°C) also yielded Neu4,8an5Ac (sodium salt) as a major degradation product, probably in two tautomeric forms [64].

Hyperexcretion of free Neu5Ac in urine, defined as sialuria, has been observed in several mentally retarded patients [65–68] (see section 10.5).

3. *Occurrence of sialic acids in biomolecules*

Members of the sialic acid family occur mainly in bound form in higher animals, from the echinoderms onwards in evolution, but also in some viruses, various bacteria, protozoa, and pathogenic fungi [8,13,69]. Generally, they are constituents of glycoproteins [20,21,25,40,46–48,51,70–107] (Table 2), glycolipids [13,31,44,108–124] (Table 3), and oligosaccharides [16,19,25,76,125–155] (Table 4), usually occurring as terminal monosaccharide units, and of homo- and heteropolysaccharides [24,26,27,49,156–185] (Table 5). However, glycosylphosphatidylinositol membrane anchors have also been shown to contain sialic acid [186]. Neu5Ac- and Neu5Gc-containing glycans frequently occur together, whereby ratio differences reflect species specificity, tissue specificity or physiological fluid specificity. Also Kdn has been found together with Neu5Ac/5Gc in specific glycoconjugates (Table 2). It should be noted that the presence of Neu5Gc in normal human tissue and soluble glycoproteins has not been established conclusively [13,187,188].

As is evident from Table 2, Neu5Ac/5Gc- and Kdn-containing elements in N- and O-glycoprotein glycans do occur in many different microenvironments, however, with a restricted glycosidic linkage specificity. In general, *N*-acylneuraminic acids are α-2,3- or α-2,6-linked with D-galactose (Gal), α-2,3- or α-2,6-linked with *N*-acetyl-D-galactosamine (GalNAc), α-2,6-linked with *N*-acetyl-D-glucosamine (GlcNAc), or α-2,8-linked with other *N*-acylneuraminic acids. These types of glycosidic linkages have been firmly established by different analytical methods, including NMR spectroscopy [76,77]. The sialic-acid-containing N-linked carbohydrate chains form part of the complex (*N*-acetyllactosamine and *N,N′*-diacetyllactosediamine subtypes; mono-, di-, tri-, tri′-, and tetraantennae) or the hybrid type of structures [189]. For the mucin-type O-glycans, the *N*-acylneuraminic-acid-containing sequences are extensions of most of the well-established core types [190]. In addition, terminal Neu5Ac(α2–4)Gal [75], terminal Neu5Ac(α2–4)GlcNAc [96], and internal Fuc(1–4)Neu5Gc [97] sequences have been reported, and only one example of a terminal Neu5Ac(α2–9)Neu5Ac(α2– dimer has

Table 2
Sialic-acid-containing elements in N- and O-glycoproteins[a]

Partial structure	N	O	Ref(s).
Neu5Ac(α2–3)Gal(β1–3)GlcNAc(β1-	+		[70,71]
Neu5Ac(α2–3)Gal(β1–3)[Neu5Ac(α2–6)]GlcNAc(β1-	+		[70,72,73]
Neu5Ac(α2–3)Gal(β1–3)[Fuc(α1–4)]GlcNAc(β1-		+	[74]
Neu5Ac(α2–4)Gal(β1–3)[Neu5Ac(α2–6)]GlcNAc(β1-	+		[75]
Gal(β1–3)[Neu5Ac(α2–6)]GlcNAc(β1-	+		[70,73]
Neu5Ac(α2–3)Gal(β1–4)GlcNAc(β1-	+	+	[76,77]
Neu5Ac(α2–3)Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1-	+	+	[77,78]
Neu5Ac(α2–3)[Gal(β1–4)]Gal(β1–4)GlcNAc(β1-	+		[79,80]
Neu5Ac(α2–3)[GalNAc(β1–4)]Gal(β1–4)GlcNAc(β1-	+	+	[77,81]
Neu5Ac(α2–3)[6S]Gal(β1–4)GlcNAc(β1-	+		[82]
Neu4,5Ac_2(α2–3)Gal(β1–4)GlcNAc(β1-	+		[83]
Neu4,5Ac_2(α2–3)[Gal(β1–4)]Gal(β1–4)GlcNAc(β1-	+		[79]
Neu5,9Ac_2(α2–3)Gal(β1–4)GlcNAc(β1-	+		[25,84]
Neu5Gc(α2–3)Gal(β1–4)GlcNAc(β1-	+		[25,84]
Neu5Ac(α2–4)Gal(β1–4)GlcNAc(β1-	+		[75]
Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1-	+	+	[76,77]
Neu5Ac(α2–6)Gal(β1–4)[6S]GlcNAc(β1-	+		[85]
Neu5Ac(α2–6)[GalNAc(α1–3)]Gal(β1–4)GlcNAc(β1-		+	[86]
Neu4,5Ac_2(α2–6)Gal(β1–4)GlcNAc(β1-	+		[20,21,87,88]
Neu5Gc(α2–6)Gal(β1–4)GlcNAc(β1-	+		[40]
Neu5Ac(α2–3)$\{Gal(\beta1–4)\}_{1-2}$Gal(β1–4)GlcNAc(β1-	+		[80,89]
±Neu5Ac(α2–3)Gal(β1–4)[±Neu5Ac(α2–3)]Gal(β1–4)[±Fuc(α1–3)]GlcNAc(β1-	+		[90]
Neu5Ac(α2–3)Gal(β1–3)GalNAc(α1-		+	[77]
Neu5Ac(α2–3)Gal(β1–3)GalNAc(α1–O)Ser/Thr		+	[77]
Neu5Ac(α2–8)Neu5Ac(α2–3)Gal(β1–3)GalNAc(α1–O)Ser/Thr		+	[91]
Neu5Ac(α2–3)[GalNAc(β1–4)]Gal(β1–3)GalNAc(α1–O)Ser/Thr		+	[77]
Neu5Gc(α2–3)Gal(β1–3)GalNAc(α1–O)Ser/Thr		+	[77]
Neu5Ac(α2–3)GalNAc(β1–4)GlcNAc(β1-	+		[92]
Neu5Ac(α2–6)GalNAc(β1–4)GlcNAc(β1-	+		[93,94]
Neu5Ac(α2–3)[GalNAc(β1–4)]GalNAc(β1–3)Gal(β1–4)Gal(β1-		+	[95]
Neu5Gc(α2–3)[GalNAc(β1–4)]GalNAc(β1–3)Gal(β1–4)Gal(β1-		+	[77]
Neu5Ac(α2–6)GalNAc(α1–O)Ser/Thr		+	[77]
Neu5Gc(α2–6)GalNAc(α1–O)Ser/Thr		+	[77]
Neu5Ac(α2–4)GlcNAc(β1–3)[Neu5Ac(α2–6)]Gal(β1-		+	[96]
-Fuc(1–4)Neu5Gc(2-		+	[97]
Neu5Ac(α2–9)Neu5Ac(α2–3/6)Gal(β1–4)GlcNAc(β1-	+		[98]
$\{Neu5Ac(\alpha2–8)\}_n$Neu5Ac(α2–3)Gal(β1–4)GlcNAc(β1-	+		[99]
$\{Neu5Ac(\alpha2–8)\}_n$Neu5Ac(α2–6)GalNAc(α1–O)Ser/Thr		+	[77]

continued on next page

Table 2, *continued*

Partial structure	N	O	Ref(s).
{Neu5Gc(α2–8)}$_n$Neu5Gc(α2–6)GalNAc(α1–O)Ser/Thr		+	[77]
{Neu5Ac(α2–8)}$_n$- with 4Ac, 7Ac, 9Ac or 9Lt		+	[100]
{Neu5Gc(α2–8)}$_n$- with 4Ac, 7Ac or 9Ac		+	[51,100]
{Neu5Ac/5Gc(α2–8)}$_n$- with 4Ac, 7Ac or 9Ac		+	[100]
{Neu5Gc(α2–O5)}$_n$-		+	[101]
Kdn(α2–3)Gal(β1–4)GlcNAc(β1-	+		[102]
Kdn(α2–3)Gal(β1–3)GalNAc(α1–3)GalNAc(α1–O)Ser/Thr		+	[47]
Kdn(α2–3)[GalNAc(β1–4)]GalNAc(β1–3)Gal(β1–4)Gal(β1-		+	[77]
Kdn(α2–6)GalNAc(α1–O)Ser/Thr		+	[48,77,103]
{Kdn(α2–8)}$_n$Kdn(α2–6)GalNAc(α1–O)Ser/Thr		+	[47]
Fuc(α1–4)[Fuc(α1–5)]Kdn(α2–6)GalNAc(α1–O)Ser/Thr		+	[104]
-Fuc(α1–4)Kdn(α2–6)GalNAc(α1–O)Ser/Thr		+	[105]
Kdn(α2–6)[Kdn(α2–3)]GalNAc(α1–O)Thr		+	[106]
Kdn(α2–3)GalNAc(α1–O)Thr		+	[106a]
Fuc(α1–4)[{Fuc(α1–5)}$_{0-1}$]Kdn(α2–3)Gal(β1-		+	[104]
{Kdn(α2–8)}$_n$- with 4Ac, 7Ac or 9Ac		+	[107]
Kdn(α2–8){Neu5Ac(α2–8)}$_n$- with ±Ac		+	[107]
Kdn(α2–8){Neu5Gc(α2–8)}$_n$- with ±Ac		+	[46]
Kdn(α2–8){Neu5Ac/5Gc(α2–8)}$_n$- with ±Ac		+	[107]
Kdn9Ac(α2–8){Neu5Gc(α2–8)}$_n$-		+	[51]

[a] In the case of a specific fragment being established by ^{1}H NMR spectroscopy, the reference refers to such a study or to a review that includes the fragment. S means sulfate.

been described [98]. In a number of structures not only sialic acid is responsible for the acidic character of the carbohydrate chain, but also sulfate. Here, an unusual example is the Neu5Ac(α2–3)[6S]Gal(β1–4)GlcNAc element [82]. The list of sialic-acid-containing sequences, in which *N*-acylneuraminic acid is replaced by Kdn is continuously growing (Table 2). Interestingly, also structural elements occur which have not been found so far for *N*-acylneuraminic acids, e.g. Kdn(α2–6)[Kdn(α2–3)]GalNAc(α1–O)Thr [106].

General structural information with respect to glycoprotein glycans is presented in volume 29a of the New Comprehensive Biochemistry series, and detailed information with respect to poly-*N*-acylneuraminyl-containing glycoproteins and Kdn-containing glycoproteins in the present volume 29b. Several of the sialic-acid-containing sequences present in N- and O-glycoprotein glycans, also occur in glycolipids, milk and urinary oligosaccharides, and in (lipo)polysaccharides of different biological origin. An impression of this overlap in structures can be obtained from an inspection of the structural data in Tables 3–5, summarizing sialic-acid-containing elements of glycolipids, structures of milk and urinary sialo-oligosaccharides, and structures or elements of sialic-acid-containing (lipo)polysaccharides, respectively. It is interesting to note that

Table 3
Compilation of sialic-acid-containing fragments in glycolipids[a]

Structure	References
Neu5Ac(α2–3)Gal(β1–1′)Cer	[108]
{Neu5Ac(α2–8)}$_{0-1}$Neu5Ac(α2–6)Glc(β1–1′)Cer	[13]
Neu5Gc/5Gc8S(α2–6)Glc(β1–1′)Cer	[13,109]
Neu5Ac(α2–6)Glc(β1–8)Neu5Ac(α2–6)Glc(β1–1′)Cer	[13]
Neu5Ac(α2–8)Neu5Ac(α2–6)Glc(β1–6)Glc(β1–1′)Cer	[13]
Neu5Ac/5Gc/4Ac5Gc/5Gc8Me/5GcMe(α2–3)Gal(β1–4)Glc(β1–1′)Cer	[44,108]
Neu5Ac/5,7Ac$_2$/5,9Ac$_2$/5,7,9Ac$_3$/5Ac8S/5Gc(α2–8)Neu5Ac(α2–3)Gal(β1–4)Glc(β1–1′)Cer	[13,108,110]
Neu5Ac/5Gc(α2–8)Neu5Gc(α2–3)Gal(β1–4)Glc(β1–1′)Cer	[108]
Neu5Ac(α2–8)Neu5Ac(α2–8)Neu5Ac(α2–3)Gal(β1–4)Glc(β1–1′)Cer	[108]
Neu5,9Ac$_2$(α2–8)Neu5Ac(α2–8)Neu5Ac(α2–3)Gal(β1–4)Glc(β1–1′)Cer	[111]
GalNAc(β1–4)[Neu5Ac/5Ac8S/5Gc/5Gc8S(α2–3)]Gal(β1–4)Glc(β1–1′)Cer	[108]
Gal(β1–3)GalNAc(β1–4)[Neu(α2–3)]Gal(β1–4)Glc(β1–1′)Cer	[112]
[R-3]GalNAc(β1–4)[Neu5Ac/5Gc(α2–3)]Gal(β1–4)Glc(β1–1′)Cer[b]	[108]
[R-3]GalNAc(β1–4)[{Neu5Ac(α2–8)}$_{1-2}$Neu5Ac(α2–3)]Gal(β1–4)Glc(β1–1′)Cer[c]	[108]
[R-3]GalNAc(β1–4)[Neu5,9Ac$_2$(α2–8){Neu5Ac(α2–8)}$_{0-1}$Neu5Ac(α2–3)]Gal(β1–4)Glc(β1–1′)Cer[c]	[108,113]
[R-3]GalNAc(β1–4)[{Neu5Gc(α2–8)}$_{1-2}$Neu5Gc(α2–3)]Gal(β1–4)Glc(β1–1′)Cer[b]	[108]
[R-3]GalNAc(β1–4)[Neu5Ac(α2–8)Neu5Gc(α2–3)]Gal(β1–4)Glc(β1–1′)Cer[b]	[114]
Ara(1–6)Gal(β1–4)[{Gal(β1–8)}$_{0-1}$]Neu5Gc/5Gc8Me(α2–3)Gal(β1–4)Glc(β1–1′)Cer	[13]
Neu5Gc8Me(2–3)GalNAc(1–3)Gal(1–4)Glc(1–1′)Cer	[115]
Neu5Gc8Me(α2–3)[Neu5Gc8Me(α2–6)]GalNAc(β1–3)Gal(β1–4)Glc(β1–1′)Cer	[44,115]
Neu5Ac/5,9Ac$_2$/5Gc(α2–3)Gal(β1–3)GalNAc(β1-	[108]
{Neu5Ac(α2–8)}$_{1-2}$Neu5Ac(α2–3)Gal(β1–3)GalNAc(β1-	[108,116]
Neu5Gc(α2–8)Neu5Gc(α2–3)Gal(β1–3)GalNAc(β1-	[117]
Neu5Ac(α2–3)[Neu5Ac(α2–6)]Gal(β1–3)GalNAc(β1-	[108]

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Table 3, *continued*

Structure	References
Neu5Ac(α2–3)[[R-3]GalNAc(β1–4)]Gal(β1–3)GalNAc(β1-[c]	[108]
Neu5Gc(α2–3)[GalNAc(β1–4)]Gal(β1–3)GalNAc(β1-	[108]
{Neu5Ac(α2–8)}$_{0-1}$Neu5Ac(α2–3)Gal(β1–3)[{Neu5Ac(α2–8)}$_{0-1}$Neu5Ac(α2–6)]GalNAc(β1-	[108]
Neu5Ac(α2–3)Gal(β1–3)[Neu5Ac(α2–6)]GalNAc(β1–4)[{Neu5Ac(α2–8)}$_{0-1}$Neu5Ac(α2–3)]Gal(β1–4)Glc(β1–1′)Cer	[118,119]
Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1-	[108]
Neu5Ac/5Gc(α2–3)Gal(β1–4)GlcNAc(β1-	[108]
{Neu5Ac(α2–8)}$_{1-2}$Neu5Ac(α2–3)Gal(β1–4)GlcNAc(β1-	[108]
Neu5Ac/5Gc(α2–8)Neu5Gc(α2–3)Gal(β1–4)GlcNAc(β1-	[120]
Neu5Ac(α2–3)[GalNAc(β1–4)]Gal(β1–4)GlcNAc(β1-	[108]
Neu5Ac(α2–3)Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1-	[108]
Neu5Ac(α2–3)Gal(β1–3)GlcNAc(β1-	[108]
Neu5Ac(α2–3)[GalNAc(β1–4)]Gal(β1–3)GlcNAc(β1-	[121]
Neu5Ac(α2–3)Gal(β1–3)[Fuc(α1–4)]GlcNAc(β1-	[108]
Neu5Ac(α2–3)Gal(β1–3)[Neu5Ac(α2–6)][R-4]GlcNAc(β1-[c]	[108]
Neu5Ac(α2–3)GalNAc(β1–3)Gal(α1-	[108]
Neu5Ac(α2–3)GalNAc(β1–3)Gal(β1-	[108]
{Neu5Gc8Me(α2–O5)}$_n$-	[31]
Kdn(α2–3)Gal(β1–4)Glc(β1–1′)Cer	[122]
[R-3]GalNAc(β1–4)[Kdn(α2–3)]Gal(β1–4)Glc(β1–1′)Cer[b]	[123,124]
Kdn/Kdn9Ac(α2–3)Gal(β1–3)GalNAc(β1-	[123,124]
Kdn(α2–3)Gal(β1–3)[Neu5Ac/Kdn(α2–6)]GalNAc(β1-	[124]

[a] S, sulfate; Cer, ceramide.
[b] R, saccharide.
[c] R, H or saccharide.

Table 4
Sialic-acid-containing human milk oligosaccharides

Structures	References
Neu5Ac(α2–3)Gal(β1–4)Glc[a–e]	[16,125–127]
Neu5Ac(α2–6)Gal(β1–4)Glc[a,b]	[16,125–127]
Neu5Ac(α2–3)Gal(β1–3)GlcNAc(β1–3)Gal(β1–4)Glc	[125,126,128,129]
Neu5Ac(α2–3)Gal(β1–3)[Neu5Ac(α2–6)]GlcNAc(β1–3)Gal(β1–4)Glc[a,f]	[125,128–130]
Neu5Ac(α2–3)Gal(β1–3)[Fuc(α1–4)]GlcNAc(β1–3)Gal(β1–4)Glc	[128,131,132]
Neu5Ac(α2–3)Gal(β1–3)[Fuc(α1–4)][Neu5Ac(α2–6)]GlcNAc(β1–3)Gal(β1–4)Glc	[129,130]
Neu5Ac(α2–3)[Fuc(α1–2)]Gal(β1–3)GlcNAc(β1–3)Gal(β1–4)Glc	[133]
Gal(β1–3)[Neu5Ac(α2–6)]GlcNAc(β1–3)Gal(β1–4)Glc[a]	[125,126,128]
Gal(β1–3)[Neu5Ac(α2–4)]GlcNAc(β1–3)Gal(β1–4)Glc	[134]
Fuc(α1–2)Gal(β1–3)[Neu5Ac(α2–6)]GlcNAc(β1–3)Gal(β1–4)Glc	[128,131,135]
Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)Glc[a]	[125,126,128]
Neu5Ac(α2–3)Gal(β1–3)GlcNAc(β1–3){Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–6)}Gal(β1–4)Glc	[136]
Neu5Ac(α2–3)Gal(β1–3)GlcNAc(β1–3)[Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–6)]Gal(β1–4)Glc	[129]
Neu5Ac(α2–3)Gal(β1–3)[Neu5Ac(α2–6)]GlcNAc(β1–3)[Gal(β1–4)GlcNAc(β1–6)]Gal(β1–4)Glc	[129]
Neu5Ac(α2–3)Gal(β1–3)[Neu5Ac(α2–6)]GlcNAc(β1–3)[Fuc(α1–3)Gal(β1–4)GlcNAc(β1–6)]Gal(β1–4)Glc	[137]
Neu5Ac(α2–3)Gal(β1–3)[Neu5Ac(α2–6)]GlcNAc(β1–3){Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–6)}Gal(β1–4)Glc	[129,130,137]
Neu5Ac(α2–3)Gal(β1–3)[Neu5Ac(α2–6)]GlcNAc(β1–3)[Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–6)]Gal(β1–4)Glc	[138]
Neu5Ac(α2–3)Gal(β1–3)[Fuc(α1–4)]GlcNAc(β1–3)[Gal(β1–4)GlcNAc(β1–6)]Gal(β1–4)Glc	[139]
Neu5Ac(α2–3)Gal(β1–3)[Fuc(α1–4)]GlcNAc(β1–3){Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–6)}Gal(β1–4)Glc	[139]
Neu5Ac(α2–3)Gal(β1–3)[Fuc(α1–4)]GlcNAc(β1–3)[Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–6)]Gal(β1–4)Glc	[139]
Neu5Ac(α2–3)Gal(β1–3)[Fuc(α1–4)]GlcNAc(β1–3)[Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)GlcNAc(β1–6)]Gal(β1–4)Glc	[140]
Neu5Ac(α2–3)Gal(β1–3)[Fuc(α1–4)]GlcNAc(β1–3){Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–3)Gal(β1–4)GlcNAc(β1–6)}Gal(β1–4)Glc	[141]
Neu5Ac(α2–3)Gal(β1–3)[Fuc(α1–4)]GlcNAc(β1–3)[Gal(β1–3)GlcNAc(β1–3)Gal(β1–4)GlcNAc(β1–6)]Gal(β1–4)Glc	[141]
Neu5Ac(α2–3)Gal(β1–3)[Fuc(α1–4)]GlcNAc(β1–3){Gal(β1–3)[Fuc(α1–4)]GlcNAc(β1–3)Gal(β1–4)GlcNAc(β1–6)}Gal(β1–4)Glc	[141]

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Table 4, *continued*

Structures	References
Neu5Ac(α2–3)Gal(β1–3)[Fuc(α1–4)]GlcNAc(β1–3){Gal(β1–3)GlcNAc(β1–3)Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–6)}Gal(β1–4)Glc	[141]
Neu5Ac(α2–3)Gal(β1–3)[Fuc(α1–4)]GlcNAc(β1–3){Fuc(α1–2)Gal(β1–3)GlcNAc(β1–3)Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–6)}Gal(β1–4)Glc	[140]
Gal(β1–3)GlcNAc(β1–3)[Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–6)]Gal(β1–4)Glc	[125,126]
Gal(β1–3)[Neu5Ac(α2–6)]GlcNAc(β1–3){Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–6)}Gal(β1–4)Glc	[136]
Gal(β1–3)[Neu5Ac(α2–4)]GlcNAc(β1–3)[Gal(β1–3)GlcNAc(β1–6)]Gal(β1–4)Glc	[134]
Gal(β1–3)[Neu5Ac(α2–4)]GlcNAc(β1–3)[Fuc(α1–2)Gal(β1–3)GlcNAc(β1–6)]Gal(β1–4)Glc	[134]
Gal(β1–3)[Fuc(α1–4)]GlcNAc(β1–3)[Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–6)]Gal(β1–4)Glc	[136]
Gal(β1–3)[Fuc(α1–4)]GlcNAc(β1–3){Neu5Ac(α2–3)Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–6)}Gal(β1–4)Glc	[142]
Fuc(α1–2)Gal(β1–3)GlcNAc(β1–3)[Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–6)]Gal(β1–4)Glc	[126,143]
Fuc(α1–2)Gal(β1–3)GlcNAc(β1–3){Neu5Ac(α2–3)Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–6)}Gal(β1–4)Glc	[142]
Fuc(α1–2)Gal(β1–3)[Fuc(α1–4)]GlcNAc(β1–3)[Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–6)]Gal(β1–4)Glc	[136]
Gal(β1–4)GlcNAc(β1–3)[Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–6)]Gal(β1–4)Glc	[125]
Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–3)[Gal(β1–4)GlcNAc(β1–6)]Gal(β1–4)Glc	[126,144]
Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–3){Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–6)}Gal(β1–4)Glc	[126]
Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–3){Fuc(α1–2)Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–6)}Gal(β1–4)Glc	[126]
Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–3)[Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–6)]Gal(β1–4)Glc	[130]
Neu5Ac(α2–6/3)Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–3)[Neu5Ac(α2–3/6)Gal(β1–4)GlcNAc(β1–6)]Gal(β1–4)Glc	[137]
Neu5Ac(α2–3)Gal(β1–4)[Fuc(α1–3)]Glc	[126]
Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)[Fuc(α1–3)]Glc	[126,145]
Neu5Ac(α2–3)Gal(β1–3)[Neu5Ac(α2–6)]GlcNAc(β1–3)Gal(β1–4)[Fuc(α1–3)]Glc	[130]
Neu5Ac(α2–3)Gal(β1–3){Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–6)}Gal(β1–4)Glc	[136]
Neu5Ac(α2–3)Gal(β1–3)[Fuc(α1–4)]GlcNAc	[146]
Neu5Ac(α2–3)Gal(β1–3)[Fuc(α1–4)]GlcNAc(β1–3)Gal	[146]

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Table 4, notes

[a] Also occurring in feces of preterm infants fed on breast milk [147].

[b] Also occurring in bovine colostrum and human (pregnancy) urine [148].

Other sialyloligosaccharides in bovine colostrum are: Neu5Ac(α2–3)Gal, Neu5Gc(α2–3)Gal(β1–4)Glc, Neu5Ac(α2–8)Neu5Ac(α2–3)Gal(β1–4)Glc, Neu5Ac(α2–6)Gal(β1–4)GlcNAc, Neu5Ac(α2–6)Gal(β1–4)GlcNAc(α1-P, Neu5Ac(α2–6)Gal(β1–4)[6P]GlcNAc.

Other sialyloligosaccharides in human (pregnancy) urine are: Neu5Ac(α2–3)Gal(β1–4)GlcNAc, Neu5Ac(α2–6)Gal(β1–4)GlcNAc, Neu5Ac(α2–3)Gal(β1–4)GlcNAc(α1-P, Neu5Ac(α2–6)Gal(β1–4)GlcNAc(α1-P, Neu5Ac(α2–3)Gal(β1–3)[Neu5Ac(α2–6)]GalNAc, Neu5Ac(α2–3)Gal(β1–3)[Neu5Ac(α2–6)]GalNAc(α1–O)Ser, Neu5Ac(α2–3)Gal(β1–3)GalNAc(α1-P, Neu5Ac(α2–3)Gal(β1–1L)-*myo*-inositol, Neu5Ac(α2–3)Gal(β1-)*scyllo*-inositol.

The urinary carbohydrates, except the inositol derivatives and the tetrasaccharide, do also occur in hemofiltrates of patients with end-stage renal disease; in addition Neu5Ac(α2–8)Neu5Ac, Neu5Ac(α2–3)Gal(β1–3)GalNAc(α1–O)Ser-Leu and Neu5Ac(α2–3)Gal(β1–4)Xyl(β1–O)Ser have been detected [149,150]. For NMR data of Neu5Ac(α2–3)Gal(β1–4)Glc, Neu5Ac(α2–3)Gal(β1–4)GlcNAc, and Neu5Ac(α2–6)Gal(β1–4)GlcNAc, see also [151].

Urine of patients with aspartylglycosaminuria contains Neu5Ac(α2–3)Gal(β1–4)GlcNAc(β1–N)Asn, Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–N)Asn, and Neu5Ac(α2–3)Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)GlcNAc(β1–N)Asn [76,152].

Urine of patients with β-mannosidosis contains Neu5Ac(α2–6)Man(β1–4)GlcNAc [153].

[c] Neu5Ac(α2–3)[6S]Gal(β1–4)Glc occurs in rat milk and mammary gland [154].

[d] Neu5,9Ac_2(α2–3)Gal(β1–4)Glc occurs in rat urine [25].

[e] Neu4,5Ac_2(α2–3)Gal(β1–4)Glc occurs in monotreme milk [19].

[f] Occurs also in human pregnancy urine [155].

for both glycoprotein and glycolipid glycans a sequence has been found comprising the oligomerization of Neu5Gc residues through their anomeric centers and *N*-glycolyl moieties, Neu5Gc(α2–O5)Neu5Gc(α2–O5)Neu5Gc(α2– [31,101]; in the case of the glycolipid material the Neu5Gc residues are also 8-*O*-methylated [31]. In microbial polysaccharides, besides internal 8- and 9-substituted Neu5Ac residues, also internal 4- and 7-substituted Neu5Ac units have been frequently found (Table 5). It should be noted that some of the sialic-acid-containing glycan fragments and polysialic acids are specifically found in lipopolysaccharides and capsular polysaccharides of pathogenic bacteria, leading to severe problems in the development of suitable vaccines. A typical source for sialo-oligosaccharides generated from N- and O-linked glycans is the urine of sialidosis and I-cell disease patients [191–199], though this is not discussed in detail here (see section 10.5, and volume 30 of the New Comprehensive Biochemistry series). Patients with other inborn errors of metabolism, like aspartylglycosaminuria [76,152] or β-mannosidosis [153] excrete small amounts of structurally unusual sialo-oligosaccharides, of which the formulae have been included in the footnotes of Table 4. Finally, sequence information of the already mentioned glycosylphosphatidylinositol anchor is available, showing that the glycan core consists of Manα-Manα-Man-[Neu5Ac-Gal-GalNAc)Man-GlcN-inositol [186].

The phenomenon of intramolecular lactone formation, often reported for polysialic acid [200–202] and for gangliosides [203–206], has not been detected so far in glycoprotein sialoglycans. In the case of a Neu5Ac(α2–8)Neu5Ac sequence, lactonization affords a Neu5Ac(α2–8,1–9)Neu5Ac element (Fig. 4), whereby the COOH group of one residue reacts with HO9 of an adjacent residue, to give a six-membered ring. Similarly Neu5Ac(α2–9)Neu5Ac can be converted into Neu5Ac(α2–9,1–8)Neu5Ac, and Neu5Ac(α2–3)Gal into Neu5Ac(α2–3,1–2)Gal or Neu5Ac(α2–3,1–4)Gal. In a polysialic acid chain of α-2,8-linked Neu5Ac residues the α-2,8/1,9-lactonization can be effected under relatively mild conditions, like mild acid treatment [200], yielding a water-insoluble polymer. The Neu5Ac(α2–9,1–8)Neu5Ac formation in a polysialic acid chain can only be realized by carbodiimide treatment [201], illustrating a more difficult condensation with the secondary HO8 group. The difference in reactivity between the primary and the secondary OH group has been nicely illustrated for the alternating α-2,8/α-2,9-polysialic acid of *E. coli* K92 (Table 5) [202]. For gangliosides, it has been stated that ganglioside lactones occur also as such in nature [206], and that in this way the negative charge of a ganglioside under physiological conditions can be modulated. Lactonization makes the oligosaccharide chain also more rigid, which may have important biological implications. Treatment with carbodiimide or glacial acetic acid can even convert a Neu5Ac(α2–8)Neu5Ac(α2–3)Gal(β1– into a Neu5Ac(α2–8,1–9)Neu5Ac(α2–3,1–2)Gal(β1– sequence [204,205].

Over the years Neu5Ac has been prepared by a variety of methods. Several biological sources have been explored to isolate this sialic acid in high amounts. Among them are edible birds nest substance [207], urine of sialuria patients [65], colominic acid $\{Neu5Ac(\alpha2–8)\}_n$ produced by *E. coli* strains [208], and hen's egg chalaza, egg-yolk membranes and delipidated egg yolk [209,210]. The large scale organic synthesis of Neu5Ac is still complicated (see section 6.1). However, recent biotechnological routes, using sialate-pyruvate lyase (aldolase; see section 9.5), readily yield large amounts of

Table 5
Survey of structures or elements of sialic-acid-containing microbial polysaccharides

Structure/element	Species	Reference(s)
Capsular polysacharides (CPS)		
{-8)Neu5Ac(α2–8)Neu5Ac(α2-}$_n$	*Neisseria meningitidis* B	[24]
	Escherichia coli K235	[156]
	Pasteurella haemolytica A-2	[157]
	Moraxella nonliquefaciens	[158]
{-8)Neu5Ac(α2–8)Neu5Ac(α2-}$_n$ (with 7Ac or 9Ac)	*Escherichia coli* K1	[24,159]
{-9)Neu5Ac(α2–9)Neu5Ac(α2-}$_n$ (with 7Ac or 8Ac)	*Neisseria meningitidis* C	[24]
{-8)Neu5Ac(α2–9)Neu5Ac(α2-}$_n$	*Escherichia coli* K92	[160]
{-4)Neu5Ac(α2–6)Gal(α1-}$_n$	*Neisseria meningitidis* W135	[24]
{-4)Neu5Ac(α2–6)Glc(α1-}$_n$ (with Ac)	*Neisseria meningitidis* Y	[24]
{-3)GalNAc(β1–4)Gal(α1–4)Neu5,9Ac$_2$(α2–3)Gal(β1-}$_n$	*Escherichia coli* K9	[26,161]
{-4)Glc(β1–4)[Neu5Ac(α2–3)Gal(β1–4)GlcNAc(β1–3)]Gal(β1-}$_n$	Group B *Streptococcus* Ia	[24]
{-4)Glc(β1–4)[Neu5Ac(α2–3)Gal(β1–3)GlcNAc(β1–3)]Gal(β1-}$_n$	Group B *Streptococcus* Ib	[24]
{-4)GlcNAc(β1–3)[Gal(β1–6)]Gal(β1–4)Glc(β1–3)Glc(β1–2)[Neu5Ac(α2–3)]Gal(β1-}$_n$	Group B *Streptococcus* II	[24]
{-4)Glc(β1–6)[Neu5Ac(α2–3)Gal(β1–4)]GlcNAc(β1–3)Gal(β1-}$_n$	Group B *Streptococcus* III	[162]
{-4)Glc(α1–4)[Neu5Ac(α2–3)Gal(β1–4)GlcNAc(β1–6)]Gal(β1–4)Glc(β1-}$_n$	Group B *Streptococcus* IV	[163]
{-4)Glc(β1–4)[Neu5Ac(α2–3)Gal(β1–4)GlcNAc(β1–6)]Glc(α1–4)[Glc(β1–3)]Gal(β1-}$_n$	Group B *Streptococcus* V	[164]
{-4)Glc(β1–6)[Neu5Ac(α2–3)Gal(β1–3)]Glc(β1–3)Gal(β1-}$_n$	Group B *Streptococcus* VI	[165]
{-4)Glc(β1–4)[Neu5Ac(α2–3)Gal(β1–4)GlcNAc(β1–6)]Glc(α1–4)Gal(β1-}$_n$	Group B *Streptococcus* VII	[165a]
{-3)Glc(β1–2)[Kdn(α2–4)][3Ac]GlcA(α1–3)Man(α1–3)Glc(α1-}$_n$	*Klebsiella ozaenae* K4 (2211)	[49]
Lipopolysaccharides (LPS)[a]		
{-3)GalNAc(β1–7)Neu5Ac(α2–3)[Glc(α1–2)]Glc(β1-}$_n$	*Escherichia coli* O24	[166,167]
{-3)GlcNAc(β1–7)Neu5Ac(α2–3)[Gal(α1–2)]Glc(β1-}$_n$	*Escherichia coli* O56	[166,167]
{-3)GalNAc(β1–4)Gal(α1–4)Neu5,7/8,9Ac$_3$(α2–3)Gal(β1-}$_n$ (also with 9Ac)	*Escherichia coli* O104	[26,27]

continued on next page

Table 5, *continued*

Structure/element	Species	Reference(s)
{-3)[±6Ac]GlcNAc(β1–7)Neu5Ac(α2–3)[CH_3(NH)C-2]FucN(α1-}$_n$	*Salmonella arizonae* O21	[168]
{-4GalANGro(α1–4)Neu5Ac(α2–3)GalANGro(β1–3)QuiNAc(β1-}$_n$ [b]	*Vibrio cholerae* H11 (non-O1)	[169]
{-3)[Glc(α1–6)]GalNAc(β1–4)Neu5Ac(α2–6)Glc(α1–6)[Glc(α1–4)Gal(β1–6)Glc(β1–3)]Gal(β1-}$_n$	*Hafnia alvei* O2	[170]
Core regions of lipopolysaccharides (LOS) [a]		
Neu5Ac(α2–3)Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)Glc(β1–4)Hep-	*Neisseria meningitidis*	[171–173]
Neu5Ac(α2–3/6)Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)Hep-	*Haemophilus ducreyi*	[174]
Neu5Ac(α2–3)[GalNAc(β1–4)]Gal(β1–3)[Gal(α1–2)]Gal(β1–3)Hep-	*Campylobacter jejuni* O1	[175]
Neu5Ac(α2–3)Gal(β1–3)[Gal(α1–2)]Gal(β1–3)Hep-	*Campylobacter jejuni* O2	[176]
Neu5Ac(α2–3)Gal(β1–3)GalNAc(β1–4)[Neu5Ac(α2–3)]Gal(β1–3)Hep-	*Campylobacter jejuni* O4	[175]
Neu5Ac(α2–3)[GalNAc(β1–4)]Gal(β1–4)Glc(β1–2)Hep-	*Campylobacter jejuni* O23 and O36	[175]
Gal(α1–6)Glc(β1–7)Neu5Ac	*Rhodobacter capsulatus* 37b4	[177]

[a] Some other species for which it has been demonstrated that their LPS or LOS contains sialic acid are: *Rhizobium meliloti* M11S (LPS, Neu5Ac/Neu5,9Ac_2) [178]; *Salmonella toucra* (LPS, terminal and 4-linked Neu5Ac) [179]; *Salmonella djakarta* (LPS, terminal Neu5Ac) [180]; *Salmonella isaszeg* (LPS, terminal and 4-linked Neu5Ac) [180]; *Citrobacter freundii* O37 (LPS, terminal and 7-linked Neu5Ac) [179]; *Pasteurella haemolytica* 1 and 5 (LPS, Neu5Ac) [181]; *Haemophilus influenzae* (LOS, terminal Neu5Ac) [182,183]; *Neisseria gonorrhoeae* (LOS, terminal Neu5Ac) [184,185]. Note that for *E. coli* O24 and O56 and *H. alvei* O2 also terminal Neu5Ac has been found [179].

[b] GalANGro, *N*-galacturonyl-2-aminoglycerol.

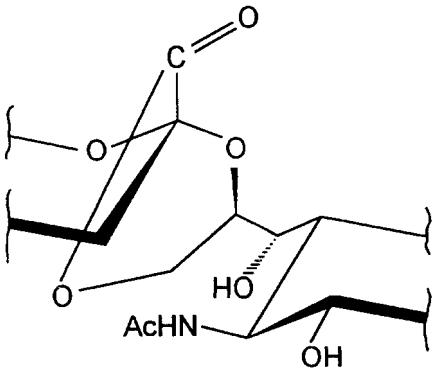

Fig. 4. Lactonization of a Neu5Ac(α2–8)Neu5Ac(α2– sequence, affording a Neu5Ac(α2–8,1–9)Neu5Ac(α2– fragment.

this sialic acid (see section 6.1). Efficient procedures to prepare Neu5Gc from colominic acid via de-*N*-acetylation, *N*-acryloylation and reductive ozonolysis, followed by acid or enzymatic hydrolysis of the formed $\{Neu5Gc(\alpha2–8)\}_n$ has appeared in refs. [211,212]. Also porcine submandibular gland is a good source for the large scale preparation of Neu5Gc, whereas $Neu5,9Ac_2$ can be prepared from bovine submandibular gland [213].

Besides the occurrence of sialic acids [(*O*-acetylated) Neu5Ac and Kdn] in microbial polysaccharides (Table 5), some lipopolysaccharides have shown to contain sialic-acid-like monosaccharides. They differ from sialic acids in the presence of an additional amino function at C7, a deoxy function at C9, and in the configuration at the chiral centers: 5,7-diamino-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-non-2-ulosonic acid and 5,7-diamino-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-non-2-ulosonic acid [214,214a]. So far, the two amino groups have been found to be substituted in different combinations, yielding acetamido, formamido, (*R*)-3-hydroxybutyramido, 4-hydroxybutyramido, or acetamidino functions. Also *O*-acetylation can occur, whereas the (*R*)-3-hydroxybutyryl group at C7 can be used to link monosaccharides in a polysaccharide chain. Typical species are *Pseudomonas aeruginosa* strains [214], *Shigella boydii* type 7 [214], *Vibrio salmonicida* [214a], *Vibrio cholerae* O2 [215], *Vibrio alginolyticus* strain 945-80 [216], *Salmonella arizonae* O61 [217], *Yersinia ruckeri* O1 [218], *Legionella pneumophila* strain 1 [219,220], and *Pseudomonas fluorescens* ATCC 49271 [220a]. For a review, see ref. [220], but it should be noted that the absolute configuration of 5,7-diamino-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-non-2-ulosonic acid was earlier assigned as D-*glycero*-L-*galacto*- [214,214a,216–219,220a].

4. *Screening of biological materials for the presence of sialic acid*

For the staining of sialic acids in tissues and cells, a great variety of techniques is available. Classical histochemistry of sialic-acid-containing glycoconjugates makes use of either binding of cationized dyes (e.g. Alcian blue, cationized ferritin, ruthenium red) or selective periodate oxidation (derivatization of generated aldehyde groups of sialic acids with e.g. *p*-dimethylaminobenzaldehyde, dansylhydrazine, rhodamine, biotin/ferritin-conjugated avidin). The second approach is strongly dependent on side-chain modifications [53]. Comprehensive reviews on this subject have appeared [6,221].

Nowadays, specific lectins are frequently used to detect the presence of glycosidically bound sialic acids in complex carbohydrates. However, the specificity of lectins is generally broad, and positive information has always to be checked in control experiments using e.g. sialidases in the presence and absence of inhibitors. For the histochemical analysis, lectins may be conjugated e.g. with gold particles, peroxidase, rhodamine or fluorescein isothiocyanate [6]. A large series of lectins which recognize sialic acid have been demonstrated to occur in nature, and most of their biological sources have been summarized in refs. [6,11,222]; see also refs. [223–227]. For updated reviews focusing on lectins, see the present volume 29b of the New Comprehensive Biochemistry series. Generally, lectins have been isolated from lower animals such as prawns, snails, crabs, spiders, scorpions, lobsters, slugs, oysters, but also from plants, rat brain and B cells. While some of these lectins bind to both Neu5Ac and Neu5Gc, others are specific for Neu5Ac. *O*-Acetylation may also influence the binding strength of the lectin, both in a positive and in a negative manner (see section 10.3).

For introductory glycoprotein analysis, in answering questions like "what monosaccharides are in the glycoprotein glycans?", two sialic-acid-specific plant lectins, having also a glycosidic linkage specificity, have been included in commercially available kits. These lectins are the agglutinins from *Maackia amurensis* and *Sambucus nigra,* being diagnostic for Neu5Ac(α2–3)Gal and Neu5Ac(α2–6)Gal/GalNAc elements, respectively. For screening purposes, the lectins are labelled with digoxigenin-succinyl-ε-amidocaproic acid hydrazide (DIG, a spacer-linked steroid hapten digoxigenin). After SDS/PAGE of the (glyco)protein mixture and Western blotting, sialoglycoprotein bands with α-2,3- and/or α-2,6-linked Neu5Ac can be labelled by one or both of these DIG-labelled lectins, whereby the detection is carried out in an enzyme immuno-assay using a digoxigenin-specific antibody conjugated to alkaline phosphatase, followed by color development with 5-bromo-4-chloro-3-indolyl-phosphate/4-nitroblue tetrazolium chloride [228,229].

Viruses and antibodies can also be used for the detection of sialic acid in complex carbohydrate systems. The sialic-acid-binding properties of a number of viruses have been established, and it has been shown that influenza C virus, bovine corona virus and encephalomyelitis virus recognize 9-*O*-acetylated sialic acids (see also sections 8.4.2, 9.1 and 10.3). Microtiter-plate and nitrocellulose-membrane assays have been developed that use the hemagglutinin (receptor) and the receptor destroying activities (sialate 9-*O*-acetylesterase) of the influenza C virus to specifically detect bound 9-*O*-acetylated sialic acids in glycoproteins [230,231]. Although the recognition site for Neu5,9Ac_2 and the esterase activity are located on the same viral glycoprotein, these activities can be separated using different temperatures for the binding (4°C) and the enzyme reaction (4-methylumbelliferyl acetate or α-naphtyl acetate as substrates; 20–37°C). Other approaches are based on binding of the virus to immobilized ligands, and detection of the virus with monoclonal antibodies, whereby the esterase was selectively inactivated by the use of diisopropylfluorophosphate (DFP) [232]. Virus particles have also been labelled with radioactive isotopes [232] or biotin [233]. The application of the assay with virus particles for the staining of *O*-acetylated sialic acids in tissue sections will be described in section 8.4.2. Recently, a new technique using a soluble chimera of the hemagglutinin-esterase portion of the hemagglutinin-esterase-fusion-glycoprotein from influenza C virus and the Fc portion of human IgG, has been reported [234,235]. Such a chimera retains

both its recognition and enzymatic functions, and also has the binding properties of the Fc portion of IgG. The probing can be carried out on blots and TLC plates taking into account precautions for the recognition and esterase activities (this has to be inhibited by DFP in the test) as discussed above.

An interesting electrochemical method for the determination of bound sialic acid has been developed, making use of a potentiometric four-channel thick-film sensor [236]. The sialidase sensor consists of a bilayer of a membrane containing *Clostridium perfringens* sialidase immobilized in a poly(vinyl acetate)–polyethylene copolymer, which is placed on top of an H^+-selective poly(vinyl chloride)–poly(vinyl acetate) indicator membrane. The enzyme-induced release of bound sialic acid leads to a concomitant decrease in pK_a of the carboxyl function of sialic acid. This decrease affords a local pH change inside the sialidase-containing sensor membrane, which is monitored by the H^+-selective indicator membrane. The pH optimum of the sialidase sensor was pH 4 for sialyllactose, mucin and colominic acid.

Finally, TLC analysis may also be of great help in the screening of biological materials for the presence of sialic acids. This item will be discussed in section 5.3.1.

5. *Isolation and analysis of sialic acids*

5.1. Liberation

The characterization of the type of sialic acid in sialoglycoconjugates is frequently carried out after release and (partial) isolation. The cleavage of sialic acid from sialic-acid-containing material is mainly performed by two methods, namely, acid hydrolysis and enzymatic hydrolysis. Both approaches have advantages and disadvantages. In the case of acid hydrolysis, problems arise with respect to de-*O*-esterification, which complicates quantitative analysis procedures. With respect to the enzymatic hydrolysis with sialidases, linkage specificity as well as a reduced or complete lack of susceptibility have to be taken into account. Moreover, in most cases much lower amounts of sialic acids are released by sialidases than by acid hydrolysis, which may be due to the different accessibility of the sialic acids in the biomolecules to be analyzed. Additionally, in work-up procedures and analyses, pH values below 4 and over 6 should be avoided to prevent migration of the *O*-acetyl group at C7/C8 and hydrolysis of *O*-acetyl groups as much as possible [23,57].

Several approaches have been reported for the effective acid hydrolysis of the labile α-2,3-, α-2,6- and α-2,8-linkages. All these procedures suffer from being not optimal in giving the real spectrum of sialic acids originally present in the sialoglycoconjugate under study, especially in the case of a mixture of (*O*-acylated) *N*-acylneuraminic acids. Terminal sialic acids are released in high yield (and low destruction) using a two-step acid hydrolysis procedure comprising treatment with formic acid (pH 2, 1 h, 70°C), followed by HCl (pH 1, 1 h, 80°C). After each step, the liberated sialic acids must be recovered by centrifugation, ultrafiltration or dialysis [11,12,213,237]. It should be noted that in the case of a spectrum of (*O*-acylated) *N*-acylneuraminic acids, the supernatant, ultrafiltrate or diffusate of the formic acid hydrolysis contains the majority of the *O*-acylated *N*-acylneuraminic acid, whereas that of the HCl hydrolysis contains

Table 6
Substrate specificity of commercially available sialidases[a]

Source of sialidase	pH optimum	α-2,3[b]	α-2,6[c]	α-2,8[d]	α-2,3[e]	Reference
Arthrobacter ureafaciens	4.4–5.5[f]	100	167	53	12	[5]
Vibrio cholerae	5.0–6.5[g]	100	53	31	25	[5]
Clostridium perfringens[h]	5.5–7.2[i]	100	44	44	20	[5]
Salmonella typhimurium	5.0–7.0[j]	100	0.4	n.d.[k,l]	n.d.	[247]
Newcastle disease virus	5.0–6.0	100	0.2	78	11	[5]

[a] Relative rates of cleavage are indicated (100 = full activity).
[b] Neu5Ac(α2–3)lactose.
[c] Neu5Ac(α2–6)lactose.
[d] Neu5Ac(α2–8)Neu5Ac(α2–3)lactose.
[e] Neu5Gc(α2–3)lactose.
[f] Sialyllactose, pH 5.0–5.5, and colominic acid, pH 4.3–4.5.
[g] Sialyllactose, pH 5.0–5.5.
[h] High-molecular-mass isoenzyme (P. Roggentin, personal communication) (see also section 9.2).
[i] Sialyllactose, pH 5.0–5.1 (acetate buffer) and pH 5.8–6.0 (phosphate buffer).
[j] Depending on the buffer system used.
[k] n.d., not determined.
[l] {-8)Neu5Ac(α2–8)Neu5Ac(α2-}$_n$, 0.1% and {-9)Neu5Ac(α2–9)Neu5Ac(α2-}$_n$, 0.08%.

mostly Neu5Ac and Neu5Gc. In the case of low-molecular-mass substances, isolations can be carried out by gel-permeation chromatography. Although these conditions do not lead to significant de-*N*-acylation, de-*O*-acylation has been shown to occur to an extent of about 30–50%. One has to consider that milder acidic conditions result in incomplete release of sialic acids. Acid hydrolysis with acetic acid (2 M, 3 h, 80°C) as suggested in ref. [238] did not improve the yield of *O*-acylated *N*-acylneuraminic acids [11]. When focusing on sialic acid analysis, the use of H_2SO_4 (0.05 M) is not recommended because of work-up problems. In connection with the HPLC analysis of Neu5Ac and Neu5Gc, microwave hydrolysis in 2 M acetic acid has shown to be an interesting alternative [239].

In the methanolysis procedure, as used for the standard quantitative monosaccharide GLC analysis of glycoconjugates [240,241], 1 M methanolic HCl (24 h, 85°C) is applied. However, under these conditions released sialic acids are completely de-*N,O*-acylated, which makes this approach unsuitable for the characterization of different types of sialic acid. It is, however, a reliable approach for the determination of the total amount of a mixture of (*O*-acetylated) *N*-acylneuraminic acids. When using a milder methanolysis procedure (0.05 M methanolic HCl, 1 h, 80°C) the de-*N*-acylation but not the de-*O*-acylation is strongly reduced [242].

In the quantitative determination of *N*-acylneuraminic acids in poly-*N*-acylneuraminic-acid-containing glycoproteins, the release of free sialic acid was shown to be optimal using a combined mild acid hydrolysis (0.1 M TFA, 3 h, 80°C)/subsequent mild methanolysis (0.05 M methanolic HCl, 1 h, 80°C) [243]. This method is also advised for the analysis of the Kdn content in Kdn-containing glycoproteins. In contrast, optimal release of Kdn from poly-Kdn-containing glycoproteins is obtained by mild methanolysis with a longer incubation time [243]. In the latter case the standard conditions of methanolysis also give good results.

The enzymatic release of *N*-acylneuraminic acids can be carried out under such mild conditions (low temperature, pH 5–6), that destruction, migration, and de-*O*-acylation is kept at a minimum. In Table 6 the substrate specificity of commercially available sialidases from *Arthrobacter ureafaciens, Vibrio cholerae, Clostridium perfringens, Salmonella typhimurium,* and Newcastle disease virus, using simple *N*-acylneuraminyllactose substrates is compared (for comprehensive reviews on sialidases, see refs. [5,33,244–246]). It is evident that the sialidases show different ratios of cleavage rates for the α-2,3-, α-2,6- and α-2,8-linkages. The finding of a strong preference of the Newcastle disease virus sialidase for α-2,3-linkages holds also for other viral sialidases, such as those from fowl plague virus and influenza A_2 virus. The latter enzyme also has a low specificity for α-2,8-linkages [5]. Among the bacterial sialidases, *A. ureafaciens* sialidase has a certain preference for α-2,6-linkages. The *S. typhimurium* sialidase is the only bacterial sialidase with a viral sialidase-like pronounced preference for α-2,3-linkages [247]. Recently, two sialidases from *Bacteroides fragilis* having a higher preference for the cleavage of α-2,8-linkages, when compared with α-2,3- and α-2,6-linkages, were isolated [248]. The sialidase, recently isolated from *Macrobdella* leech, cleaves only Neu5Ac(α2–3)Gal linkages [61]. In general, Neu5Ac residues are released faster than Neu5Gc residues. In a study using different N- and O-glycoproteins with α-2,3- and/or α-2,6-linked *N*-acylneuraminic acids as substrates [antifreeze glycoprotein, ovine submandibular gland glycoprotein, α_1-acid glycoprotein; Neu5Ac(α2–6)GalNAc(α1–O)Thr/Ser, Neu5Ac/5Gc(α2–6)[Gal(β1–3)]GalNAc(α1–O)Thr, Neu5Ac/5Gc(α2–3)Gal(β1–3)GalNAc(α1–O)Thr, Neu5Ac(α2–3)Gal(β1–4)GlcNAc(β1–O)R; Neu5Ac/5Gc(α2–6)Gal(β1–4)GlcNAc(β1–O)R] and sialidases from *A. ureafaciens, V. cholerae, C. perfringens,* Newcastle disease virus, fowl plague virus, and influenza A_2 virus, roughly similar patterns of substrate specificity as for sialyllactoses were found. However, it was demonstrated that the core oligosaccharide and/or the protein structure may also influence the rate of release for different glycosidic linkages [249]. In the case of *S. typhimurium* sialidase, also N-glycoprotein α-2,3-sialoglycans were susceptible to efficient cleavage, but not mucin O-glycoprotein sialoglycans [247]. The most recently discovered sialic acid, Neu5GcAc, could not be released with *V. cholerae* sialidase [43]. A comparison of the different commercially available sialidases shows that the *C. perfringens* sialidase iso-enzyme with a molecular mass of about 63 kDa has the broadest specificity. It should be noted that *C. perfringens* in fact produces two sialidases, the larger of which (63 kDa) is commercially available (P. Roggentin, personal communication) (see also section 9.2). As described for the acid hydrolysis procedure, the work-up of enzymatically released sialic acids can be achieved employing various methods, depending on the starting sialoglycoconjugate material.

4-*O*-Acetylated neuraminic acids in any glycoconjugate are resistant to most sialidases tested so far; only viral sialidases show a low but significant activity. With the exception of *Streptococcus sanguis* sialidase [250], the presence of *O*-acetyl substituents at C7–C9 leads to a reduced rate of cleavage by all sialidases, so that prolonged incubations are necessary for an efficient release [251]. More information has been collected in a detailed study with bacterial and viral sialidases and 4-methylumbelliferyl (MU) α-glycosides of 4-, 7-, and 9-*O*-acetylated *N*-acetylneuraminic acids as substrates [252]. In contrast to

the other sialidases tested, the fowl plague virus sialidase catalyzes a slow release of Neu4,5Ac$_2$ from α-Neu4,5Ac$_2$-MU.

The recent finding of {Neu5Gc(α2–O5)}$_n$ elements in a glycoprotein [101] initiated a kinetic study of the enzymatic and non-enzymatic hydrolysis of Neu5Gc(α2–O5)Neu5Gc and Neu5Gc(α2–8)Neu5Gc [253]. It turned out that at pH < 3.8 the rate of acid hydrolysis of the unusual α-2,O5-linkage was greater than that of the normal α-2,8-linkage. However, at pH > 3.8 reverse results were obtained; Neu5Gc(α2–8)Neu5Gc released a small but detectable amount of Neu5Gc even at pH 6. The α-2,O5-linkage was only partially cleaved by *C. perfringens* and *V. cholerae* sialidases, and was essentially resistant to *A. ureafaciens* sialidase.

The detection of sialidases that can release Kdn is so far highly limited. The liver of the loach *Misgurnus fossilis* was found to contain a sialidase capable of releasing both Neu5Ac and Kdn from sialoglycoconjugates [39]. While the sialidases investigated so far in detail require an NH-acyl group at C5 for full activity, the loach enzyme can handle both NH-acyl and OH functions at C5. The rainbow trout also turned out to be a useful source for the isolation of a sialidase, active in releasing both Neu5Ac and Kdn [254]. Recently, a sialidase was isolated from *Sphingobacterium multivorum* that specifically released α-2,3-, α-2,6- and α-2,8-linked Kdn; Neu5Ac and Neu5Gc were not liberated [255].

Before fractionation, pools of free sialic acids can be freed from contaminants by several methods, including ion-exchange chromatography, cellulose chromatography, reversed-phase chromatography, preparative TLC [6,11,12]. Generally, one of the purification procedures for the pool of sialic acids comprises Dowex ion-exchange chromatography at low temperature. After passage through a cation-exchange resin (Dowex 50W-X8, H^+-form), the pool of sialic acids is adsorbed to an anion-exchange resin (Dowex 2-X8 or 1-X8, $HCOO^-$-form). Elution from the anion-exchange resin is generally carried out with 0–2 M formic acid. The ion-exchange chromatography should be carried out rapidly, as prolonged contact of acylneuraminic acids with the resin or the solvent systems used may result in degradation, *O*-acetyl migration and/or de-*O*-acetylation. After rotary evaporation or lyophilization, the sialic acid pools are stored at –20°C.

In general, mixtures of sialo-oligosaccharides from {Neu5Ac(α2–8)}$_n$, {Neu5Ac(α2–9)}$_n$, {–8)Neu5Ac(α2–9)Neu5Ac(α2–}$_n$, {Neu5Gc(α2–8)}$_n$, and {Kdn(α2–8)}$_n$, are generated by limited acid hydrolysis [211,256,257]. Depending on the polysialic acid, different conditions have been applied. Also attention has been paid to the intramolecular self-cleavage of polysialic acids such as {Neu5Ac(α2–8)}$_n$ [258]. Adjacent COOH groups with a high pK_a (3.9–5.5) act as proton donors for general acid catalysis. The lability is seen under mild acidic conditions, that can be encountered in various physiological situations. {Neu5Ac(α2–8)}$_{>3}$ is substantially more unstable than {Neu5Ac(α2–8)}$_{2-3}$.

A highly useful enzyme for the depolymerization of polysialyl carbohydrate chains, yielding oligosialyl compounds, is endo-sialidase (endo-N) produced by infection of *E. coli* K1 with a lytic bacteriophage [9,259,260]. The enzyme is specific in cleaving α-2,8-linkages, and requires at least five Neu5Ac or Neu5Gc residues for activity. A limited digestion of {Neu5Ac(α2–8)}$_n$-R affords mainly {Neu5Ac(α2–8)}$_4$, with some {Neu5Ac(α2–8)}$_{1-3}$. Alternating α-2,8/α-2,9-polysialyl chains, as present in some

bacterial polysaccharides, are also cleaved, but α-2,9-linked polysialyl chains are resistant. Poly-Kdn and $\{Neu5Gc(\alpha 2–O5)\}_n$ are not substrates for endo-N. A similar endo-sialidase associated with phage particles, namely, *E. coli* bacteriophage ϕ92, has been reviewed in ref. [261].

5.2. Colorimetry

One of the oldest methods used to detect and to quantitate sialic acids is colorimetry [3,6,12,237,262]. When carried out on non-purified samples, the influence of contaminants interfering with the assays has to be taken into account. Greatest problems are encountered when using cells or tissue extracts, as the level of contamination is inevitably high. Moreover, factors such as non-identical reactions of different sialic acids in the same assay and the non-specificity of the reactions for the sialic acids are important.

Although several colorimetric methods have been developed in the past, only two main procedures are currently routinely applied, namely the orcinol/Fe^{3+}/HCl assay, known as the "Bial" reaction, and the periodic acid/thiobarbituric acid assay. For microadaptations of these two different tests, see ref. [11].

In the first assay, the sample is mixed with orcinol, $FeCl_3$ and concentrated HCl and heated at 96°C. The formed purple to red-violet chromophore is extracted with isoamyl alcohol and its absorbance measured at 572 nm. Because of the use of HCl, the method can be used to quantitate the total amount of both free and glycosidically bound sialic acids. Due to the strongly acidic conditions, ester groups are released. As the assay does not discriminate between bound and free sialic acids, it is widely used to monitor the presence of sialic acids in either form during fractionation of biological material. It should be noted that other monosaccharides, especially pentoses, hexoses and uronic acids interfere with the assay, which is of importance when small amounts of sialic acid are present.

In the second assay, only suitable to quantitate free sialic acids, sialic acid is oxidized by periodate at 37°C under strongly acidic conditions. The oxidation leads to the formation of a prechromogen, a six carbon aldehyde, which then yields the chromogen β-formyl pyruvic acid by aldol cleavage between C4 and C5. The chromogen reacts with thiobarbituric acid to give a red chromophore, the absorbance of which is measured at 549 and 532 nm. In principle two approaches can be followed, called the Warren method and the Aminoff method. Differences between the methods lie in the acidity of the initial periodate oxidation and in the solvent used for the extraction of the pigment (cyclohexanone, Warren; acidic 1-butanol, Aminoff). It should be noted that substituents in the glycerol side chain severely influence the periodate oxidation. Therefore, in the case of ester substituents, a prior saponification is necessary (0.1 M NaOH, 37°C). Types of free sialic acid which do not yield the chromogen are negative in this test. Several compounds have been shown to interfere with the periodate/thiobarbituric acid assay, most especially 2-deoxyribose, 2-keto-3-deoxyaldonic acids other than Kdn, disaccharides such as lactose and maltose, and unsaturated fatty acids. The greatest errors arise in the quantitation of sialic acids from cellular extracts or homogenates containing membrane and nucleic acid material. Therefore, prior ion-exchange column chromatography and removal of lipids

by ether extraction are of advantage. Special attention to the periodic acid/thiobarbituric acid assay of Kdn has been paid in ref. [243].

When both tests are used in combination, a differentiation between total and free sialic acid is possible, allowing the calculation of the amount of glycosidically bound sialic acid.

In a new approach for the direct determination of free and bound sialic acid, an acidic ninhydrin assay has been proposed [263]. Heating of solutions of sialic-acid-containing material with ninhydrin/acetic acid/37% HCl at 100°C yields a stable chromophore, the absorbance of which can be measured at 470 nm.

In view of the comments made in section 5.1 with respect to the release of sialic acid by sialidases, a quantification procedure for bound sialic acid based on the enzymatic analysis of pyruvate, formed after sialidase/aldolase treatment [6,264], should be handled with care.

Of the various fluorimetric assays available for sialic acid analysis, the method which allows the discrimination between sialic acids with or without *O*-acyl groups at C8 and/or C9 may be of interest [6,265]. After mild periodate oxidation, formaldehyde, derived from C9 in the case of non-substituted HO9 and HO8 is derivatized with acetylacetone in the presence of ammonium acetate, leading to the fluorigen 3,5-diacetyl-1,4-dihydro-2,6-dimethylpyridine (410 nm excitation, 510 nm emission). It is evident that all contaminants producing formaldehyde under the influence of periodate will interfere with this sialic acid analysis.

Finally, for the quantitative estimation of the *O*-acyl content of sialic-acid-containing samples (Hestrin assay), also a colorimetric assay is routinely used. The method is based on the reaction with hydroxylamine in alkaline medium yielding hydroxamates, which form with $Fe(ClO_4)_3$ red chromophores, the absorbance of which is measured at 520 nm [262].

5.3. Chromatography

5.3.1. Thin-layer chromatography

From the beginning of free sialic acid analysis, TLC has played a major role in screening and tentative assignment procedures, and over the years several solvent systems for both cellulose and silicagel plates have been reported [6,237,262]. One of the most popular TLC methods for the analysis of (substituted) *N*-acylneuraminic acids comprises the use of plastic HPTLC plates precoated with cellulose and 1-propanol/1-butanol/0.1 M HCl (2:1:1, v/v/v) as solvent system. It shows the best and most reproducible separation of different sialic acids, and is less sensitive to interfering substances when compared with other systems. Generally, the visualization of the different sialic acids is carried out by spraying with the orcinol/Fe^{3+}/HCl reagent [237], yielding typical purple bands. For quantitative purposes densitometry is also used. It should be noted that due to differences in cellulose quality (impurities), even after pre-washing of the plates, the reproducibility of R_f values is relatively low. Therefore, analyses should be carried out in the presence of an appropriate reference sialic acid mixture on a separate lane. To give an impression of the separation capacity of cellulose plates, Table 7 summarizes the R_f values of a series

Table 7
Thin-layer chromatographic migration rates (R_f) of sialic acids on 0.1 mm cellulose plates using 1-propanol/1-butanol/0.1 M HCl (2:1:1, v/v/v) [6]

Compound	R_f	Compound	R_f
Neu5Ac	0.45	Neu2en5Ac	0.55
Neu4,5Ac_2	0.60	Neu5Gc	0.35
Neu5,7Ac_2	0.54	Neu4Ac5Gc	0.65
Neu5,9Ac_2	0.63	Neu9Ac5Gc	0.55
Neu5,7,9Ac_3	0.70	Neu7,9$Ac_2$5Gc	0.70
Neu5,8,9Ac_3	0.75	Neu5GcAc	0.51
Neu5,7,8,9Ac_4	0.80	Neu2en5Gc	0.45
Neu5Ac9Lt	0.56		

of *N,O*-acyl-neuraminic acids from one experiment [6]. In the case of radio-labelled sialic acids, the bands can also be traced by radio-TLC-scanning.

Application of the TLC method in a two-dimensional procedure with intermediate ammonia treatment gives information about the type of *N*-acylneuraminic acid of the constituting *N,O*-acylneuraminic acids. In this way a differentiation is possible for example between co-migrating Neu9Ac5Gc and Neu5,7Ac_2 [6]. In principle, de-*O*-acetylations can also be carried out by sialate *O*-acetylesterases [266].

For the analysis of oligomers of α-2,8-linked Neu5Ac, Neu5Gc or Kdn, a TLC procedure on silicagel with the solvent system 1-propanol/25% ammonia/water (12:2:5, v/v/v) has shown good results [267]. In this way, mixtures of $\{Neu5Ac(\alpha2\text{–}8)\}_2$ to $\{Neu5Ac(\alpha2\text{–}8)\}_{14}$, $\{Neu5Gc(\alpha2\text{–}8)\}_2$ to $\{Neu5Gc(\alpha2\text{–}8)\}_{10}$, or $\{Kdn(\alpha2\text{–}8)\}_2$ to $\{Kdn(\alpha2\text{–}8)\}_7$ are well separated. It should be noted that $\{Neu5Ac(\alpha2\text{–}8)\}_n$ and $\{Neu5Gc(\alpha2\text{–}8)\}_n$ can be visualized also with a resorcinol spray reagent, whereas for $\{Kdn(\alpha2\text{–}8)\}_n$ the orcinol spray reagent is needed.

5.3.2. High-performance liquid chromatography

Initially, the separation of sialic acids was mainly carried out by cellulose chromatography at low temperature [6,237]. However, nowadays HPLC fractionations using different column materials, elution protocols and detection techniques have replaced this approach [6,11,268]. The application of HPLC has also introduced a rapid method for tentative assignments of sialic acids in complex mixtures, based on elution times of known standards, being more reliable when more than one HPLC procedure is followed. Moreover, a rapid method for quantification of released sialic acids has become available. Due to the relatively short HPLC runs, also fast transitions between members of the sialic acid family due to migration of substituents, introduction of substituents, cleavage of substituents, or other (enzymatic) modifications can easily be monitored.

First detailed reports on the separation of non-derivatized sialic acids deal with the application of Aminex A-28 or A-29 anion-exchange chromatography using 0.75 mM [269, 270] or 0.5 mM [268] Na_2SO_4 as eluting system and UV monitoring at 200 nm (nanomole range). In a different approach, fluorigenic derivatives of sialic acids, prepared by reaction

with 1,2-diamino-4,5-methylenedioxybenzene (DMB) in the presence of 2-mercaptoethanol and sodium hydrogensulfite, have been separated by C_{18} reversed-phase HPLC [271–273], using acetonitrile/methanol/water (9:7:84, v/v/v) as solvent system and fluorescence monitoring at 373 nm excitation and 448 nm emission wavelengths. An appropriate cutoff filter may be used instead. The fluorescence labelling makes a relatively specific and highly sensitive (femto- to picomole range) detection possible. However, using radiolabelled sialic acids, it was found that the derivatization reaction is not quantitative [268]. For an adapted protocol, see ref. [11]. Another interesting approach is the conversion of Neu5Ac/5Gc into chemiluminescent quinoxalinone derivatives using 4,5-diaminophthalhydrazide dihydrochloride (α-keto acid derivatization) [274]. These derivatives are analyzed by reversed-phase HPLC (femtomole range), whereby the chemiluminescence detection follows the reaction of the derivatives with hydrogen peroxide in the presence of potassium hexacyanoferrate(III) in alkaline solution. Other conversions of Neu5Ac, useful for HPLC separations, include the derivatization with 4,4′-dicarboxy-2,2′-biquinoline [275], 2-cyanoacetamide [276], periodate-thiobarbituric acid [277], benzoic anhydride [278], 4′-hydrazino-2-stilbazole [279] and 1,2-diamino-4,5-dimethoxybenzene [280].

Taking advantage of the separation capacity of the anion-exchange resin CarboPac PA-1, fractionation of non-derivatized sialic acids at neutral pH, using sodium acetate as eluent and pulsed amperometric detection (PAD) following postcolumn addition of alkali, has shown excellent results in terms of the wide array of sialic acids that can be separated, the sensitivity of the detection method (picomole range), and the relative ease of use for preparative work (without PAD detection) [268]. The general problem of quantification in PAD analyses, due to differences in detector response attributed to differences in the number of free hydroxyl groups of the various components separated, holds also for sialic acids. So far, only for a limited number of sialic acids relative detector response factors have been calculated (e.g. Neu5Ac, 30 500; $Neu5,9Ac_2$, 14 500; Neu5Gc, 35 400). It is important to note that pH values >11, as usually applied in CarboPac-PAD analyses of oligosaccharides (for such a Neu5Ac/Neu5Gc separation, see ref. [281]), will lead to rapid de-*O*-acylation of the *O*-acylated sialic acids [282]. This phenomenon can even occur between the point of postcolumn alkali addition and the entry into the PAD detector [268].

In an evaluation of five different HPLC methods, it turned out that no single method is adequate to completely separate and quantitate complex mixtures of sialic acids [268], and the use of multi-dimensional HPLC is advised. As a clear illustration of this statement, Table 8 is included. This evaluation also compares a series of essential features of the five HPLC methods, namely, sensitivity, specificity of detection, separation by number of hydroxyl groups or substituents, separation of isomers, preparative use, avoiding of ester migration during purification, and avoiding of ester loss during purification. In this comparison [268,270,271,283,284] the HPLC systems I (CarboPac PA-1) and III (TSK-ODS 120T, DMB derivatives) gave the highest averaged scores in terms of applicability. A major advantage of HPLC system V (Aminex A-29) is the short running time, only 5–6 min, which makes this approach highly attractive for studying enzymatic conversions.

In order to obtain information about the structure of sialic acids, HPLC is a very useful

Table 8
High-performance liquid chromatographic elution times of sialic acids in five different HPLC systems I to V, relative to Neu5Ac [268]

Compound	I[a]	II[b]	III[c]	IV[d]	V[e]
Neu2Me	0.17	0.13		0.23	
Neu1,2Me$_2$	0.40	0.85		0.46	
Neu5Ac	1.00	1.00	1.00/1.00[f]	1.00	1.00
Neu4,5Ac$_2$	0.76	0.39	1.68/1.71[f]	1.22	1.41
Neu5,7Ac$_2$	0.74	0.36	1.06	0.95	0.87
Neu5,9Ac$_2$	0.95	0.35	1.57/1.62[f]	1.30	1.47
Neu5,7(8),9Ac$_3$	0.74	0.23	1.90	1.17	1.76
Neu5,7,8,9Ac$_4$	0.62		1.98	1.62	2.05
Neu5Ac2Me	0.73	1.02		0.34	0.73
Neu4,8an5Ac	2.15	0.58	1.39	2.04	
Neu2en5Ac	2.21	0.66		1.54	1.68
Neu5Gc	1.17	1.50	0.84/0.78[f]	1.20	1.33
Neu4Ac5Gc	0.86	0.54	1.53	1.59	1.69
Neu9Ac5Gc	1.06	0.49	1.30[f]	1.57	1.70
Neu5Gc8Me	0.82	0.75	1.03/0.98[f]	0.94	0.89
Neu9Ac5Gc8Me	0.67	0.36	1.70	1.21	
Neu7,9Ac$_2$5Gc8Me	0.59	0.29	2.20	1.47	
Kdn			0.73[f]		
Acetic acid		0.08			1.70

[a] System I: Anion-exchange chromatography on CarboPac PA-1 with PAD detection at room temperature; 5 mM sodium acetate for 5 min, then a linear gradient to 50% 5 mM sodium acetate/50% 5 mM acetic acid in 30 min; mixing of the column effluent with 300 mM NaOH; running time 18–24 min [268].
[b] System II: Amine adsorption/ion suppression chromatography on Micropak AX-5 with UV monitoring (200 nm) at room temperature; acetonitrile/water/0.25 M sodium dihydrogenphosphate (72:18:6, v/v/v); running time 21–24 min [283].
[c] System III: Reversed-phase chromatography on TSK-ODS 120T and fluorescence monitoring (373 nm excitation and 448 nm emission) at room temperature; DMB derivatives; acetonitrile/methanol/water (9:7:84, v/v/v); running time 12–13 min [271].
[d] System IV: Anion-exchange chromatography on Aminex HPX-72S with UV monitoring (200 nm) at 40°C; 0.1 M sodium sulfate; running time 15–17 min [284].
[e] System V: Anion-exchange chromatography on Aminex A-29 with UV monitoring (200 nm) at room temperature; 0.75 mM sodium sulfate; running time 5–6 min [36,270].
[f] Taken from [11], RP-18 column.

technique to be applied in combination with mild chemical or enzymatic degradation methods. For instance, HPLC before and after alkaline treatment of a mixture of *O*-acylated sialic acids can give information about the de-*O*-acylated sialic acids present, e.g. in terms of their *N*-acyl substituents. Also the linkage specificity of sialidases in releasing sialic acids from sialoglycoconjugates (see section 5.1) can be monitored by HPLC. Furthermore, the specificity of enzymes involved in sialic acid metabolism can be studied in this way. In this respect, interesting results have been obtained

with aldolase, cleaving sialic acids to *N*-acylmannosamine derivatives and pyruvate, and with sialate 9-*O*-acetylesterase, hydrolyzing *O*-acetyl groups from C9 of sialic acids. The aldolase degrades Neu5Ac faster than Neu5Gc; a slow degradation has been observed for *O*-acylated sialic acids, not affecting 4-*O*-acetylated sialic acids at all [5]. A typical example of the HPLC analysis of enzyme reactions, in which esterase and aldolase are involved, and including the non-enzymatic conversion of an *O*-acetyl group from C7 to C9, is presented in Fig. 5 [6,7,36]. Other examples are the determination of sialidase activity (sialyllactose as substrate, Neu5Ac as product, and Neu2en5Ac as inhibitor), CMP-Neu5Ac synthase activity (disappearance of Neu5Ac, appearance of CMP-Neu5Ac), CMP-Neu5Ac phosphodiesterase activity (appearance of Neu5Ac, disappearance of CMP-Neu5Ac) [36]. Recently, 5-*N*-acetyl-9-*O*-acetyl-2-(*N*-dansyl-4-aminophenylthio)-α-neuraminic acid has been proposed as a highly sensitive fluorescent substrate for the HPLC measurement of sialate 9-*O*-acetylesterase (334 nm excitation, 564 nm emission) [285]. As a thioglycoside, the compound is very stable in acidic aqueous solution and towards enzymatic hydrolysis by sialidases. In this context, it is also worthwhile mentioning that a sensitive HPLC assay has been developed for the tracing of sialyltransferase activity, making use of the synthetic fluorigenic acceptor lactose 2-[(2-pyridyl)amino]ethyl glycoside [286]. Details for a HPLC separation of CMP-Neu5Ac, CMP-Neu5Gc and CMP-Kdn on a DC-613 cation-exchange column are reported in ref. [107].

In addition to the fractionation procedures described for free sialic acids, several approaches have been reported for the separation of sialyl-oligomers. These compounds with a degree of polymerization up to 16 have been fractionated with varying results using conventional gel-filtration, TLC, DEAE-Sephadex A-25, and HPLC methods (see section 5.1 for preparation; see section 5.3.1 for TLC). A survey of literature has been included in ref. [287]. In general, mixtures of sialo-oligomers from $\{Neu5Ac(\alpha2\text{–}8)\}_n$, $\{Neu5Ac(\alpha2\text{–}9)\}_n$, $\{Neu5Gc(\alpha2\text{–}8)\}_n$, or $\{Kdn(\alpha2\text{–}8)\}_n$ can be isolated on a preparative scale via convential DEAE-Sephadex A-25 [256,257] or DEAE-Toyopearl 650M [267] anion-exchange chromatography. HPLC procedures comprise anion-exchange and adsorption-partition chromatography. A mixture of $\{Neu5Ac(\alpha2\text{–}8)\}_{2\text{–}16}$ has been efficiently separated on a Zorbax SAX anion-exchange column using 0.2–1 M NaCl in 10 mM phosphate buffer pH 3.5 [287]. Also adsorption-partition chromatography on polystyrene DC-613 using mixtures of 0.02–0.025 M sodium phosphate buffer pH 7.4 and acetonitrile as solvent system, has shown good results [267]. On Mono Q anion-exchange columns, excellent results were obtained in the separation of $\{Neu5Ac(\alpha2\text{–}8)\}_{2\text{–}14}$, $\{Neu5Gc(\alpha2\text{–}8)\}_{2\text{–}10}$, or $\{Kdn(\alpha2\text{–}8)\}_{2\text{–}7}$ after conversion into alditols with $NaBH_4$ (or $NaBT_4$), and using a NaCl gradient in Tris-HCl buffer pH 8 as elution system [267,288]. In this context, several studies have focused on the determination of the chain lengths of sialo-oligomers and -polymers (for a review of methods currently employed in the analysis of polysialic acids, see ref. [289]), and recently a highly detailed adapted methodology for the analysis of α-2,8-linked sialo-oligomers and -polymers has appeared [290]. Using three variable assay procedures, providing overlapping information, details could be provided with respect to the degree of polymerization, the simultaneous identification of Neu5Ac, Neu5Gc and Kdn when present in a single preparation, and the ability to distinguish qualitatively between

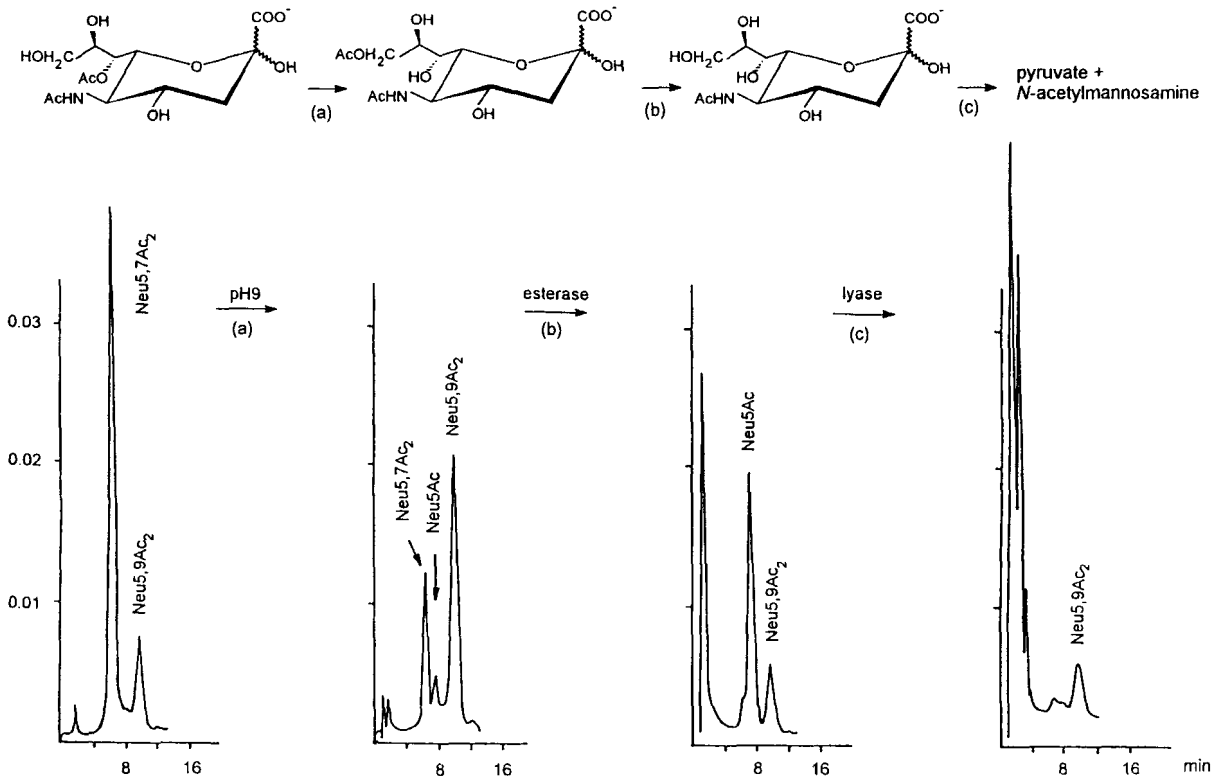

Fig. 5. HPLC profiling (Aminex A-28, 0.75 mM Na_2SO_4) of the chemo-enzymatic conversion of Neu5,7Ac_2 into pyruvate and *N*-acetyl-D-mannosamine (ManNAc). (a) Intramolecular migration of the *O*-acetyl group from C7 to C9 under slightly alkaline conditions, yielding Neu5,9Ac_2, accompanied by some de-*O*-acetylation; (b) enzymic release of the *O*-acetyl group at C9 with the aid of sialate-9-*O*-acetyl-esterase, yielding Neu5Ac; (c) aldolase (lyase)-catalyzed degradation of Neu5Ac, yielding pyruvate and ManNAc.

reducing and non-reducing polymers. The developed approach may include mild periodate oxidation (degradation of non-reducing terminal unit) in combination with reduction (degraded glycerol side chain yielding C7-sialic acid; reducing unit if present affording the corresponding stereoisomeric alditols), whereas monomer analysis is carried out after sialidase or acid hydrolysis on CarboPac PA-1 with pulsed amperometric detection.

In the structural analysis of glycoprotein-derived N- and O-linked sialic-acid-containing carbohydrate chains, fractionation procedures based on HPLC play a major role. As this aspect is outside the scope of this chapter, no details are included. Typical examples, making use of anion-exchange chromatography (Mono Q, CarboPac), and normal (e.g. Lichrosorb-NH_2) or reversed-phase chromatography, are found in refs. [25,81,84,133,291–300]. In this context, it is also worth noting the recent use of high-performance capillary electrophoresis for the separation of glycoprotein-derived N-glycan chains [301] and O-glycan chains [302].

5.3.3. Gas–liquid chromatography combined with mass spectrometry

As discussed in section 5.1, methanolysis of free and glycosidically bound sialic acids gives rise to the formation of methyl ester β- and α-methyl glycosides. Using the conditions of the standard quantitative monosaccharide analysis of glycoconjugates, de-*N*-acylation/de-esterification takes place, which means that *N,O*-acylneuraminic acid residues are converted into neuraminic acid methyl ester methyl glycoside (β, ~96%; α, ~4%). For the characterization by GLC the sialic acid methyl ester methyl glycoside is derivatized via *N*-acetylation/trimethylsilylation or pertrifluoroacetylation [241]. It should be noted that during the *N*-acetylation step HO9 of Neu5Ac, when not substituted, is partially *O*-acetylated (~4%). Using milder methanolysis conditions [242], an *N*-acetylation step is not necessary, yielding a method to determine both Neu5Ac and Neu5Gc by GLC. In principle, the latter approach is also suitable to determine sialic acids bearing only *O*-alkyl groups. GLC analysis is generally carried out on SE-30 type column materials.

Starting from free sialic acids (purified or as a pool), mainly present in their β-anomeric forms, volatile sialic acid derivatives are generated using mild derivatization procedures such as esterification with diazomethane followed by trimethylsilylation [15,303] or pertrimethylsilylation [11,304]. With respect to silylation cocktails, it should be noted that *N*-methyl-*N*-trimethylsilyl-2,2,2-trifluoroacetamide/pyridine leads to the formation of *N*-trimethylsilyl derivatives, yielding two different peaks for each sialic acid [36,305]. Subsequent GLC analysis is generally carried out on SE-30 or OV-17 type column materials. Both types of derivatives are highly suitable for MS analysis, and GLC coupled with electron impact (EI) MS formed the basis for the development of a highly reliable mass spectrometric method for the identification of sialic acids. Originally set up for the GLC-EI MS analysis of mixtures of *N,O*-acylneuraminic acids [303], the method has also proved to be useful for the analysis of other naturally occurring sialic acids, of (partially) *O*-methylated sialic acid methyl ester methyl glycosides as obtained in methylation analyses, and of synthetic sialic acid(s) (derivatives) [6,11,15].

In the following the principles of the EI MS identification procedure will be explained. Typical derivatives are trimethylsilylated methyl ester or pertrimethylsilylated derivatives of *N,O*-acylneuraminic acids or of *N*-acyl-*O*-alkylneuraminic acids, acetylated *N*-acyl-

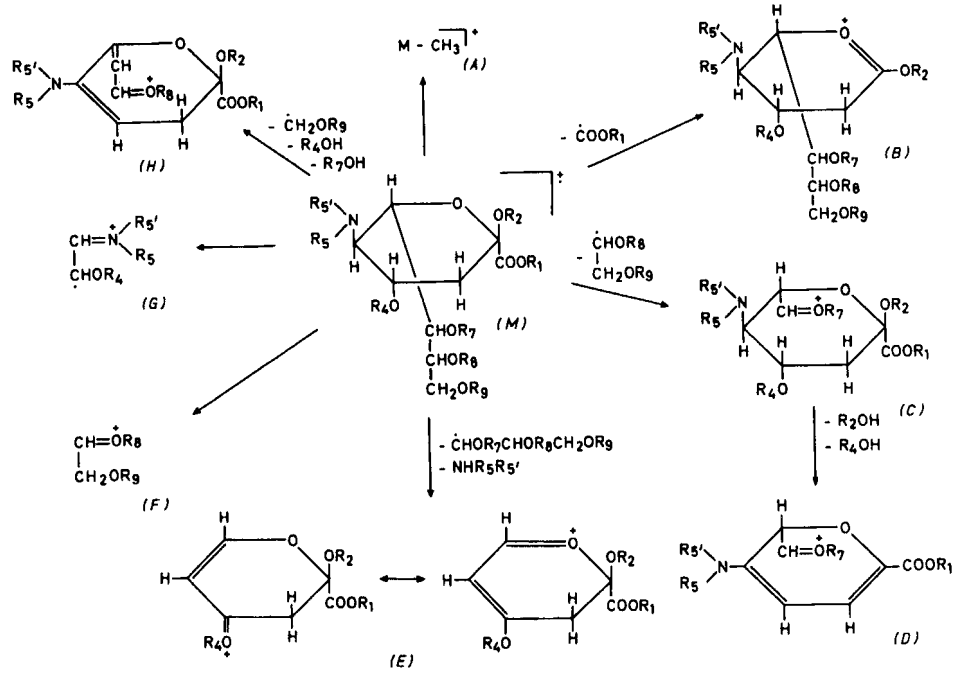

Fig. 6. Survey of the selected fragment ions A–H worked out for the following derivatives: trimethylsilylated methyl ester or pertrimethylsilylated derivatives of *N,O*-acylneuraminic acids or of *N*-acyl-*O*-alkylneuraminic acids, and trimethylsilylated/methylated *N,N*-acyl,methyl-neuraminic acid methyl ester methyl glycosides [15].

O-alkylneuraminic acid methyl ester methyl glycosides, trimethylsilylated/methylated *N,N*-acyl,methyl-neuraminic acid methyl ester methyl glycosides (methylation analysis), and acetylated/methylated *N,N*-acyl,methyl-neuraminic acid methyl ester methyl glycosides (methylation analysis). The determination of the type, number, and position of the *O*-acyl or *O*-alkyl groups as well as the type of the *N*-acyl group in neuraminic acids is facilitated by the highly specific EI mass spectra of the derivatized compounds. In Fig. 6, a schematic survey is depicted showing the selected fragment ions A–H, which furnish the information (abundances and *m/z* values of the ions) necessary to deduce the complete structure of the sialic acids. Fragments A and B indicate the molecular mass of the sialic acid derivatives and thereby the type and the number of substituents. Fragments C–H can be used for the determination of the positions of the different substituents. Fragment A is formed from the molecular ion by the elimination of a methyl group originating from a trimethylsilyl substituent in trimethylsilylated (*O*-acylated/*O*-alkylated) *N*-acylneuraminic acid derivatives. When $R_{5'} = CH_3$ (methylation analysis), the eliminated methyl group can

also originate from the *N,N*-acyl,methyl group. Fragment B is formed by elimination of the C1 part of the molecule. Eliminations of $OCOCH_3$ in *O*-acylated sialic acid derivatives and of NH_2COCH_3 in *N*-acetylneuraminic acid derivatives, which in principle give rise to the same *m/z* value as fragment B in the case of $R_1 = CH_3$, can be neglected. For *O*-trimethylsilylated *N,O*-acylneuraminic acids (β-anomers) it holds that, when compared to their methyl esters, in their trimethylsilyl esters the intensity of fragment A decreases relative to B. Fragment C is formed by elimination of C8–C9, with localization of the charge on position 7. In general, cleavage occurs between two alkoxylated carbon atoms, or between an acetoxylated and an alkoxylated carbon atom, rather than between two acetoxylated carbon atoms. In accordance with the fragmentation rules for partially methylated alditol acetates [306], the charge is preferentially located on an ether oxygen instead of on an ester oxygen. Therefore, fragment C has only significant abundance if C7 bears an ether group. When an ester group is present at C7, this fragment ion is absent or hardly observable. Fragment D is formed from fragment C by consecutive eliminations of R_2OH and R_4OH. It is evident that the occurrence of this fragment ion is dependent on the presence of fragment C. Fragment E is formed by elimination of the whole side-chain C7–C8–C9 and the substituent at C5. This fragment ion is not observed if an *O*-acyl group is attached to C4, illustrating that the transition state in the McLafferty rearrangement is more favored when the substituent at C4 is an ether group rather than an ester group. For *O*-trimethylsilylated *N,O*-acylneuraminic acids (β-anomers) it holds that, when compared to their methyl esters, in their trimethylsilyl esters the intensity of fragment E is much reduced but still present; instead, an additional fragment derived from fragment E by loss of Me_3SiOH is clearly present. Fragment F contains C8–C9. Based on the same fragmentation rules as mentioned above for fragments C and D, this ion can only readily be formed if an ether group is attached to C8. Fragment G consists of the C4–C5 part of the molecule. Fragment H, necessary to use for derivatives containing only *O*-alkyl substituents, is formed by elimination of the C9 part, followed by elimination of R_4OH and R_7OH. For instance, this fragment is useful to discriminate between an $OSiMe_3$ group at C8 or C9 in trimethylsilylated partially methylated *N*-acylneuraminic acids. Finally, for quadrupole analyzers, in the high mass range the fragment ions A, B and C often are of low intensity, especially when only small amounts of material are available.

In Table 9 a survey is presented of GLC retention times and of characteristic EI MS fragment ions for a series of naturally occurring sialic acids, analyzed as their trimethylsilylated methyl ester or as their pertrimethylsilylated derivatives [6,11,15,31,38,43,307]. Although the sialic acids predominantly occur in the β-anomeric form, the α-anomer could occasionally be detected separately from the β-anomer, in most cases as a small shoulder. As a typical example, in Figs. 7a,b the EI mass spectra of the trimethylsilylated methyl ester of β-Neu5Ac and of the pertrimethylsilylated derivative of β-Neu5Ac, respectively, are depicted. Additional spectra have been published in refs. [15,43,304,307]. For a detailed survey of fragment ions of other derivatives mentioned, including mass spectra, and those obtained from periodate-oxidized sialic acids (C7-Neu5Ac, C8-Neu5Ac, C7-Neu5Gc and C8-Neu5Ac), see refs. [15,308]; for EI MS data of Neu4,8an5Ac, see ref. [63]; for EI MS data of permethylated Kdn, see ref. [46].

In additional studies, the suitability of chemical ionization (CI) for the GLC-MS analysis of pertrimethylsilylated *N,O*-acylneuraminic acids has been investigated. Isobu-

Table 9

GLC and characteristic EI MS fragment ions (70 eV) of (i) trimethylsilylated methyl ester (TM) and (ii) pertrimethylsilylated (PT) derivatives of naturally occurring sialic acids (β-anomers)[a]

Sialic acid	R_{Neu5Ac}		Fragment (*m/z*)														Reference(s)
	TM	PT	A		B		C		D		E		F		G		
			TM	PT	TM	PT	TM	PT	TM	PT	TM	PT	TM	PT	TM	PT	
Neu5Ac	1.00	1.00	668	726	624	624	478	536	298	356	317	375	205	205	173	173	[11,15]
Neu4,5Ac_2	1.18	1.05	638	696	594	594	448	506	298	356	–	–	205	205	143	143	[11,15]
Neu5,7Ac_2	1.04		638		594		–		–		317		205		173		[15]
Neu5,8Ac_2	1.05		638		594		478		298		317		–		173		[15]
Neu5,9Ac_2	1.13	1.02	638	696	594	594	478	536	298	356	317	375	175	175	173	173	[11,15]
Neu4,5,9Ac_3	1.31		608		564		448		298		–		175		143		[15]
Neu5,7,9Ac_3	1.14		608	666	564	564	–	–	–	–	317	375	175	175	173	173	[6,15]
Neu5,8,9Ac_3	1.19	1.04	608	666	564	564	478	536	298	356	317	375	–	–	173	173	[11,15]
Neu5,7,8,9Ac_4	1.15		578		534		–		–		317		–		173		[11,15]
Neu5Ac9Lt	2.55		740	798	696	696	478	536	298	356	317	375	277	277	173	173	[15,307]
Neu4,5$Ac_2$9Lt	3.01		710		666		448		298		–		277		143		[15]
Neu5Ac8Me		0.98		668		566		536		356		375		147		173	[11,31]
Neu5,9$Ac_2$8Me		1.00		638		536		536		356		375		117		173	[11,31]
Neu2en5Ac	1.09	1.01	578	636	–	–	388	446	298	356	227	285	205	205	–	–	[11,15]
Neu2,7an5Ac				564		462		374		–		–		205		173	[6,38]
Neu5Gc	1.81	1.19	756	814	712	712	566	624	386	444	317	375	205	205	261	261	[11,15]
Neu4Ac5Gc	2.02		726	784	682	682	536	594	386	444	–	–	205	205	231	231	[6,15]
Neu7Ac5Gc	1.83		726		682		–		–		317		205		261		[15]
Neu9Ac5Gc	2.04	1.21	726	784	682	682	566	624	386	444	317	375	175	175	261	261	[11,15]
Neu7,9$Ac_2$5Gc	2.01		696		652		–		–		317		175		261		[15]
Neu8,9$Ac_2$5Gc	1.99		696		652		566		386		317		–		261		[15]

continued on next page

Table 9, *continued*

Sialic acid	R_{Neu5Ac}		Fragment (*m/z*)														Reference(s)
	TM	PT	A		B		C		D		E		F		G		
			TM	PT	TM	PT	TM	PT	TM	PT	TM	PT	TM	PT	TM	PT	
Neu7,8,9Ac$_3$5Gc	1.93		666		622		–		–		317		–		261		[15]
Neu5Gc8Me		1.14		756		654		624		444		375		147		261	[11,31]
Neu9Ac5Gc8Me		1.17		726		624		624		444		375		117		261	[11,31]
Neu5GcAc		1.21		784		682		594		414		375		205		231	[43]
Neu2en5Gc				724		–		534		444		285		205		–	[6]

[a] The R_{Neu5Ac} values of the TM derivatives on 3.8% SE-30 at 215°C are given relative to the TM derivative of β-Neu5Ac.
The R_{Neu5Ac} values of the PT derivatives on CP-Sil 5 (capillary column), using the program 5 min/140°C; 2°C/min up to 220°C; 15 min/220°C, are given relative to the PT derivative of β-Neu5Ac.
For the preparation of TM derivatives, see [15]; for the preparation of PT derivatives, see [304]. For an explanation of the minus signs, see text.

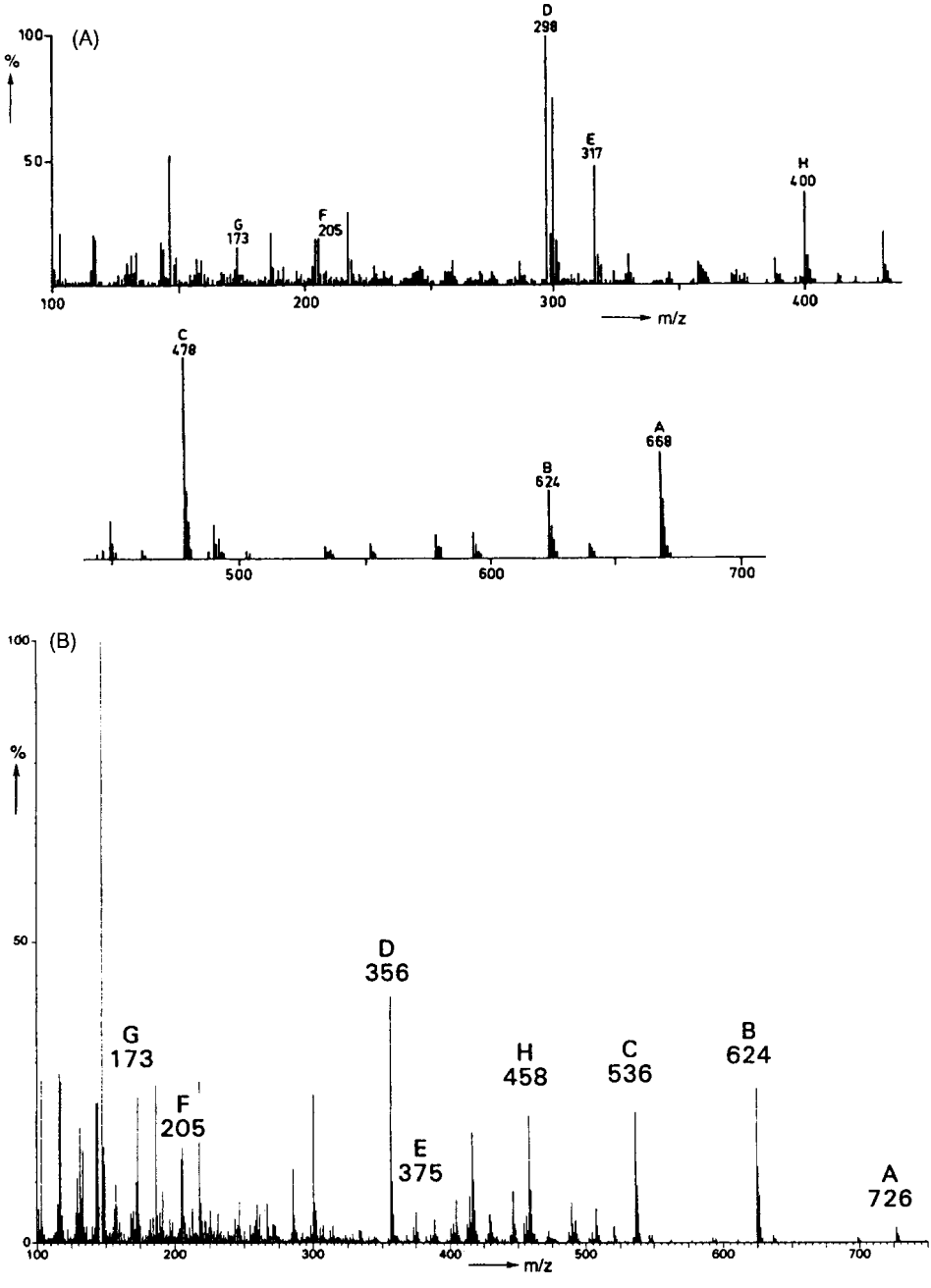

Fig. 7. (A) EI mass spectrum (70 eV) of the trimethylsilylated methyl ester of β-Neu5Ac; (B) EI mass spectrum (70 eV) of the pertrimethylsilylated derivative of β-Neu5Ac.

tane [304], as well as methane and ammonia [11] were used as reactant gases. The CI mass spectra are characterized in the high mass range by $[M+H]^+$ pseudomolecular ions, and typical major fragment ions derived from $[M+H]^+$ by loss of R_2OH (fragment I), R_4OH (fragment I'), and R_2OH+R_4OH (fragment II). It was found that methane in particular gave CI spectra that also include several of the typical fragment ions observed in the EI spectra.

In addition to GLC-MS, HPLC-CI MS with Aminex A-29 as column material and ammonium formate in water/acetonitrile as solvent system has been explored for the analysis of underivatized *N,O*-acylneuraminic acids [6,309]. Although the positive-ion mass spectra allow the discrimination between different *N*-acylneuraminic acids (Neu5Ac, Neu5Gc) and the determination of the degree of *O*-acetylation (Neu5,9Ac_2, Neu5,7,9Ac_3, Neu5,7,8,9Ac_4), the position of the *O*-acetyl groups (Neu4,5Ac_2, Neu5,7Ac_2, Neu5,9Ac_2) could not be established. For the latter assignment, the combination with specific elution positions of standards on the HPLC column is advised. In the interpretation of the various fragment ions, the open-chain structure of the sialic acids has been generally used.

Neu5Ac and Neu5Gc have also been converted into phosphatidylethanolamine dipalmitoyl derivatives, and after separation by HPTLC and subsequent isolation, the sialic acid derivatives were analyzed by liquid secondary ion MS [281]. In both cases intense $[M-H]^-$ ions together with sodium attachment ions were detected. For the detection of Neu5Ac on human tumor mucin, after liberation with sialidase, electrospray MS has been used [310].

5.3.4. Fast atom bombardment mass spectrometry

Free sialic acids, isolated after cleavage from glycoconjugate starting material, have been investigated, without derivatization, by FAB MS using 5% aqueous acetic acid solutions for loading into glycerol on the FAB target [41]. The positive and negative FAB mass spectra of each sialic acid showed clear $[M+H]^+$ and $[M-H]^-$ pseudomolecular ions, respectively. Sialic acid mixture analysis (μg range) made the recognition of subgroups of sialic acids with the same molecular mass possible (e.g. Neu5Ac$(Ac)_1$, Neu5Ac$(Ac)_2$, Neu5Ac$(Ac)_3$, Neu5Gc$(Ac)_3$). However, a differentiation between positional isomers was not possible.

Sialic acids were also studied after derivatization, which improves the sensitivity [41]. Direct peracylation failed to produce suitable derivatives, but reduction under acidic conditions followed by peracylation (perdeuteroacetylation or perpropionylation) gave good results. Generally, the sialic acids give rise to two major pseudomolecular ions, corresponding to the peracylated open chain form and an open-chain-derived lactone form, and a minor pseudomolecular ion corresponding to an open-chain-derived anhydroform (2,6 and/or 4,8). As the lactone peak is markedly reduced in the spectrum of Neu4,5Ac_2, HO4 seems to be mainly involved in the lactonization. In the case of sialic acid mixtures, a fast sialic acid subgroup analysis based on molecular masses is possible; again, a differentiation between positional isomers cannot be achieved. Careful analysis of the negative FAB spectra of reduced and perpropionylated sialic acids in mixtures demonstrated that these spectra could also be used for quantitative purposes. As worked out for mixtures of Neu5Ac, Neu5,9Ac_2 and Neu5Gc, an estimate of the relative amounts of these sialic acids can be given with an error of 10–15%, when the sum of the intensities

of the $[M+H]^+$ ions of the linear and the lactone forms of each component is compared, taking into account that the molar response of Neu5Gc is approximately 50% of that of Neu5Ac.

In order to generate sialic-acid-derived compounds, which can be used to differentiate between positional isomers, use has been made of the rather difficult periodate oxidation under mild or more rigorous conditions [53,308]. Under both conditions, the resulting aldehyde groups were derivatized with *p*-amino-benzoic acid ethyl ester, reductively introduced at acidic pH without loss of the native *O*-acetyl functions [41]. Sialic acids treated in this way were additionally reduced and peracylated, and then analyzed by FAB MS. Mixtures of products with different ring sizes (original, lactonized, anhydro) and/or open chain forms, depending on the substitution pattern, are often obtained. Of the mono-*O*-acetylated *N*-acylneuraminic acids Neu4,5Ac_2, Neu5,7Ac_2, Neu5,9Ac_2, Neu4Ac5Gc and Neu9Ac5Gc were investigated, however, no attention was paid to the behavior of Neu5,8Ac_2. Neu5,7(8),9Ac_3 and Neu7,9$Ac_2$5Gc were also included in these investigations.

Although not discussed in this chapter, FAB MS is widely used in the characterization of glycoprotein-derived N- and O-linked sialic-acid-containing carbohydrate chains. Typical information can be found in refs. [311,312].

5.3.5. ^1H NMR spectroscopy

Since the introduction of high-resolution ^1H NMR spectroscopy for the structural analysis of glycoprotein-derived glycans, a huge amount of NMR data have been generated, and highly detailed reviews on N-linked [76] and O-linked [77] carbohydrate chains have appeared. The continuous expansion in the amount of data has made it necessary to develop computerized search programs, and, in connection with the still growing Complex Carbohydrate Structural Database (CARBBANK), attention has been paid to the development of a NMR-spectroscopic data base of carbohydrate structures, called SUGABASE [313]. Sialic-acid-containing oligosaccharides/glycopeptides constitute a considerable majority of the glycoprotein glycans. In addition to the two reviews mentioned above, a specific review focusing on the NMR spectroscopy of sialic acids has also been published [16].

Free as well as glycosidically bound sialic acid give rise to highly characteristic ^1H NMR parameters. The ^1H NMR spectra are generally recorded in D_2O, and because of the pH dependency of the proton chemical shifts, the spectral data are standardized at pD 6–7. The choice of the pH is also of importance in view of the earlier discussed de-*O*-acylation, *O*-acyl migration, and autohydrolysis.

As a typical example of a free sialic acid, in Fig. 8 the 500 MHz ^1H NMR spectrum of Neu5Ac in D_2O at pD 7 is depicted. The spectrum shows a minor and a major set of protons, reflecting the subspectra of the α- and β-anomer of Neu5Ac, respectively (α:β = 7:93), and especially the H3e,3a signals, resonating outside the bulk signal, can be used for the differentiation between both anomers. The effect of pH on the proton chemical shifts is clearly illustrated by the positions of the Neu5Ac H3e,3a resonances. At pD 1.4, the H3e and H3a signals of β-Neu5Ac resonate at δ 2.313 and δ 1.880, respectively, whereas these values are δ 2.208 and δ 1.827 at pD 7. In the case of α-Neu5Ac, at pD 1.4, the resonances are found at δ 2.718 and δ 1.705,

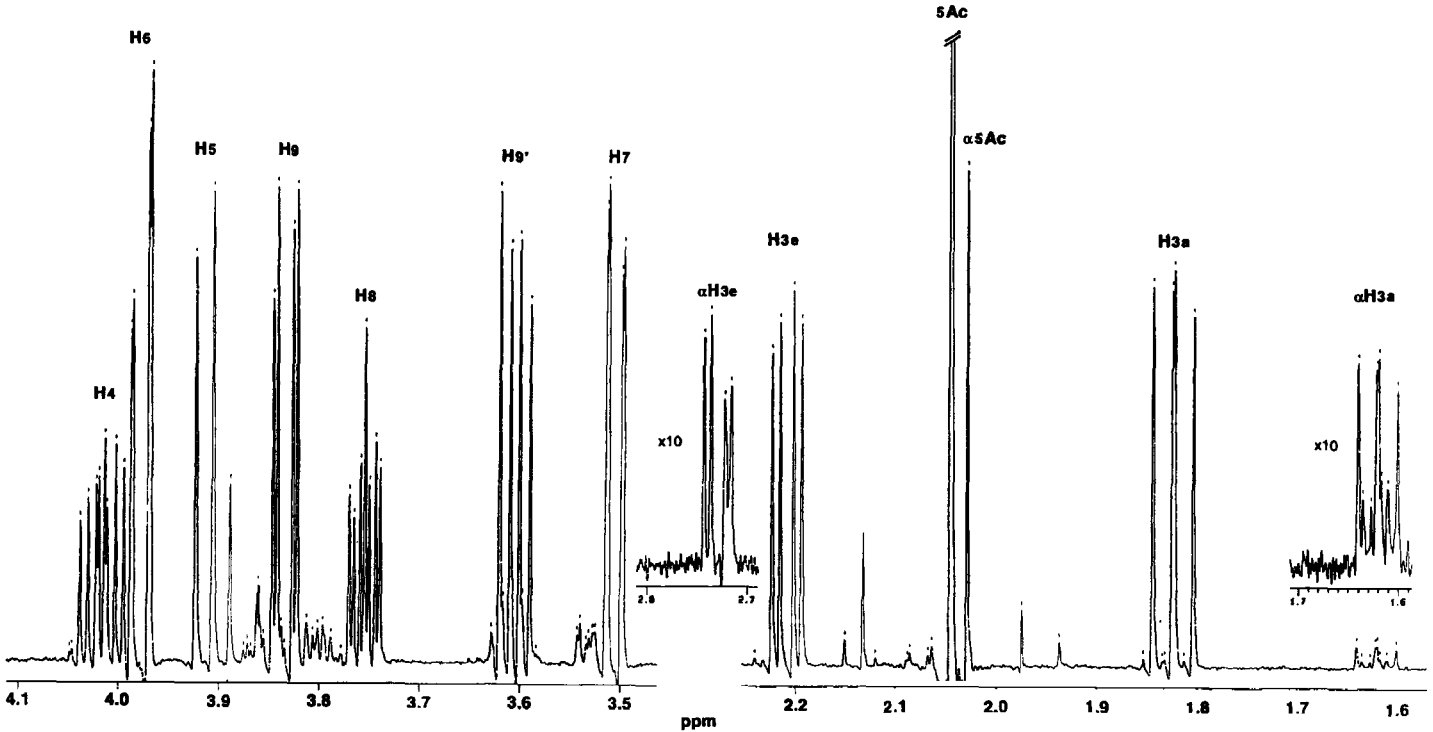

Fig. 8. Resolution-enhanced 500 MHz ^{1}H NMR spectrum of Neu5Ac dissolved in D_2O recorded at pD 7 and 27°C.

respectively, and at pD 7.0 at δ 2.730 and δ 1.621, respectively. Over the years, a large number of (naturally occurring) sialic acids and related derivatives have been analyzed, and in Table 10 a survey of the chemical shift values of a selected group of compounds, including naturally occurring sialic acids and CMP-sialic acids, is presented [16,31,38,51,64,107,308,314,315]. Inspection of these data reveals the typical influences of the substituents on the chemical shift values of the skeleton H-atoms. Comparison of the H3a,3e resonances of Neu5Ac and Neu5Gc indicate more downfield positions for those of Neu5Gc ($\Delta\delta \approx +0.02$). Additional data for Neu5Ac, Neu5,7Ac_2, Neu5,9Ac_2, Neu5,7,9Ac_3, and Neu5,8,9Ac_3 in 0.1 M sodium phosphate/D_2O at 37°C and pD 7–7.5 are reported in ref. [23]. The ^{1}H NMR studies of free *N,O*-acylneuraminic acids have shown that the anomeric equilibrium of a 7-*O*-acetylated sialic acid differs strongly from that of the sialic acids not substituted at C7. In contrast to the normal equilibrium values of $\alpha:\beta \approx 7:93$, both Neu5,7$Ac_2$ and Neu5,7,9Ac_3 have an equilibrium ratio of about 23:77. Additional ^{1}H NMR data of methyl glycosides and methyl ester methyl glycosides of *N,O*-acylneuraminic acids and some sialyllactoses can be found in ref. [16]. The ^{1}H NMR data of the (methyl ester) methyl glycoside of β-Kdn have been reported in ref. [46], and those of CMP-9amino-Neu5Ac and CMP-9NAc-Neu5Ac in ref. [316].

^{1}H NMR spectroscopy has shown to be an excellent method to monitor chemical and biochemical conversions of sialic acids, directly in the NMR-tube or by analysis of isolated reaction products. A typical example is the demonstration of the release of α-Neu5Ac as the primary product of bacterial and viral sialidase action on Neu5Ac(α2-glycosides and oligosaccharides [317–320]. The initial formation of α-Neu5Ac, as traced by ^{1}H NMR spectroscopy, formed an excellent probe to investigate the kinetics of the mutarotation of Neu5Ac by means of ^{1}H NMR analysis in dependency of the pH [321]. At pD 5.4 the establishment of the equilibrium of mutarotation turned out to be rather slow, but at higher and lower pD values a more rapid establishment was observed, so that at pD 1.3 and pD 11.7 mutarotation was too fast to be measured. With the ability to generate α-Neu5Ac *in situ,* the aldolase-catalyzed degradation of Neu5Ac to pyruvate and *N*-acetylmannosamine (ManNAc) could also be investigated in more detail using ^{1}H NMR spectroscopy [322]. Using sialidase (pH optimum 5.4) and aldolase (pH optimum 7.2) from *C. perfringens* and *N*-acetyl-2-azido-2-deoxy-α-neuraminic acid as substrate at pH 5.4, only released α-Neu5Ac was found to be consumed by the aldolase, yielding specifically α-ManNAc followed by a fast mutarotation to α,β-ManNAc. These findings confirmed earlier work using Neu5Ac(α2–3)lactose as α-Neu5Ac-generating system and crystalline β-Neu5Ac [319]. In the reversed reaction α-ManNAc is the substrate [322]. For more details about the aldolase-catalyzed degradation, see section 9.3. In connection with these studies, it has to be noted that under comparable conditions the activities of sialidase and aldolase in D_2O are only about 50% of those in H_2O. With respect to *O*-acetyl migrations, also the earlier mentioned (see section 2) spontaneous conversion at physiological pH of Neu5,7Ac_2 into Neu5,9Ac_2 and of Neu5,7,9Ac_3 into Neu5,8,9Ac_3 has been monitored by ^{1}H NMR spectroscopy [23]. Furthermore, the NMR approach has shown its value in the determination of substrate specificities of various sialidases using substrates with differently linked sialic acid residues [323–325].

Table 10

^{1}H Chemical shifts for different types of sialic acids. Chemical shifts are given in ppm relative to internal acetone in D_2O (δ 2.225) at 300 K, unless indicated otherwise

Sialic acid	pD	Chemical shift														Ref.
		H3a	H3e	H4	H5	H6	H7	H8	H9	H9′	4Ac	5Ac	7Ac	8Ac, 8Me	9Ac	5Gc	
β-Neu5Ac	2	1.880	2.313	4.067	3.931	4.056	3.556	3.750	3.841	3.619	–	2.053	–	–	–	–	[16]
α-Neu5Ac	2	1.705	2.718	n.d.[a]	3.85	3.684	3.53	3.75	3.85	3.62	–	2.036	–	–	–	–	[16]
β-Neu5Ac	7	1.827	2.208	4.024	3.899	3.984	3.514	3.753	3.835	3.608	–	2.050	–	–	–	–	[16]
α-Neu5Ac	7	1.621	2.730	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	2.030	–	–	–	–	[16]
β-Neu4,5Ac_2	7	1.951	2.249	5.274	4.15	4.15	3.570	3.775	3.844	3.619	2.065	1.992	–	–	–	–	[16]
β-Neu5,7Ac_2	4	1.905	2.236	3.950	3.767	4.246	5.045	3.911	3.629	3.444	–	1.976[b]	2.144[b]	–	–	–	[16]
α-Neu5,7Ac_2	4	1.649	2.757	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	1.947[b]	2.128[b]	–	–	–	[16]
β-Neu5,9Ac_2	7	1.833	2.221	4.024	3.913	3.991	3.571	3.977	4.365	4.187	–	2.057	–	–	2.119	–	[16]
α-Neu5,9Ac_2	7	1.624	2.720	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	n.d.	–	–	n.d.	–	[16]
β-Neu5,7,9Ac_3	2	1.924	2.303	3.978	3.775	4.293	5.162	4.140	4.106	4.106	–	1.981[b]	2.134[b]	–	2.106	–	[16]
α-Neu5,7,9Ac_3	2	1.686	2.751	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	1.956[b]	n.d.[b]	–	n.d.	–	[16]
β-Neu5,8,9Ac_3	7	1.838	2.189	3.978	3.903	3.780	3.838	5.114	4.528	4.287	–	2.057	–	2.089	2.105	–	[16]
β-Neu5,8,9Ac_3	2	1.862	2.250	4.006	3.912	3.830	3.866	5.115	4.545	4.287	–	2.059	–	2.091	2.107	–	[16]
Neu2en5Ac	6	–	5.690	4.470	4.051	4.213	3.601	3.936	3.885	3.646	–	2.068	–	–	–	–	[16]
Neu2,7an5Ac[c]	7	2.167	2.007	3.953	3.919	4.543	4.434	3.537	3.592	3.755	–	2.035	–	–	–	–	[38]
Neu4,8an5Ac[d]	7	2.983	2.844	4.188	4.333	3.852	3.496	3.363	3.814	3.734	–	2.041	–	–	–	–	[64]
		2.143	1.723	n.d.	4.249	n.d.	3.439	3.268	3.690	n.d.	–	2.041	–	–	–	–	[64]

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Table 10, *continued*

Sialic acid	pD	Chemical shift															Ref.
		H3a	H3e	H4	H5	H6	H7	H8	H9	H9′	4Ac	5Ac	7Ac	8Ac, 8Me	9Ac	5Gc	
β-Neu5Ac2P	7	1.548	2.403	4.093	3.888	4.239	3.386	4.028	3.883	3.581	–	2.045	–	–	–	–	[16]
CMP-β-Neu5Ac	8	1.639	2.484	4.066	3.92	4.141	3.456	3.92	3.90	3.622	–	2.054	–	–	–	–	[16]
CMP-β-Neu5Ac9Ac[e]	7	1.64	2.48	n.d.	3.96	n.d.	3.51	4.07	n.d.	n.d.	–	2.05	–	–	2.08	–	[314]
C8-β-Neu5Ac	6	1.814	2.203	3.998	3.892	3.772	3.722	3.655[f]	–	–	–	2.055	–	–	–	–	[308]
								3.586[f]									
C7-β-Neu5Ac	6	1.821	2.238	3.992	3.710	3.800	3.671[g]	–	–	–	–	2.044	–	–	–	–	[308]
							3.634[g]										
C7-α-Neu5Ac	6	1.599	2.630	n.d.	n.d.	n.d.	n.d.	–	–	–	–	2.026	–	–	–	–	[308]
β-Neu5Gc	7	1.840	2.243	4.127	4.002	4.106	3.549	3.777	3.821	3.613	–	–	–	–	–	4.143	[16]
α-Neu5Gc	7	1.644	2.749	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	–	–	–	–	4.12	[16]
β-Neu9Ac5Gc	7	1.842	2.234	4.14	4.006	4.109	3.570	3.970	4.365	4.183	–	–	–	–	2.115	4.144	[16]
α-Neu9Ac5Gc	7	1.649	2.751	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	–	–	–	n.d.	4.123	[16]
β-Neu5Gc8Me	7	1.863	2.219	4.110	3.983	4.017	3.559	3.432	3.932	3.652	–	–	–	3.425	–	4.133	[31]
α-Neu5Gc8Me	7	1.643	2.551	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	–	–	n.d.	–	4.125	[31]
CMP-β-Neu5Gc[e]	7	1.64	2.48	3.92	4.03	n.d.	3.42	3.60	3.87	3.60	–	–	–	–	–	4.11	[314]
C8-β-Neu5Gc	6	1.832	2.218	4.097	3.976	3.887	3.709	3.657[f]	–	–	–	–	–	–	–	4.144	[308]
								3.589[f]									

continued on next page

Table 10, *continued*

Sialic acid	pD	Chemical shift														Ref.
		H3a	H3e	H4	H5	H6	H7	H8	H9	H9′	4Ac	5Ac	7Ac	8Ac, 8Me	9Ac	5Gc	
C8-α-Neu5Gc	6	1.622	2.665	n.d.	n.d.	n.d.	n.d.	n.d.	–	–	–	–	–	–	–	4.129	[308]
C7-β-Neu5Gc	6	1.840	2.253	4.096	3.798	3.906	3.674[g]	–	–	–	–	–	–	–	–	4.133	[308]
							3.632[g]										
C7-α-Neu5Gc	6	n.d.	2.650	n.d.	n.d.	n.d.	n.d.	–	–	–	–	–	–	–	–	4.116	[308]
β-Kdn	7	1.837	2.265	4.015	3.603	3.995	3.881	3.772	3.883	3.675	–	–	–	–	–	–	[106]
β-Kdn9Ac[h]	7	1.768	2.158	3.94	3.575	3.94	3.91	3.91	4.376	4.238	–	–	–	–	2.124	–	[51]
CMP-β-Kdn[h]	7	1.568	2.379	3.968	3.538	4.046	3.863	3.896	3.731	3.618	–	–	–	–	–	–	[107]

[a] n.d., not determined.
[b] On one line values may have to be interchanged.
[c] Sialic acid occurs in $^5C_2$ conformation.
[d] Sialic acid occurs in $^7C_4$ conformation and is present in two tautomeric forms; chemical shifts are relative to HOD at δ 4.750; H3a = H3 and H3e = H3′.
[e] Values are assigned relative to the HOD signal at δ 4.81 at 296 K.
[f] H8 and H8′.
[g] H7 and H7′.
[h] Personal communication Y. Inoue; values are assigned relative to 2,2-dimethyl-2-silapentane-5-sulphonate in D_2O (set to 0 ppm). Note that in CMP-β-Kdn the H8 and H9 signals have been interchanged when compared with ref. [315].

Table 11

^{1}H-Chemical shifts for the H3e and H3a signals of sialic acids as part of N- and O-linked glycoprotein glycans. Chemical shifts are given in ppm relative to internal acetone in D_2O (δ 2.225) at 300 K, or relative to internal 2,2-dimethyl-2-silapentane-5-sulphonate in D_2O set to 0 ppm (marked [a])

Structural element	H3a	H3e
Neu5Ac(α2–3)Gal(β1-	1.78–1.81	2.75–2.78
Neu4,5Ac_2(α2–3)Gal(β1-	1.93	2.77
Neu5,9Ac_2(α2–3)Gal(β1-	1.80	2.76
Neu5Gc(α2–3)Gal(β1-	1.81–1.82	2.77–2.79
Kdn(α2–3)Gal(β1–4)GlcNAc(β1-	1.75–1.76[a]	2.71–2.72[a]
Kdn(α2–3)Gal(β1–3)GalNAc(α1–3)GalNAc-ol	1.72[a]	2.69[a]
Neu5Ac(α2–3)[Gal(β1–4)]Gal(β1-	1.82[a]	2.73[a]
Neu5Ac(α2–3)[GalNAc(β1–4)]Gal(β1-	1.92–1.94	2.66–2.68
Neu5Ac(α2–6)Gal(β1-	1.70–1.72	2.67
Neu4,5Ac_2(α2–6)Gal(β1-	1.85	2.68
Neu5,9Ac_2(α2–6)Gal(β1-	1.71	2.67
Neu5Gc(α2–6)Galβ1-	1.73–1.74	2.69–2.70
Gal(β1–3)[Neu5Ac(α2–6)]GlcNAc(β1–2)Man(α1-	1.71–1.72	2.73–2.74
Gal(β1–3)[Neu5Ac(α2–6)]GlcNAc(β1–4)Man(α1-	1.76–1.77	2.72–2.73
Neu5Ac(α2–6)GalNAc(β1-	1.70–1.72	2.66
Neu5Gc(α2–3)[GalNAc(β1–4)]GalNAc(β1-	1.85–1.86	2.55–2.56
Kdn(α2–3)[GalNAc(β1–4)]GalNAc(β1-	1.79[a]	2.48[a]
Neu5Ac(α2–6)GalNAc-ol	1.69–1.71	2.72–2.74
Neu5Gc(α2–6)GalNAc-ol	1.71–1.72	2.74–2.75
Kdn(α2–6)GalNAc-ol	1.64–1.66	2.66–2.68

Another interesting pH phenomenon, for the first time demonstrated by ^{1}H NMR spectroscopy, is the complete replacement of H3a by a D-atom, when free Neu5Ac is kept in an alkaline D_2O solution at pD 9.0 [326]. In fact, H3a can be exchanged in the pH range 6.5–9.0, and the H–D exchange is reversible. In the ^{1}H NMR spectrum the H3a signal disappears, and the coupling patterns of H3e and H4 alter. At pD 12.4, H3e can also be replaced by a D-atom [327]. On the basis of this finding, the exchange experiments were also carried out in T_2O, yielding T-labelled Neu5Ac, which was converted enzymatically into T-labelled CMP-Neu5Ac [326]. In glycosidically linked Neu5Ac the H3 atoms are not exchangeable, rendering this specific labelling technique suitable for sialyltransferase experiments.

In the ^{1}H NMR structural-reporter-group concept, developed for the structural analysis of glycoprotein N- and O-glycans, advantage is taken of the fact that a number of the H-atoms of the constituting monosaccharides, occurring in special microenvironments, resonate outside the bulk-signal region [76,77,328]. In the case of glycosidically linked α-sialic acids the structural reporters are the H3a- and H3e-atoms, and the *N*-acetyl or *N*-glycolyl groups. The positions of the H3a,3e signals reflect

not only structural information with respect to the type of sialic acid present, but also with respect to the coupled monosaccharide in terms of type of linkage and type of monosaccharide. Furthermore, *O*-acyl substituents induce additional shifts for other H-atoms. In Table 11 a survey is presented of chemical shifts of H3e and H3a signals of sialic acid residues occurring in different linkage types as part of N- and O-glycans. For a further fine-tuning of the chemical shifts within the presented ranges, influenced by the different microenvironments wherein the sialic acid residues occur, see refs. [76,77] and the references cited in Table 2. It should be noted that the presence of a certain sialic acid in a certain linkage also influences the structural-reporter-group signals of other monosaccharide residues [76,77]. More detailed information with respect to the H3e,3a chemical shifts of sialic acids in sialo-oligomers and -polymers can be obtained from refs. [31,47,77,329,330]. A series of H3a,3e signals of sialic acids in milk- and glycolipid-derived oligosaccharides have been included in ref. [77]. More general NMR data of glycosidically linked sialic acids in glycolipids, milk and urinary oligosaccharides and (lipo)polysaccharides, if available, can be found in the references cited in Tables 3–5 (see also ref. [331]). For a series of sialocarbohydrates, it has been shown that H6 of Neu5Ac, easily traced from the TOCSY H3a,H6 correlation, also has potential value for discriminating between α-2,3- (δ 3.63 ± 0.011) and α-2,6- (δ 3.70 ± 0.017) linked Neu5Ac in Neu5Ac(α2–3/6)Gal(β1–O)R/Neu5Ac(α2–6)GalNAc(α1–O)Ser/Thr [331]. However, for branched oligosaccharides, this rule is not valid if the monosaccharide in the branching position is in the alditol form [332].

The H3a,3e/NAc structural reporters of Neu5Ac have proved to be suitable in studying sialylation reactions in terms of positional specificity and branching (N-linked carbohydrate chains) specificity, using different sialyltransferases and CMP-Neu5Ac as donor [333–335] (see also section 6.3).

In more biophysical studies, several aspects of sialic acids have been investigated by NMR spectroscopy. Although so far mainly α-forms of bound sialic acid have been detected, good differentiation systems for α- and β-forms are essential, and a number of empirical rules have been reported [336]. In a heteronuclear 2D-approach it could be demonstrated that the determination of the geminal C,H coupling constant ^{2}J(C2,H3a) offers a unique criterion for the anomeric assignment in sialic acid glycosides (α, −8 Hz; β, −3 to −4 Hz) [337]. Also the values of the vicinal C,H coupling constants ^{3}J(C1,H3a) can be applied for this differentiation (α, ~6 Hz; β, ~1 Hz) [55,336,338]. More details with respect to anomeric determinations have been reviewed in ref. [339].

^{13}C NMR data of sialic acids and glycoprotein-derived sialocarbohydrates have been reviewed in ref. [16]. In several sialic-acid-related investigations, e.g. synthetic studies, ^{13}C NMR spectroscopy forms part of the analysis techniques, and will not be reviewed in detail. For some additional data, more directly related with the glycoprotein glycan character of this chapter, see refs. [48,63,64,103,105,152,331,332,337,340–343].

6. *Chemo-enzymatic highlights in sialic acid chemistry*

During the last ten years, activity in sialic acid chemistry has grown exponentially. Synthetic as well as biosynthetic routes for the preparation of sialic acids, sialic acid

derivatives, analogues, glycosides and sialoglycoconjugates have been explored. The main reason for this considerable interest in preparing sialic acids and sialic-acid-containing compounds lies in the fact that sialic acids were found to be among the most biologically important carbohydrate units in glycoconjugates. The progress in organic synthetic protocols and the availability of relevant enzymes in suitable amounts made it realistic to develop the sialic acid field from a preparative synthetic side. Initially, the main tools were to prepare suitable derivatives to study the properties of sialic acids, or to prepare substrates and inhibitors for sialidases, sialyltransferases or for sialic acid converting enzymes. Although these tools are still highly relevant, the preparation of sialo-oligosaccharides using strictly organic synthetic or enzymatic methods, or a mixture of both, are also receiving considerable attention. For relevant reviews on preparative (bio)synthetic aspects, see refs. [339,344–353].

6.1. Free sialic acids

Several protocols have been followed for the organic chemical synthesis of Neu5Ac. At first, the approaches were based on condensation reactions of (derivatives of) *N*-acetyl-D-mannosamine (ManNAc) or *N*-acetyl-D-glucosamine (GlcNAc) with (derivatives of) oxaloacetic acid (for a review, see ref. [3]), however, the yields were very low. In this context, a procedure was worked out, that allowed modifications at C1–C3 [354]. A total synthesis of Neu5Ac from non-carbohydrate precursors has been reported in ref. [355]. Using a protocol for indium-mediated allylations of aldehydes, Neu5Ac was prepared in good yields from a ManNAc precursor [356]. Furthermore, synthetic routes for Neu5Ac have been proposed, based upon 1-deoxy-1-nitro-sugar chemistry, that should also allow the preparation of Neu5Ac analogues, modified at several carbon atoms of the skeleton [357–359]. A separate route also yielded Neu5Ac [360]. A synthesis starting with the aldol condensation of D-glucose (Glc) and oxaloacetic acid, followed by adaptation of the substituent at C5 has been described in ref. [361]. More recently, another approach for the organic synthesis of Neu5Ac and Neu5Ac derivatives, based on the *cis*-selective Wittig reaction of benzoyl 2,3-*O*-isopropylidene-α-D-*lyxo*-pentodialdo-1,4-furanoside with [(3S)-3,4-(isopropylidenedioxy)butyl]-triphenylphosphonium iodide as a first step, has been reported in ref. [362]. Comments on the acetylation of Neu5Ac and its methyl ester have been published in ref. [363]. Starting from Neu5Ac, in some derivatization reactions 1,4- as well as 1,7-lactone formation has been observed [349]. For the organic synthesis of Kdn, several routes were employed [364–368], among them procedures starting from Neu5Ac or from D-mannose (Man).

Neu5Ac-aldolase-catalyzed condensations of ManNAc and pyruvate [3,33], initially only investigated to understand sialic acid metabolism, have been optimized for preparative purposes. In principle, ManNAc can be generated from the cheaper GlcNAc in an alkaline epimerization process, yielding an epimeric mixture of which only the monosaccharide with the D-*manno*-configuration is recognized by the aldolase [369,370]. However, ManNAc can also be generated from GlcNAc in a GlcNAc-epimerase-catalyzed isomerization [371]. A multigram-scale enzymatic synthesis based on the aldol condensation of ManNAc and pyruvate in the presence of phosphate, catalyzed by immobilized microbial Neu5Ac-aldolase, has been reported [372]. A similar approach,

using the aldolase enclosed in a dialysis membrane instead of being immobilized, has also been described [373]. Although *in vivo,* the conversion of Neu5Ac into Neu5Gc occurs exclusively on the level of activated sialic acid (see section 8.4.1), Neu5Gc can be prepared *in vitro* by incubating a mixture of *N*-glycolyl-D-mannosamine/*N*-glycolyl-D-glucosamine and pyruvate with immobilized aldolase [374]. Interestingly, a series of other sugars also turned out to be accepted by the aldolase, and Man and 2-deoxy-D-glucose in particular are excellent substrates [372,375–377]. In the case of Man as starting material, relatively moderate amounts of Kdn have been prepared [254,376].

A series of free *O*-acetylated sialic acids, i.e., Neu4,5Ac_2, Neu5,9Ac_2, Neu4,5,9Ac_3, and Neu5,7,8,9Ac_4, together with the benzyl ester α-glycosides of Neu5,7Ac_2 and Neu5,7,9Ac_3, have been synthesized by organic synthetic routes using protecting group techniques [378,379]. Partially *O*-acetylated sialic acid derivatives have also been prepared using more simple synthetic routes. β-Neu5,9$Ac_2$1,2Me_2, β-Neu4,5,9$Ac_3$1,2Me_2, and β-Neu4,5,8,9$Ac_4$1,2Me_2 were obtained from β-Neu5Ac1,2Me_2 by using *N*-acetylimidazole [53]. To realize 9-*O*-acetylations, also other acetylating reagents were applied, such as trimethyl orthoacetate [380,381], acetyl chloride [378], and dimethylacetamide dimethyl acetal [382]. In a recent comprehensive study, in particularly the use of trimethyl orthoacetate and dimethylacetamide dimethyl acetal was explored using the 4-aminophenylthio, 4-nitrophenylthio, and 4-nitrophenyl glycosides of COOH-esterified α-Neu5Ac as acceptors, and depending on the acetylating reagent a range of partially *O*-acetylated derivatives could be generated [57].

One of the naturally occurring *O*-acetylated sialic acids, Neu5,9Ac_2, has also been synthesized in an enzymatic way [383,384] on a gram-scale [372]. After the enzymatic acetylation of O6 of ManNAc, using isopropenyl acetate and protease N as a catalyst, 2-*N*-acetyl-6-*O*-acetyl-D-mannosamine was condensed with pyruvate as catalyzed by the aldolase. These two enzymatic steps turned out to be highly regio- and stereoselective. Following another route, Neu5,9Ac_2 has been synthesized enzymatically by incubating Neu5Ac with trichloroethyl acetate in pyridine using porcine pancreas lipase as a catalyst [385]. An enzymatic synthesis of Neu5Ac9Lt has also been worked out [370,384].

For the study of biochemical pathways, several isotopically labelled sialic acids and sialic acid derivatives have been prepared, both by enzyme-catalyzed synthesis and by organic synthesis. A survey of labelled sialic acids is presented in Table 12 [107,344,386]. In enzymatic procedures, use is generally made of the aldolase-catalyzed condensation of *N*-acyl-D-mannosamines and (phosphoenol)pyruvate, suitably labelled in one or both of the two synthons. For the preparation of *N*-[1-^{14}C]acetyl- and *N*-[1-^{14}C]glycolyl-neuraminic acid, as well as *O*-[^{14}C]acetylated sialic acids, surviving slices of submaxillary salivary glands incubated with [1-^{14}C]acetate, followed by isolation of the glycoprotein fraction and mild acid hydrolysis, have been used. A number of these labelled sialic acids have been converted into their CMP-glycosides, and subsequently incorporated into glycoconjugates (see section 6.3). Of course, labelling of glycoconjugates can also be carried out by periodate oxidation/tritiated borohydride reduction, thereby converting sialic acids, if chemically possible, into their radiolabelled C7 and C8 analogues. Using the latter approach, fluorescent probes (dansylhydrazine, dansylethylenediamine, fluoresceinamine) and EPR spin labels can also be incorporated (see references cited in ref. [344]). The same holds for glycine [387].

Table 12
Survey of radiolabelled sialic acids[a]

Sialic acid	Reference
N-[^{3}H]Acetylneuraminic acid	[344]
N-[1-^{14}C]Acetylneuraminic acid	[344]
N-Acetyl-[3-^{3}H]neuraminic acid	[344]
N-Acetyl-[9-^{3}H]neuraminic acid	[386]
N-Acetyl-[1-^{14}C]neuraminic acid	[344]
N-Acetyl-[4-^{14}C]neuraminic acid	[344]
N-Acetyl-[2-^{14}C,9-^{3}H]neuraminic acid	[344]
5-*N*-Acetyl-9-azido-9-deoxy-[1-^{14}C]neuraminic acid	[344]
5-*N*-Acetyl-4-*O*-methyl-[3-^{3}H]neuraminic acid	[344]
5-*N*-[1-^{14}C]Acetyl-2-deoxy-2,3-didehydro-neuraminic acid	[344]
N-[1-^{14}C]Glycolylneuraminic acid	[344]
N-Glycolyl-[1-^{14}C]neuraminic acid	[344]
N-Glycolyl-[2-^{14}C,9-^{3}H]neuraminic acid	[344]
[^{14}C]-2-Keto-3-deoxynononic acid	[107]

[a] For specific references, see [344].

Several interesting sialic acid variants and sialic acid derivatives have been synthesized, and a list is presented in Table 13 [316,344,347,349,357–359,368,370,376–380,384,388–446]. A number of these compounds have been surveyed in ref. [350]. Both organic synthetic and aldolase-catalyzed routes have been followed. The major part of these compounds were prepared to study sialic acid metabolism (aldolase, CMP-Neu5Ac synthase), sialic acid transfer (sialyltransferases), sialic acid release (sialidases), inhibition phenomena, or hemagglutinin–sialic acid interactions, and biological details are presented in sections 8–10. Compounds reported up to 1982 have been reviewed earlier [344]. Of special interest are the fluorescent and photoactivatable sialic acid derivatives [390,419], which can be applied, after conversion into their corresponding CMP-glycosides, to detect enzyme activities or to follow biological processes (see sections 6.2 and 8.2). In the context of sialic acid variants, the following compounds are also of interest. In view of the similarity in acidity of a tetrazole group and a carboxyl function, a variant of Neu5Ac has been prepared containing a CN_4H instead of a COOH group [447]. Also the synthesis of a series of Neu5Ac derivatives with specifically introduced *tert*-butyldimethylsilyl groups have been reported [400,448]. Furthermore, variants of 2d-2H_{ax}-Neu5Ac and 2d-2H_{eq}-Neu5Ac, in which the carboxyl function has been replaced by a phosphono (PO_3H_2) group [449], and a phosphonic acid analogue of Neu2en5Ac [450], have been synthesized. In addition to 6-amino-2,6-dideoxy-sialic acids, as mentioned in Table 13, the preparation of 2-*C*-hydroxymethyl derivatives [451], and C6 and C7 analogues [452] have also been reported.

Table 13
List of sialic acids, prepared along organic chemical or aldolase-catalyzed routes for use in biochemical studies[a]

Compound	Abbreviation	Reference(s)
N-Acetylneuraminic acid	Neu5Ac	see text
5-*N*-Acetyl-2-deoxy-2,3-didehydro-neuraminic acid	Neu2en5Ac	[344]
2-Deoxy-2,3-didehydro-neuraminic acid	Neu2en	[350]
N-Glycolylneuraminic acid	Neu5Gc	see text
2-Deoxy-2,3-didehydro-5-*N*-glycolyl-neuraminic acid	Neu2en5Gc	[344]
5-Azido-neuraminic acid	$Neu5N_3$	[388]
5-Azido-2-deoxy-2,3-didehydro-neuraminic acid	$Neu2en5N_3$	[350]
2-Keto-3-deoxynononic acid	Kdn	see text
2,3-Didehydro-2,3-dideoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid	Kdn2en	[389]
N-Acetyl-[1-^{13}C]neuraminic acid	[1-^{13}C]Neu5Ac	[344]
N-Acetyl-[3-^{2}H]neuraminic acid	[3-^{2}H]Neu5Ac	[344]
N-Acetyl-[6-^{2}H]neuraminic acid	[6-^{2}H]Neu5Ac	[359]
N-Aminoacetyl-neuraminic acid	$Neu5AcNH_2$	[390]
N-Thioacetyl-neuraminic acid	Neu5AcSH	[390,391]
5-*N*-Acetyl-2,7-anhydro-neuraminic acid	Neu2,7an5Ac	[349]
5-*N*-Acetyl-2-deoxy-2-H_{ax}-neuraminic acid	2d-2H_{ax}-Neu5Ac	[392–394]
5-*N*-Acetyl-2-deoxy-2-H_{eq}-neuraminic acid	2d-2H_{eq}-Neu5Ac	[393–395]
5-*N*-Acetyl-2-deoxy-2-H_{eq}-4-oxo-neuraminic acid	2d-2H_{eq}-4oxo-Neu5Ac	[396]
5-*N*-Acetyl-2-deoxy-4-epi-neuraminic acid	2d-4epi-Neu5Ac	[397]
5-*N*-Acetyl-2-deoxy-7-epi-2-H_{eq}-neuraminic acid	2d-7epi-2H_{eq}-Neu5Ac	[394,398]
5-*N*-Acetyl-2-deoxy-8-epi-2-H_{eq}-neuraminic acid	2d-8epi-2H_{eq}-Neu5Ac	[394,398]
5-*N*-Acetyl-2-deoxy-7,8-diepi-2-H_{eq}-neuraminic acid	2d-7,8epi$_2$-2H_{eq}-Neu5Ac	[394,398]

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Table 13, *continued*

Compound	Abbreviation	Reference(s)
5-*N*-Acetyl-3-fluoro-neuraminic acid	$3F_{eq}$-Neu5Ac	[344,392]
5-*N*-Acetyl-3-hydroxy-neuraminic acid	$3OH_{eq}$-Neu5Ac	[344,399]
5-*N*-Acetyl-4-*O*-acetyl-neuraminic acid	$Neu4,5Ac_2$	[378,380]
5-*N*-Acetyl-4-*O*-acetyl-4-epi-β-neuraminic acid methyl ester methyl glycoside	$4epi\text{-}\beta Neu4,5Ac_2 1,2Me_2$	[400]
5-*N*-Acetyl-4-deoxy-neuraminic acid	4d-Neu5Ac	[358,401–403]
5-*N*-Acetyl-4-deoxy-4-iodo-neuraminic acid	4I-Neu5Ac	[404]
5-*N*-Acetyl-4-deoxy-4-(R)-*C*-methyl-neuraminic acid		[405]
5-*N*-Acetyl-4-deoxy-4-(S)-*C*-methyl-neuraminic acid		[405]
5-*N*-Acetyl-4-epi-neuraminic acid	4epi-Neu5Ac	[357,406]
5-*N*-Acetyl-4-epi-4-*O*-methyl-neuraminic acid ethyl ester	4epi-Neu5Ac1Et4Me	[344]
5-*N*-Acetyl-4-*O*-methyl-β-neuraminic acid (ethyl ester/ethyl glycoside)	Neu5Ac4Me	[344,407]
5-*N*-Acetyl-4-oxo-neuraminic acid (methyl β-glycoside or ethyl α-glycoside)	4oxo-Neu5Ac	[408,409]
5-*N*-Acetyl-7-deoxy-neuraminic acid	7d-Neu5Ac	[377,410]
5-*N*-Acetyl-7-epi-neuraminic acid	7epi-Neu5Ac	[411,412]
5-*N*-Acetyl-7-*O*-methyl-neuraminic acid	Neu5Ac7Me	[377]
5-*N*-Acetyl-7-oxo-β-neuraminic acid methyl glycoside	7oxo-βNeu5Ac2Me	[408]
5-*N*-Acetyl-8-deoxy-neuraminic acid	8d-Neu5Ac	[410]
5-*N*-Acetyl-8-epi-neuraminic acid	8epi-Neu5Ac	[411,412]
5-*N*-Acetyl-8-*O*-methyl-neuraminic acid	Neu5Ac8Me	[344]
5-*N*-Acetyl-8-oxo-neuraminic acid methyl α- and β-glycoside	8oxo-Neu5Ac2Me	[394,408,413]
5-*N*-Acetyl-9-*O*-acetyl-neuraminic acid	$Neu5,9Ac_2$	see text
5-*N*-Acetyl-9-*S*-acetyl-9-thio-neuraminic acid		[414]

continued on next page

Table 13, *continued*

Compound	Abbreviation	Reference(s)
5-*N*-Acetyl-9-amino-9-deoxy-neuraminic acid	9amino-Neu5Ac	[316,390, 415–417]
5-*N*-Acetyl-9-azido-9-deoxy-neuraminic acid (methyl α-glycoside)	9azido-Neu5Ac	[316,384,390, 417,418]
5-*N*-Acetyl-9-(4-azidobenzamido)-9-deoxy-neuraminic acid		[419]
5-*N*-Acetyl-9-(4-azidosalicylamido)-9-deoxy-neuraminic acid		[419]
5-*N*-Acetyl-9-benzamido-9-deoxy-neuraminic acid	9NBz-Neu5Ac	[316,390,415, 417]
5-*N*-Acetyl-9-(4-benzoylbenzamido)-9-deoxy-neuraminic acid		[419]
5-*N*-Acetyl-9-cyano-9-deoxy-α-neuraminic acid benzyl glycoside	9cyano-αNeu5Ac2Bn	[394]
5-*N*-Acetyl-9-*O*-(*N*-dansylglycyl)-neuraminic acid		[420]
5-*N*-Acetyl-9-deoxy-neuraminic acid (methyl α-glycoside)	9d-Neu5Ac	[394,410,421]
5-*N*-Acetyl-9-deoxy-9-(3-fluoresceinylthioureido)-neuraminic acid	9fluoresceinyl-Neu5Ac	[390,415,422, 423]
5-*N*-Acetyl-9-deoxy-9-fluoro-neuraminic acid	9F-Neu5Ac	[384,424–426]
5-*N*-Acetyl-9-deoxy-9-iodo-neuraminic acid	9I-Neu5Ac	[344,427]
5-*N*-Acetyl-9-deoxy-9-thioacetamido-neuraminic acid		[390,391]
5-*N*-Acetyl-9-*O*-(dimethylphosphinyl)-neuraminic acid		[384]
5-*N*-Acetyl-9-*O*-glycyl-neuraminic acid methyl ester	Neu5Ac9Gly1Me	[344]
5-*N*-Acetyl-9-hexanoylamido-9-deoxy-neuraminic acid	9NHx-Neu5Ac	[316,390,417]
5-*N*-Acetyl-9-*O*-lactyl-neuraminic acid	Neu5Ac9Lt	[370,384]
5-*N*-Acetyl-9-*O*-methyl-neuraminic acid	Neu5Ac9Me	[347]
5-*N*-Acetyl-9-*O*-phosphoro-neuraminic acid	Neu5Ac9P	[344]

continued on next page

Table 13, *continued*

Compound	Abbreviation	Reference(s)
5-*N*-Acetyl-9-thio-neuraminic acid		[414]
5-*N*-Acetyl-4,9-di-*O*-acetyl-neuraminic acid	Neu4,5,9Ac_3	[378]
5-*N*-Acetyl-2,7-dideoxy-2-H_{eq}-neuraminic acid	2,7d_2-2H_{eq}-Neu5Ac	[394,398]
5-*N*-Acetyl-2,8-dideoxy-2-H_{eq}-neuraminic acid	2,8d_2-2H_{eq}-Neu5Ac	[394,398]
5-*N*-Acetyl-4,7-dideoxy-neuraminic acid	4,7d_2-Neu5Ac	[410]
5-*N*-Acetyl-7,9-dideoxy-neuraminic acid	7,9d_2-Neu5Ac	[421]
5-*N*-Acetyl-7,8-diepi-neuraminic acid (methyl α-glycoside)	7,8epi$_2$-Neu5Ac	[394,411,412]
5-*N*-Acetyl-7,7-dimethoxy-β-neuraminic acid methyl glycoside		[408]
5-*N*-Acetyl-8,8-dimethoxy-neuraminic acid methyl α- and β-glycoside		[394,408,413]
5-*N*-Acetyl-7,8,9-tri-*O*-acetyl-neuraminic acid	Neu5,7,8,9Ac_4	[379]
5-*N*-Acetyl-4,7,9-trideoxy-neuraminic acid	4,7,9d_3-Neu5Ac	[421]
5-*N*-Acetyl-4-allylamino-2,3-didehydro-2,4-dideoxy-neuraminic acid		[428]
5-*N*-Acetyl-4-amino-2,3-didehydro-2,4-dideoxy-neuraminic acid	4amino-Neu2en5Ac	[428–430]
5-*N*-Acetyl-4-azido-2,3-didehydro-2,4-dideoxy-neuraminic acid	4azido-Neu2en5Ac	[430,431]
5-*N*-Acetyl-9-*S*-(4-azido-2-nitrophenyl)-2,3-didehydro-2,9-dideoxy-9-thio-neuraminic acid		[432]
5-*N*-Acetyl-2-deoxy-2,3-didehydro-4-epi-neuraminic acid (methyl ester)	4epi-Neu2en5Ac	[344,433,434]
5-*N*-Acetyl-2-deoxy-2,3-didehydro-4-oxo-neuraminic acid	4oxo-Neu2en5Ac	[409,433,435]
5-*N*-Acetyl-2-deoxy-2,3-didehydro-6-thio-neuraminic acid		[436]
5-*N*-Acetyl-2-deoxy-2,3-didehydro-7-epi-neuraminic acid	7epi-Neu2en5Ac	[350]
5-*N*-Acetyl-2-deoxy-2,3-didehydro-8-epi-neuraminic acid	8epi-Neu2en5Ac	[437]
5-*N*-Acetyl-2-deoxy-2,3-didehydro-7,8-diepi-neuraminic acid	7,8epi$_2$-Neu2en5Ac	[350]
5-*N*-Acetyl-2-deoxy-2,3-didehydro-8,8-dimethoxy-neuraminic acid		[413]

continued on next page

Table 13, *continued*

Compound	Abbreviation	Reference(s)
5-*N*-Acetyl-2,3-didehydro-2,4-dideoxy-4-dimethylamino-neuraminic acid		[428]
5-*N*-Acetyl-2,3-didehydro-2,4-dideoxy-4-guanidinyl-neuraminic acid	4guanidino-Neu2en5Ac	[428,429]
5-*N*-Acetyl-4-(*N*-hydroxy-*N*-allylamino)-2,3-didehydro-2,4-dideoxy-neuraminic acid		[428]
5-*N*-Acetyl-4-(2-hydroxyethylamino)-2,3-didehydro-2,4-dideoxy-neuraminic acid		[428]
5-*N*-acetyl-2,3-didehydro-2,4-dideoxy-neuraminic acid	4d-Neu2en5Ac	[438]
5-*N*-acetyl-2,3-didehydro-2,7-dideoxy-neuraminic acid	7d-Neu2en5Ac	[350]
5-*N*-acetyl-2,3-didehydro-2,8-dideoxy-neuraminic acid	8d-Neu2en5Ac	[350]
5-*N*-acetyl-2,3-didehydro-2,9-dideoxy-neuraminic acid	9d-Neu2en5Ac	[350]
5-*N*-acetyl-2,3-didehydro-2,4,7-trideoxy-neuraminic acid	4,7d_2-Neu2en5Ac	[350]
4-Acetamido-5-*N*-acetyl-2,3-didehydro-2,4-dideoxy-neuraminic acid		[428]
5-*N*-Acetyl-6-amino-2-deoxy-2-H_{ax}-neuraminic acid		[439]
5-*N*-Acetyl-6-amino-2-deoxy-2-H_{ax}-4-epi-neuraminic acid		[439]
5-*N*-Acetyl-6-amino-2-deoxy-2-H_{eq}-4-epi-neuraminic acid		[439]
5-*N*-Acetyl-6-thio-neuraminic acid		[436]
5-*N*-Acetyl-5-epi-6-thio-neuraminic acid		[436]
5-*N*-Acetyl-4,5-diepi-6-thio-neuraminic acid		[436]
9-*O*-Acetyl-5-*N*-glycolyl-neuraminic acid	Neu9Ac5Gc	[380]
4-Acetamido-5-*N*-acetyl-4-deoxy-neuraminic acid	4NAc-Neu5Ac	[440]
4-Acetamido-3,4-dideoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid	iso-Neu4Ac	[441]
5-Acetamido-2,6-anhydro-3,5-dideoxy-2,3-difluoro-D-*arabino*-L-*talo*-nononic acid	2d-2F_{ax}-3F_{eq}-Neu5Ac	[392]
5-Acetamido-2,6-anhydro-3,5-dideoxy-4-*C*-methyl-D-*erythro*-L-*altro*-nononic acid		[396]
5-Acetamido-2,6-anhydro-3,5-dideoxy-4-*C*-methyl-D-*erythro*-L-*manno*-nononic acid		[396]

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Table 13, *continued*

Compound	Abbreviation	Reference(s)
5-Acetamido-2,6-anhydro-4-*C*-methyl-3,4,5-trideoxy-D-*erythro*-L-*altro*-nononic acid		[396]
5-Acetamido-2,6-anhydro-4-*C*-methyl-3,4,5-trideoxy-D-*erythro*-L-*manno*-nononic acid		[396]
5-Acetamido-2,6-anhydro-4-*C*-methylene-3,4,5-trideoxy-D-*glycero*-D-*galacto*-nononic acid		[396]
5-Acetamido-2,5-dideoxy-2,3-difluoro-D-*erythro*-L-*gluco*-non-2-ulopyranosonic acid		[442,443]
5-*C*-Acetamidomethyl-5-deamino-neuraminic acid		[444]
9-Acetamido-5-*N*-acetyl-9-deoxy-neuraminic acid	9NAc-Neu5Ac	[316,390,415, 417]
5-*N*-Glycolyl-9-*O*-phosphoro-neuraminic acid	Neu5Gc9P	[344]
5-Bromo-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid	5Br-Kdn	[446]
3-Deoxy-D-*glycero*-D-*gulo*-non-2-ulopyranosonic acid	5epi-Kdn	[376]
3-Deoxy-D-*glycero*-D-*talo*-non-2-ulopyranosonic acid	4epi-Kdn	[368]
3,5-Dideoxy-5-fluoro-D-*glycero*-D-*gulo*-non-2-ulopyranosonic acid	5epi-5F-Kdn	[384]
3,5-Dideoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid	5d-Kdn	[376,384,445]
3,7-Dideoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid	7d-Kdn	[376]
3,7,9-Trideoxy-7,9-difluoro-D-*glycero*-L-*altro*-non-2-ulopyranosonic acid	7epi-7,9F_2-Kdn	[384]

[a] For literature references before 1982 and additional lists of sialic acid(s) (derivatives), see ref. [344].

6.2. Glycosides of sialic acids

The organic synthesis of a long series of alkyl and aryl α-glycosides of *N*-acylneuraminic acids has been previously reported (for reviews, see refs. [339,344]). One of the famous condensation reactions (classical Koenigs–Knorr method) comprised the silver carbonate-promoted condensation of 5-*N*-acetyl-4,7,8,9-tetra-*O*-acetyl-2-chloro-2-deoxy-β-neuraminic acid or the corresponding methyl ester (Fig. 9, structure A) with the appropriate alcohol, followed by removal of the protecting groups. In order to improve the yields, much attention has been paid to better catalysts. In these glycosidation reactions typical side reactions are the formation of unsaturated sialic acid derivatives (elimination of HCl) and of β-glycosides (see section 6.3). For the preparation of simple β-glycosides, *N*-acylneuraminic acids are often heated with the appropriate alcohol in the presence of an acid catalyst, followed by saponification of the formed ester. However, complex alcohols give rise to problems (for a review, see ref. [344]). A mild and efficient Raney nickel-catalyzed deuteration procedure has been reported for Neu5Ac glycosides, with a rate of exchange at C8 > C9 > C7 ≫ C4 [453].

Attention has also been paid to the synthesis of N-, S- and Se-glycosides, which are sialidase stable [339,454,455]. Specific S-glycosides are used as sialic acid donors in sialoglycoconjugate organic synthesis (see section 6.3). Early examples are the syntheses of the 4-nitrophenyl N- and S-glycosides of α-Neu5Ac [456]. Of special interest are the syntheses of 5-*N*-acetyl-2-azido-2-deoxy-α- and β-neuraminic acids [322,457,458]. The azides can readily be converted into the corresponding 2-amino derivatives, and used in e.g. *N*-acylation reactions [459]. For a preparation of the 6-thioanalogue of 2azido-α-Neu5Ac, see ref. [436]. In further investigations, a series of S-glycosides of α-Neu5Ac was synthesized (thiophenyl, 4-nitrothiophenyl, 4-aminothiophenyl, 2-mercaptopyridyl), starting from 5-*N*-acetyl-4,7,8,9-tetra-*O*-acetyl-2-chloro-2-deoxy-β-neuraminic acid methyl ester and using triethylbenzylammonium chloride as a phase transfer catalyst [454]. These compounds turned out to be effective sialidase inhibitors.

For the detection of sialidase activity both (naturally occurring) oligosaccharides and simple α-glycosides are used. In these assays, two approaches can be followed, namely, determination of the released (modified) sialic acid or identification of the released aglycon. In the case of α-glycosides, in which the released aglycon concentration is measured spectrophotometrically or detected on solid supports, substrates with synthetically introduced aglycons having specific chromogenic properties, are used. Among such substrates, released aglycons can be detected directly or after condensation with specific reagents. One of the oldest substrates is the 4-nitrophenyl glycoside of α-Neu5Ac [456], whereby released 4-nitrophenol is estimated by absorption at 400 nm. In an adapted synthetic version, the compound has been prepared by coupling of 5-*N*-acetyl-4,7,8,9-tetra-*O*-acetyl-2-chloro-2-deoxy-β-neuraminic acid methyl ester with sodium-nitrophenoxide in *N,N*-dimethylformamide, and subsequent deprotection [460]. Another suitable substrate is the 3-methoxyphenyl glycoside of α-Neu5Ac, synthesized by coupling of 5-*N*-acetyl-4,7,8,9-tetra-*O*-acetyl-2-chloro-2-deoxy-β-neuraminic acid with 3-methoxyphenol in the presence of silver carbonate, followed by de-*O*-acetylation. Liberated 3-methoxyphenol is determined after coupling

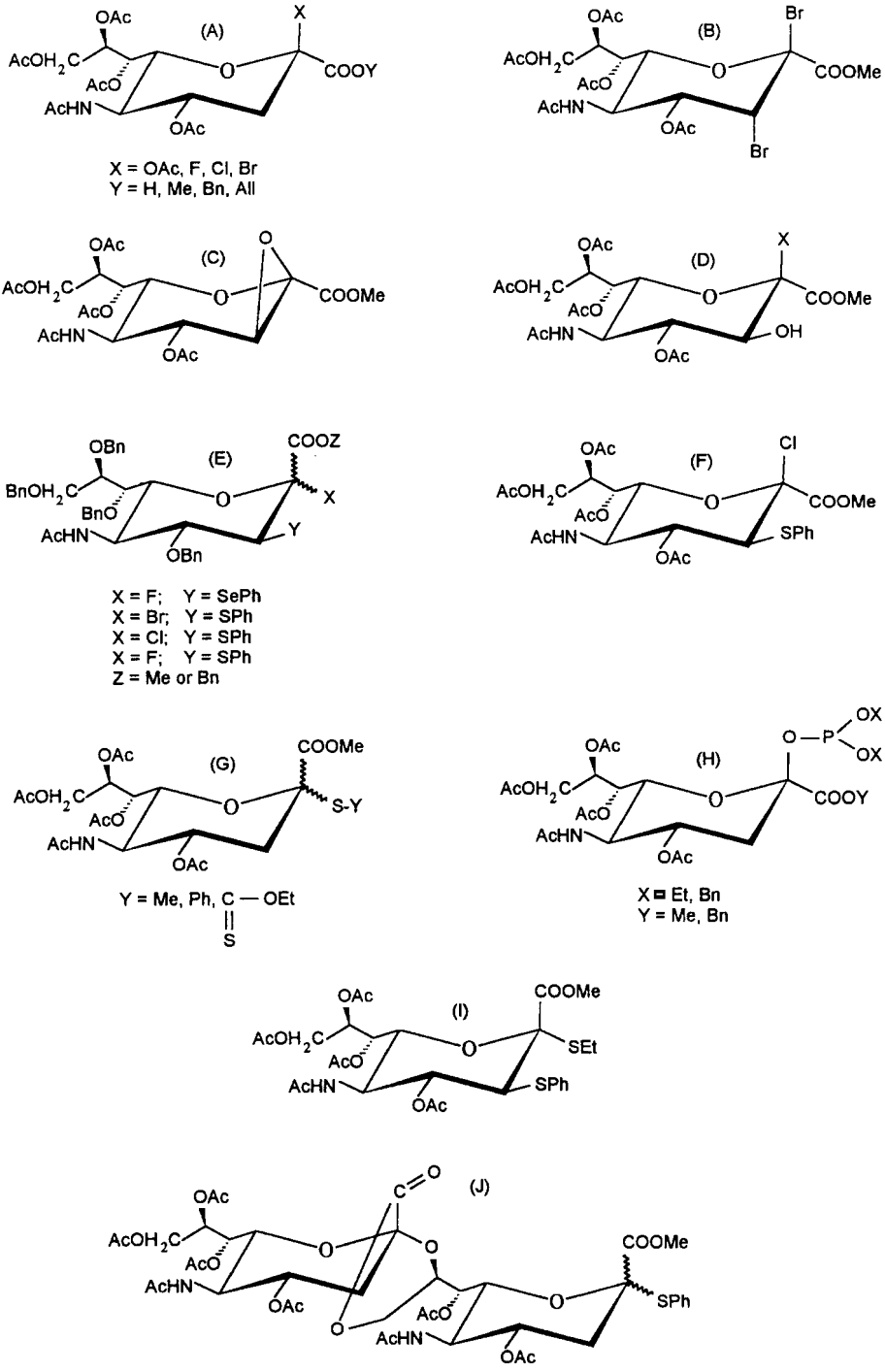


Fig. 9. Frequently used Neu5Ac donors in the organic synthesis of sialo-oligosaccharides.

with the diazonium salt of 4-amino-2,5-dimethoxy-4′-nitroazobenzene (red colored product) [461] or with 4-aminoantipyrine in the presence of the oxidizing agent potassium ferricyanide (colored quinone) [462]. The most popular fluorigenic substrate is the 4-methylumbelliferyl glycoside of α-Neu5Ac, which is prepared by different methods [344]. A convenient synthesis is the condensation of 5-*N*-acetyl-4,7,8,9-tetra-*O*-acetyl-2-chloro-2-deoxy-β-neuraminic acid methyl ester with the sodium salt of 4-methylumbelliferone in *N,N*-dimethylformamide, followed by deprotection [463]. Released 4-methylumbelliferone is measured at 360 nm (excitation)/440 nm (emission). Although the 4-methylumbelliferyl glycoside of α-Kdn has been synthesized starting from Neu5Ac [445], also a direct route using the glycosyl chloride of peracetylated β-Kdn methyl ester and the sodium salt of 4-methylumbelliferone has been explored [254]. In addition, several 4-methylumbelliferyl α-glycosides of sialic acid variants and substituted sialic acids, including partial *O*-acetylated ones, have been synthesized (e.g. refs. [252,350,399,445,464]. To develop a sensitive assay for the analysis of the linkage specificity of bacterial and viral sialidases, Neu5Ac(α2–3)- and Neu5Ac(α2–6)Gal(β1–O)$C_6H_4NO_2$ were synthesized enzymatically by using α-2,3- and α-2,6-sialyltransferase, respectively, CMP-Neu5Ac (see section 6.3), and *p*-nitrophenyl-β-Gal*p* [465]; after cleavage of Neu5Ac, *p*-nitrophenol can be released by additional treatment with β-galactosidase.

For the localization of sialidase on electropherograms or for histochemistry, the chromogenic 5-bromo-indol-3-yl glycoside of α-Neu5Ac has been synthesized by coupling of 5-*N*-acetyl-4,7,8,9-tetra-*O*-acetyl-2-chloro-2-deoxy-β-neuraminic acid methyl ester with 1-acetyl-5-bromo-3-hydroxyindole, and subsequent deprotection [466]. The unstable intermediate 5-bromo-indoxyl, released by sialidase, is readily transformed into insoluble blue-green 5,5′-dibromo-indigo, which marks the sites of enzyme activity. To facilitate the screening of bacterial colonies or plaques for sialidase activity, the 5-bromo-4-chloro-indol-3-yl glycoside variant has also been synthesized [467]. Using the same sialic acid synthon as starting product, the 4-azido-2-nitrophenyl S-glycoside of α-Neu5Ac has been prepared, which is a potential photoaffinity probe reagent for the screening of sialidases in tissues and the purification of sialic-acid-binding proteins [468]. The sialidase-resistant thioglycosyl linkage also makes the incorporation of ^{35}S possible.

In order to detect sialate 9-*O*-acetylesterase activity, a highly sensitive fluorescent substrate, 5-*N*-acetyl-9-*O*-acetyl-2-[4-(dansylamino)phenylthio]-α-neuraminic acid, has been synthesized (see also sections 5.3.2 and 9.1) [285]. The regioselective acetylation at O9 of the dansylated S-glycoside was carried out with trimethyl orthoacetate. Other useful fluorescent substrates for sialate *O*-acetylesterase assays comprise 5-*N*-acetyl-7,8,9-tri-*O*-acetyl-2-[4-(dansylamino)phenylthio]-α-neuraminic acid and 5-*N*-acetyl-4-*O*-acetyl-2-[4-(dansylamino)phenylthio]-α-neuraminic acid [469], and the 4-[3-(fluoresceinyl)thioureido]phenyl S-glycoside of α-Neu5,9Ac_2 [57]. In all cases the fluorescent groups have been coupled to the glycosidic 4-aminophenylthio group of *O*-acetylated Neu5Ac derivatives.

In connection with the generation of a monoclonal antibody to free Neu5Ac for the purpose of establishing a simple and specific assay of Neu5Ac in serum and urine, a broad series of sialic acid α- and β-glycosides have been synthesized using substituted glycerol, substituted sphinganine and cholesterol as aglycons [470].

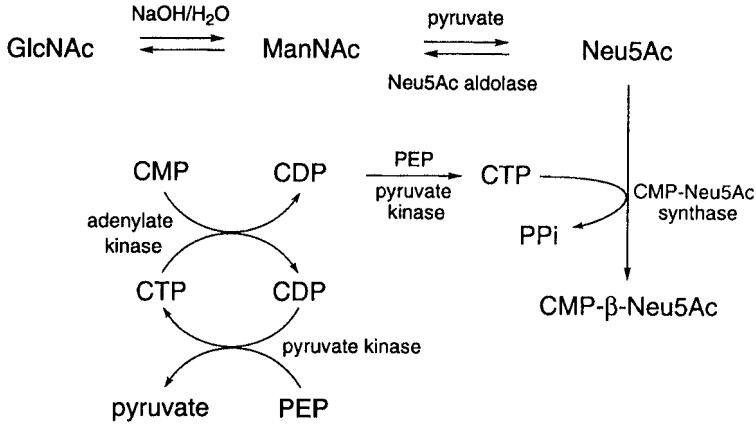


Fig. 10. One-pot synthesis of CMP-β-Neu5Ac from ManNAc and CMP [473]. PEP, phosphoenolpyruvate; PPi, pyrophosphate.

The synthesis of CMP-sialic acids is generally carried out enzymatically using CTP and CMP-sialic acid synthase as a catalyst [33,314,370,471,472]. A multigram-scale one-pot synthesis of CMP-β-Neu5Ac has been reported in ref. [473]. ManNAc, prepared by base-catalyzed epimerization of GlcNAc, was reacted with sodium pyruvate in the presence of Neu5Ac-aldolase to yield Neu5Ac (see section 6.1). For the formation of CMP-Neu5Ac, CTP was generated *in situ* from CMP by using adenylate kinase, pyruvate kinase, and phosphoenolpyruvate, and reacted with Neu5Ac in the presence of CMP-Neu5Ac synthase (Fig. 10). Instead of a one-pot synthesis, for practical reasons it is easier to generate and store crude solutions of Neu5Ac and CTP. For the use of GlcNAc in combination with GlcNAc-epimerase, see ref. [474]. Experiments with cloned CMP-Neu5Ac synthases from *E. coli* systems with Neu5Ac and Kdn showed a high specificity for Neu5Ac, thereby suggesting that in this case the 5-acetamido group is critical [384]. Chemical syntheses of CMP-Neu5Ac, applying the phosphoramidite method [475] or using sialyl phosphites [476], have also been described. Furthermore, a synthetic approach for the preparation of CMP-Neu5Gc based on the phosphite method has appeared [477].

In addition to CMP-β-Neu5Ac, CMP-β-Neu5,9Ac_2, CMP-β-Neu5Gc, and CMP-β-Kdn, a large series of artificial CMP-sialic acids have been prepared biochemically on microscale starting from the corresponding sialic acid (see references cited in Table 13) and CTP. Among them are CMP-9azido-Neu5Ac, CMP-9amino-Neu5Ac, CMP-9NAc-Neu5Ac and other C9-modified CMP-sialic acids, CMP-Neu5AcNH_2, CMP-Neu5Ac4Me, and CMP-4d-Neu5Ac [33,314,350,390,407,419,478]. The CMP-sialic acids have found a broad application in enzymatic sialylations using different sialyltransferases (see sections 6.3 and 8.3). Several of the artificial CMP-sialic acids turned out to be suitable donors for asialo-α_1-acid glycoprotein as acceptor with Gal(β1–4)GlcNAc α-2,6-sialyltransferase from rat liver as a biocatalyst [316,402,417]. The transfer of CMP-9amino-Neu5Ac is of considerable interest, as α-linked 9amino-Neu5Ac in sialoglycoconjugates is not a substrate for bacterial, viral or mammalian sialidases tested so far. CMP-9amino-Neu5Ac and CMP-Neu5AcNH_2 have also been used as synthons

to prepare fluorescent and photoactivatable analogues [419]. Because of the defined acceptor specificity, sialyltransferases in combination with fluorescent or photoactivatable donor CMP-sialic acids are excellent tools for selective introduction of a fluorescent or photoactivatable substituent to a distinct glycoconjugate. The latter reference [419] also includes kinetic data and information concerning the fluorimetric sialyltransferase assay. Typical fluorescent products comprise the CMP-sialic acids of 5-*N*-acetyl-9-deoxy-9-(3-fluoresceinylthioureido)-neuraminic acid (CMP-9fluoresceinyl-Neu5Ac), 5-*N*-acetyl-9-(7-amino-4-methylcoumarinyl)acetamido-9-deoxy-neuraminic acid (CMP-9AMCA-Neu5Ac), 5-*N*-acetyl-9-deoxy-9-(fluoresceinylaminomonochlorotriazinyl)amino-neuraminic acid (CMP-9MTAF-Neu5Ac), and *N*-(3-fluoresceinylthioureido-acetyl)neuraminic acid (CMP-Neu5fluoresceinyl). In the preparation of photoactivatable derivatives the $NH_2$9 group of CMP-9amino-Neu5Ac has been substituted with a 4-azidobenzoyl, a 4-azidosalicyl, a 4-benzoylbenzoyl or a 4-azido[T]benzoyl group. In a similar way, CMP-Neu5AcNH_2 has been labeled with a 4-azidobenzoyl group. Of special interest is the recently reported chemical synthesis of CMP-{Neu5Ac(α2–8)Neu5Ac} [479]; an attempt to prepare this compound biosynthetically with Neu5Ac(α2–8)Neu5Ac and CMP-sialic acid synthase failed so far [472]. In addition to the preparation of regular CMP-sialic acids, synthetic approaches have been worked out for the organic synthesis of a *S*-(*N*-acetylneuraminyl)nucleoside analogue [480,481] and other CMP-sialic acid variants [482].

For the immobilization of sialic acids on Sepharose solid supports, which provides potentially useful affinity materials, see the references cited in ref. [344]. The preparation of an affinity adsorbent with immobilized sialic acid through a thioglycosidic linkage has been described in ref. [483]. Synthetic sialidase-stable α-Neu5,9Ac_2 *p*-aminophenylthio glycoside has been immobilized directly or by a six-carbon long spacer group to agarose for lectin isolations [382]. The allyl glycoside of α-Neu5Ac has been applied as a starting material for the synthesis of Neu5Ac-neoglycoproteins and pseudopolysaccharides. These polymers containing multivalent sialic acid are in principle useful for various applications related with recognition/binding/inhibition processes. Reductive ozonolysis of the allyl group (O_3, then Me_2S), followed by coupling of the formed aldehyde to protein carriers (ε-aminogroup of lysine) by sodium cyanoborohydride-mediated reductive amination, yielded neoglycoproteins with varying amounts of Neu5Ac [484,485]. Copolymerization of the allyl glycoside with acrylamide generated a water-soluble pseudopolysaccharide [484]. In order to create a longer spacer arm for copolymerization with acrylamide, the allyl glycoside was converted into a 3-(2-aminoethylthio)propyl glycoside by reaction with cysteamine hydrochloride, after which the amino function was *N*-acryloylated [486]. The same principle of conjugation or copolymerization via *N*-acryloyl groups was also used for the preparation of sialo-oligosaccharide-neoglycoproteins and copolymers of sialo-oligosaccharides and acrylamide [487]. Using the strategy of reductive amination, *p*-formylphenyl glycoside of α-Neu5Ac was also conjugated with proteins [488], and starting from the *p*-nitrophenyl O- and S-glycosides, *p*-*N*-acryloylamino analogues were synthesized, which could be copolymerized with acrylamide, yielding water-soluble pseudopolysaccharides with Neu5Ac and acrylamide in different ratios [489], or directly coupled with polylysine [490]. Using trimethyl orthoacetate, the Neu5Ac residue of the

S-glycoside-containing polymer was converted into Neu5,9Ac_2 [489]. Finally, a series of interesting Neu5Ac and Neu5,9Ac_2-based dendrimers have been synthesized [491].

6.3. Sialo-oligosaccharides

The organic synthesis of oligosaccharides having terminal α-linked sialic acid has proved to be highly complex. The specific difficulties arise from three factors inherent in the sialic acid molecule. First, the carboxylic acid function at the anomeric center (C2) electronically disfavors oxonium ion formation. Secondly, from a steric point of view, the carboxyl function restricts the glycoside formation. Thirdly, the presence of a neighboring methylene group in the ring (C3), instead of a substituted carbon atom, eliminates the possible assisting and/or directing effect of an adjacent substituent [346]. This means that side reactions can be relatively important, mainly the thermodynamically favored β-glycoside and 2,3-dehydro-derivative formation, and low yields are quite often obtained. Initially, the synthesized glycosidic linkages comprised mainly Neu5Ac(α2–6)Gal(β1–, Neu5Ac(α2–6)GlcNAc(β1–, Neu5Ac(α2–6)Glc(β1–, Neu5Ac(α2–3)Gal(β1–, Neu5Ac(α2–3)GlcNAc(β1– [344], and over the years these glycosidic linkages, together with Neu5Ac(α2–6)GalNAc(α1–, still receive most of the attention. In view of the desire to prepare biologically relevant carbohydrate chains, this is understandable.

In Fig. 9 a series of typical Neu5Ac donors, introduced by different research groups with the aim of increasing both the glycosidation yield and the α-stereoselectivity, is depicted [339,346–348]. For each class of donors, some further information is presented in the following paragraphs.

The oldest approach of synthesizing sialo-oligosaccharides is the one starting from 2-deoxy-2-halo-β-Neu5Ac derivatives (Fig. 9, structure A). Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-chloro-2,3,5-trideoxy-D-*glycero*-β-D-*galacto*-non-2-ulopyranosonate, X = Cl, Y = Me, turned out to be a particularly useful donor [492], and typical promoters are silver and mercury salts. Due to poor stereoselectivity, α,β-glycoside mixtures are generally obtained, and HCl-elimination from the donor is a major side reaction. The reaction with secondary hydroxy groups in particular gave rise to problems. In the case of the aim to prepare Neu5Gc-containing oligosaccharides, also the *N*-glycolyl group in the donor analogue is *O*-acetylated [493]. For the synthesis of Neu5Ac(α2–9)Neu5Ac, see ref. [494].

In another approach, a series of 3-substituted Neu5Ac donors was prepared, starting from peracetylated [495] or perbenzylated [496,497] Neu2en5Ac methyl ester, thereby making use of the highly reactive 2,3-double bond to form adducts (Fig. 9, structures B–F) (see also ref. [498]). In the case of structure B as donor with silver triflate as a promoter, only β-glycosidic linkages were created, and among several products, the Neu5Ac(β2–8)Neu5Ac linkage was synthesized [495]. Structure C yielded mainly β-glycosidic linkages. From structure D, only the bromo variant is effective, although α,β-glycoside mixtures are still formed. The bromo variant of structure D with silver triflate as a promoter has been applied in the synthesis of Neu5Ac(α2–8)Neu5Ac and Neu5Ac(α2–9)Neu5Ac linkages [499,500]. Structures E and F form another series of donors, and E with X = Br and Y = SPh (mercury salts as promoter) has been shown to give particularly high glycosidation yields and α-stereoselectivity.

A third type of donor involves the use of *S*-methyl or *S*-phenyl α-glysosides (Fig. 9, structure G) [498,501–503]. Initially developed to synthesize S-glycosides, making use of sodium salts of the peracetylated Neu5Ac methyl ester α- or β-thioglycosides and suitable protected bromides [504–506] (see also refs. [507,508]), this type of donors has shown to be highly attractive in O-glycosidation reactions. In these couplings, frequently used promoters are dimethyl(methylthio)sulfonium triflate or *N*-iodosuccinimide/triflic acid [347]. The choice of the solvent system is very important, as it greatly influences the stereoselectivity; e.g. acetonitrile gives mainly α-glycosidation. In addition to reports dealing with the synthesis of many monosialo-oligosaccharides, including those with a Neu5Ac(α2–2)Glc, a Neu5Ac(α2–3)GlcNAc, and a Neu5Ac(α2–3)GalNAc sequence [509], typical examples are the creation of Neu5Ac(2–9)Neu5Ac [510,511] and Neu5Ac(α2–8)Neu5Ac [512] linkages, as well as sialyl Le^x sequences (native and variants) (ref. [513] and references cited therein). In this context, it is also interesting to note that several syntheses of sialo-oligosaccharides include the use of a separately prepared disaccharide donor with a terminal α-linked sialic acid [514]. Although in general the donors contain a *N*-acetyl group at C5, other examples have been reported with the phthaloyl (benzeneselenenyl triflate as a promoter [515]) or the *tert*-butoxycarbonyl function as N-protecting group, e.g. in the case of the synthesis of Neu-containing glycoconjugates [516,517]. Following similar routes, Kdn-containing oligosaccharides have also been synthesized [518]. As a variation on this theme, the application of *S*-sialyl xanthates (SCSOEt) as donors in sialo-oligosaccharide synthesis has led to interesting results, including a high α-stereoselectivity [519–522]. Here, also the use of *O*-benzoyl protection instead of *O*-acetyl protection has been proposed [523,524]. Furthermore, the preparation of sialic acid S-glycosyl donors employing *S*,*S*′-bis(1-phenyl-1*H*-tetrazol-5-yl)dithiocarbonate should be mentioned [525]. Another efficient donor combines parts of the structures F and G, yielding an α-thioglycoside with a SPh substituent at C3 (Fig. 9, structure H) [526]. The high stereoselective α-sialylation was obtained using either methyl sulfenyl bromide/silver triflate or *N*-iodosuccinimide/triflic acid as promoters.

Sialyl phosphites with trimethylsilyl triflate as a promoter have additionally been shown to be of practical use (Fig. 9, structure I), affording good yields and α-stereoselectivity [527–529], and examples include the synthesis of sialyl Le^x sequences [528]. For a detailed study on the evaluation of different sialyl phosphites, see ref. [530].

For the organic synthesis of sialo-oligosaccharides with di- or trimeric Neu5Ac elements, specific glycosyl donors have been prepared directly from Neu5Ac(α2–8)Neu5Ac or Neu5Ac(α2–8)Neu5Ac(α2–8)Neu5Ac [531–533]. Treatment of the free oligosaccharides with H^+-resin in methanol, followed by *O*-acetylation and subsequent replacement of the anomeric acetoxy group by a phenylthio function yielded the corresponding peracetylated methyl ester phenyl 2-thioglycosides, in which Neu5Ac residues are linked via a (α2–8,1–9) lactone ring (Fig. 9, structure J for a disialosyl donor). For additional data with respect to the preparation of dimeric donors with structure A at the reducing site, see ref. [534].

In terms of preparative chemistry, the use of CMP-β-Neu5Ac as a glycosyl donor and (cloned) α-2,3/6-sialyltransferases as biocatalysts have achieved a permanent position in the planning of synthetic routes for sialo-oligosaccharide chains; see e.g. refs. [314,342, 345,351,472,473,535–544]. Especially in the field of the preparation of sialyl Le^x frag-

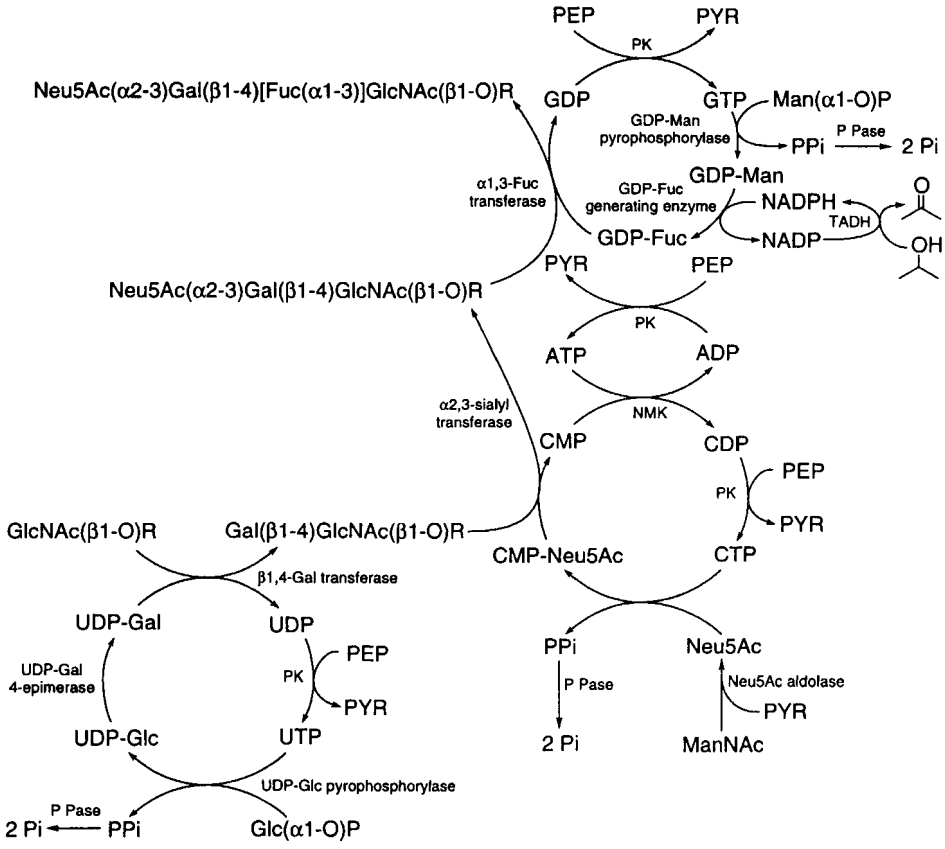


Fig. 11. Chemo-enzymic synthesis of the sialyl Le^x sequence [541]. NMK, nucleoside-monophosphate kinase; PEP, phosphoenolpyruvate; PK, pyruvate kinase; PPase, inorganic phosphatase; Pi, phosphate; PPi, pyrophosphate; PYR, pyruvate; TADH, *Thermoanaerobium brockii* alcohol dehydrogenase.

ments, excellent results have been obtained. To illustrate the enzymatic conversions, a series of typical examples will be presented. A first comprehensive example comprises the enzymatic sialylation on a microscale of oligosaccharides containing Gal(β1–3)GlcNAc, Gal(β1–4)GlcNAc, Gal(β1–4)Glc, and Gal(β1–3)GalNAc sequences by using different purified mammalian sialyltransferases [342], creating Neu5Ac(α2–6)Gal, Neu5Ac(α2–3)Gal, or Neu5Ac(α2–6)GlcNAc linkages, as verified by ^{1}H and ^{13}C NMR spectroscopy (see section 5.3.5). The generation of the Neu5Ac(α2–6)GalNAc(α1–O) sequence on a microscale has been described in ref. [536]. A second example (Fig. 11) is the chemo-enzymatic synthesis on a preparative scale of Neu5Ac(α2–3)Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–O)$CH_2CH{=}CH_2$ (and analogues) using β-1,4-galactosyltransferase and recombinant α-2,3-sialyltransferase and α-1,3-fucosyltransferase with *in situ* regeneration of UDP-Gal, CMP-Neu5Ac and GDP-Fuc [541]. A third example is the one-pot enzymatic synthesis of Neu5Ac(α2–6)Gal(β1–4)GlcNAc (and analogues) based on a β-galactosidase-catalyzed galactosylation, using lactose as a donor and GlcNAc as

an acceptor, and a pig liver α-2,6-sialyltransferase-catalyzed sialylation with *in situ* regeneration of CMP-Neu5Ac [542]. A fourth example is the enzymatic synthesis of Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–O)pent-4-ene, a precursor for the organic chemical synthesis of higher oligosaccharides [472]. The trisaccharide was synthesized starting from GlcNAc(β1–O)pent-4-ene, UDP-Gal (*in situ* generated from UDP-Glc catalyzed by UDP-Gal 4-epimerase), and Neu5Ac in a one-pot reaction employing β-1,4-galactosyltransferase and α-2,6-sialyltransferase in a complete cofactor regeneration system. The availability of specific sialyltransferases will certainly contribute to a further expansion of this area. In this context, the recent finding of a novel sialyltransferase which catalyzes the transfer of Kdn from CMP-Kdn to the non-reducing termini of oligo/polysialyl chains, thereby capping a further elongation of a $\{Neu5Gc(\alpha2–8)\}_n$ chain, is of interest [545]. In the framework of the finding that two Neu5Ac(α2–6)Gal(β1–4)GlcNAc units are the receptor determinants for the influenza virus hemagglutinin, these elements have been systematically anchored on a Gal residue in order to design structures capable of bimodal viral binding, and along chemo-enzymatic routes heptasaccharides with the general formula Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–*x*)[Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–*y*)]Gal(β1–O)$(CH_2)_5$COOMe, where *x* and *y* are 2 and 3, 2 and 4, 2 and 6, 3 and 6, and 4 and 6, respectively, have been synthesized [546]. The concept of preparing compounds with a multivalent presentation of Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1– fragments on a linear or branched (via lysine) peptide backbone has been nicely worked out in ref. [547]. After the organic synthesis of a large series of peptide backbones in which GlcNAc(β1–N)Asn units were incorporated, the oligosaccharide extensions were performed enzymatically by using β-1,4-galactosyltransferase and α-2,6-sialyltransferase.

Neu5,9Ac_2-containing oligosaccharides have been prepared along both organic chemical and enzymatic routes. In a reaction with trimethyl orthoacetate, Neu5Ac(α2–6)-Gal(β1–4)Glc could be readily converted into Neu5,9Ac_2(α2–6)Gal(β1–4)Glc [380]. Employing CMP-Neu5,9Ac_2 and immobilized porcine liver Gal(β1–4)GlcNAc α-2,6-sialyltransferase, Neu5,9Ac_2(α2–6)Gal(β1–4)GlcNAc has been synthesized [478]. The Neu5Ac4Me-, Neu5Ac9Me-, and 8epi-Neu5Ac-thioglycoside donors have been used to synthesize the corresponding sialoglycoconjugates [548], whereas also thioglycoside donors of 4d-, 7d-, 8d-, and 9d-Neu5Ac have been prepared [549].

In other enzymatic approaches, use has been made of a β-D-galactoside α-2,3-*trans*-sialidase from *Trypanosoma cruzi* as biocatalyst (see also section 9.2.3). This enzyme catalyzes the reversible transfer of Neu5Ac from a donor-substrate of the sequence Neu5Ac(α2–3)Gal(β1–O)R_1 to virtually any Gal(β1–O)R_2 acceptor substrate, affording a product Neu5Ac(α2–3)Gal(β1–O)R_2 [550–552] (Fig. 12). A frequently used donor substrate is Neu5Ac(α2–3)Gal(β1–4)Glc. In the case of Gal(β1–3/4)GlcNAc(β1–O)R sequences, the GlcNAc unit should not be substituted with a Fuc residue, as in Le^x or Le^a determinants [551]. The *trans*-sialidase reaction has been used in the chemo-enzymatic preparation of a water-soluble polyacrylamide, bearing multivalent Neu5Ac(α2–3)Gal(β1–4)GlcNAc elements [553]. To solve the problem of the poor enzymatic α-2,3-sialylation of Gal 2-(trimethylsilyl)ethyl β-glycoside using all known α-2,3-sialyltransferases and CMP-Neu5Ac, attention has been paid to the development of a sequence of enzymatic reactions, including cloned α-2,3-sialyltransferase and

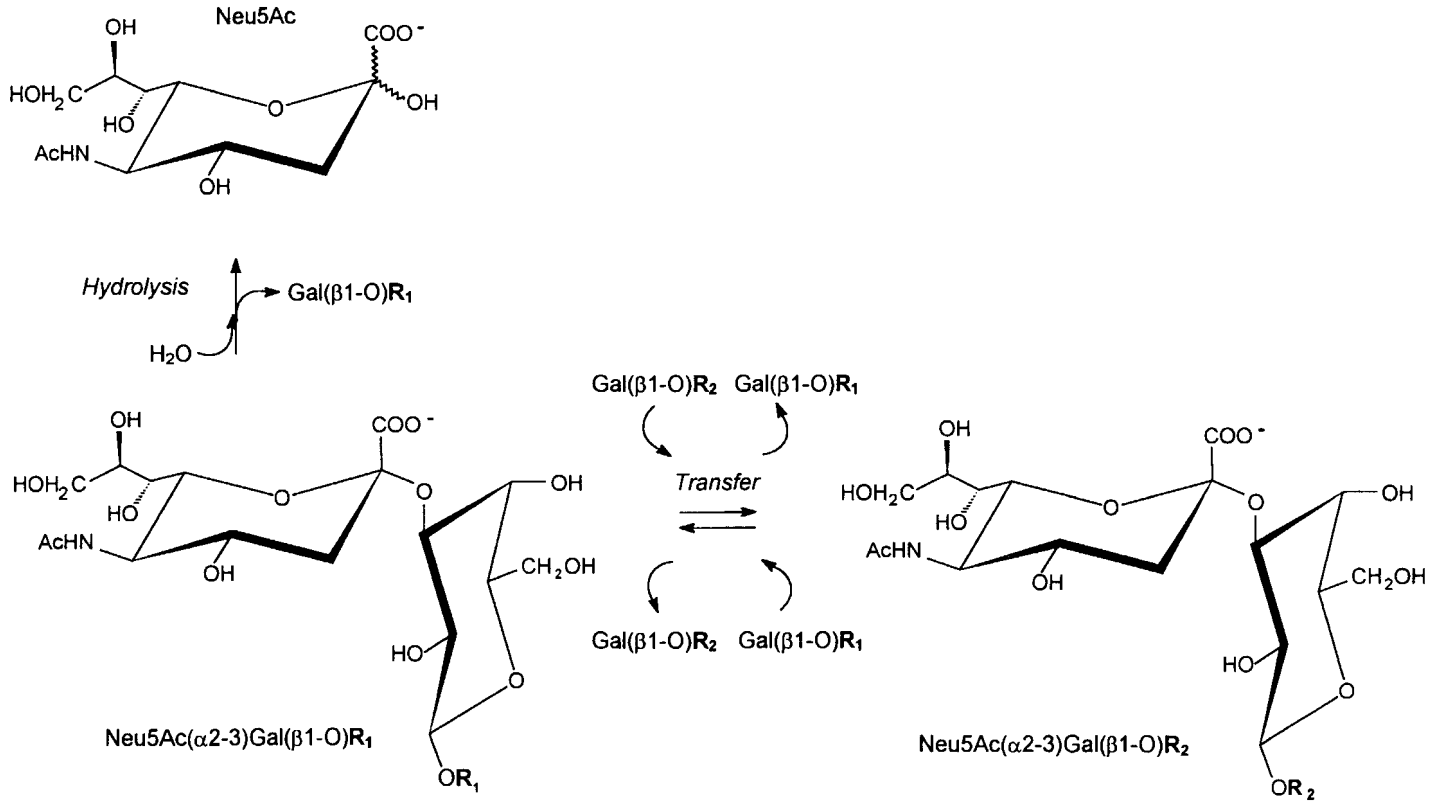


Fig. 12. Reactions catalyzed by *trans*-sialidases.

CMP-Neu5Ac synthase, yielding an alternative active sialyl-donor-substrate *in situ* (e.g. Neu5Ac(α2–3)lacto-*N*-tetraose), which can be used by *trans*-sialidase [554]. In this way it was possible to convert Gal 2-(trimethylsilyl)ethyl β-glycoside into Neu5Ac(α2–3)Gal 2-(trimethylsilyl)ethyl β-glycoside, a sialodisaccharide that can readily be transformed into a disaccharide donor, of interest for additional organic syntheses. In another study 4-MU-Neu5Ac was tested as a donor with lactose as acceptor [555]. Starting from periodate treated/reductive aminated 4-MU-Neu5Ac derivatives, interesting possibilities for the inclusion of fluorescent or photolyzable groups were demonstrated.

Bacterial sialidases have also been explored in synthetic approaches. In a reverse-enzyme reaction with *A. ureafaciens* sialidase, incubation of a concentrated solution of Neu5Ac and lactose yielded Neu5Ac(α2–6)Gal(β1–4)Glc and Gal(β1–4)[Neu5Ac(α2–6)]Glc [556]. Similar experiments were carried out with immobilized *V. cholerae* sialidase, using Neu5Ac *p*-nitrophenyl α-glycoside as a donor. In this way Neu5Ac(α2–*x*)Gal and Neu5Ac(α2–*x*)Glc linkages could be produced, in which the α-2,6-linkage dominated over the α-2,3-linkage [557]. Transglycosylation of a Neu5Ac unit using Neu5Ac(α2–8)Neu5Ac as a donor to Gal(β1–4)GlcNAc and Gal(β1–4)Glc was performed using sialidases of various origin [558]. Although the yields were low, a high regioselectivity was observed. The *C. perfringens, A. ureafaciens* and *V. cholerae* sialidases generated α-2,6-linkages, and the Newcastle disease virus sialidase α-2,3-linkages with the terminal Gal residue.

For detailed information with respect to the synthesis of C-glycosides of sialic acids, see refs. [559–563]. As an example, the synthesis of a multivalent material that consists of the C-glycoside of Neu5Ac, which is resistant to viral sialidase hydrolysis, should be mentioned [562].

7. *Conformational aspects of sialic acids*

Earlier studies have appeared on the X-ray crystallography of both crystalline β-Neu5Ac•H_2O and β-Neu5AcOMe•$1H_2O$ [56,564]. In an additional study, the crystal and molecular structure of α-Neu5AcOl,$2Me_2$ was also analyzed [565]. The C=O bond of the COOH function is approximately coplanar with the ring C–O bond in α-Neu5AcOl,$2Me_2$, whereas in both β-Neu5Ac and β-Neu5AcOMe the C=O bond is found to be nearly eclipsed with the anomeric C–O bond. In all three derivatives, the *N*-acetyl group is essentially planar, adopting the Z-conformation of a peptide bond. For α-Neu5AcOl,$2Me_2$ and β-Neu5AcOMe a hydrogen bond between the H-atom of HO7 and the carbonyl O-atom of AcNH5 was observed. The overall conformation of the glycerol side chain is the same for all three derivatives, as far as non-H atoms are concerned. In α-Neu5AcOl,$2Me_2$ a hydrogen bond between the H-atom of HO8 and the carbonyl O-atom of the COOMe group is detectable. One of the oldest NMR studies on the conformation of Neu5Ac is that focused on the spatial structure of αNeu5Ac2Me in D_2O [566,567]. On the basis of ^1H–^1H coupling constants in combination with ^{13}C spin–lattice relaxation times (T_1), a model could be constructed in which the amide H-atom of AcNH5 is hydrogen-bonded to O7, and the H-atom of HO8 is hydrogen-bonded to the ring-oxygen. A third hydrogen bond between the carbonyl O-atom of AcNH5

and the H-atom of HO4 was suggested on the basis of molecular model building. In this model, apparently, the anomeric center is not involved in any hydrogen bonding, leading to the same conformation for α- and β-anomers. Independent of the models discussed above, the results fit the observation made by ^{1}H NMR spectroscopy that HO7 and HO8 usually occur in a *trans*-orientation [53]. An NMR (H_2O-suppressed 1D TOCSY, ROESY, NOESY) study, carried out on Neu5Ac(α2–3)Gal(β1–4)GlcNAc and Neu5Ac(α2–6)Gal(β1–4)GlcNAc in 85% H_2O/15% $(CD_3)_2CO$, and aimed to detect hydroxyl and amido protons, indicated that in both compounds, thus irrespective of the type of linkage, the H-atom of HO8 of Neu5Ac is involved in a strong intramolecular hydrogen bond [568].

In view of the fact that 7epi-Neu5Ac and 7,8epi$_2$-Neu5Ac are substrates for CMP-sialic acid synthase (see section 6.1), but not 8epi-Neu5Ac [411], a conformational study on the side chain conformation of these sialic acids and Neu5Ac itself (all <10% α-anomer) was performed [569]. Use was made of ^{1}H and ^{13}C NMR spectroscopy, including NOE measurements, combined with hard-sphere calculations. From a comparison of the four side chain conformations in aqueous solution at neutral pH, it was concluded that the absence of activation of 8epi-Neu5Ac may be due to diminished binding interactions between the hydrophobic and hydrophilic regions of this sialic acid and the activating enzyme, when compared with the other three sialic acids. In additional studies 7d-, 8d-, and 9d-Neu5Ac and 4,7d$_2$-Neu5Ac were also investigated [570].

Nowadays, much attention is paid to the conformational analysis of glycoprotein glycans, including combinations of NMR spectroscopy and molecular modelling programs, and with respect to sialic-acid-containing fragments the sequences Neu5Ac(α2–6)Gal(β1–4)GlcNAcβ1–, Neu5Acα2–3Galβ1–4GlcNAcβ1–, and Neu5Acα2–3Galβ1–4[Fuc(α1–3)]-GlcNAcβ1– in particular are currently studied in detail by many research groups. As this aspect is outside the scope of this chapter, no details will be presented. In relation to glycoprotein glycans containing polysialyl elements, conformational studies on the polysaccharides of *N. meningitidis* B, $\{Neu5Ac(\alpha2–8)\}_n$, and *N. meningitidis* C, $\{Neu5Ac(\alpha2–9)\}_n$, are of interest [571–575]. Conformation and solution dynamics studies of the B- and C-polysaccharides, using NMR spectroscopy, showed an antiperiplanar orientation of H7 and H8 in the C-polysaccharide and a gauche orientation in the B-polysaccharide [571]. In a further NMR study on $\{Neu5Ac(\alpha2–8)\}_n$ it was found that both linkages in $\{Neu5Ac(\alpha2–8)\}_3$ differed in conformation from each other and from the inner linkages of the polymer colominic acid. These conformational differences extend to both terminal disaccharides of oligosaccharides larger than $\{Neu5Ac(\alpha2–8)\}_5$. It could be demonstrated that at least 5 residues are required before a linkage with a similar orientation to that of the internal α2,8-linkages of colominic acid is generated [572]. In this context, it is interesting to note that $\{Neu5Ac(\alpha2–8)\}_{10}$ at the minimum is required to bind to B-polysaccharide specific antibodies, which means that the immunologically functional moiety of the decamer is formed by the inner 6 residues. Finally, in more recent studies the helical character of $\{Neu5Ac(\alpha2–8)\}_n$ in solution has been studied, and ordered helical structures [573] as well as an inherently flexible structure, containing a wide range of energetically favorable helices, one of which is an extended helix where $n \approx 9$ [574], has been proposed.

8. *Biosynthesis of sialic acids*

8.1. General

Cells from higher animals and various microorganisms produce sialic acids in a long pathway starting from glucose [5,8,33]. Only mammalian erythrocytes, which have lost their nucleus during maturation, can no longer synthesize sialic acids, although they are still sialylated on their surface. The presence of sialic acids on the outer cell membrane is considered to be a prerequisite for the viability of mammalian cells, at least in tissues or fluids of organisms. After the loss of cell surface sialic acids by sialidase, these monosaccharides are rapidly restored [576].

Since the last reviews on sialic acid metabolism [5,33], little progress has been made on the enzymatic and regulatory mechanisms as well as on the molecular biology involved in the biosynthesis of *N*-acetylneuraminic acid (Neu5Ac). However, further insight has been gained into the biosynthesis, degradation and role of modified sialic acids, especially *N*-acetyl hydroxylation, *O*-acetylation and *O*-methylation. An overview on the reactions involved, including catabolic enzymes, is shown in Fig. 13. The reactions, subcellular site, regulation, pathobiochemical role and molecular genetics of the enzymes activating sialic acids with CTP and transferring them onto nascent glycoconjugate molecules have also been intensively studied in animal and bacterial cells and much progress has been made. These new aspects of sialic acid metabolism will be discussed below.

The regulation of sialic acid biosynthesis and consequently the sialylation of glycoproteins and gangliosides of cells seem to be governed in a rather variable manner, resulting in characteristic qualitative and quantitative differences during cell differentiation, growth, functional changes, ageing and malignant transformation of cells. Details will be given in section 10 on the biological role of sialic acids.

In order to influence these processes, inhibitors of sialic acid biosynthesis or breakdown e.g. by sialidases are required. Various metal ions such as Zn^{2+}, Cu^{2+} and selenite were shown to inhibit enzymes of sialic acid biosynthesis in rat liver homogenates [578]. They impair the activity of the key regulatory enzyme UDP-*N*-acetylglucosamine-2′-epimerase. In the same tissue, 3-*O*-methyl-*N*-acetyl-D-glucosamine efficiently inhibits *N*-acetylglucosamine and *N*-acetylmannosamine kinases involved in Neu5Ac formation [579]. Consequently, the incorporation of *N*-acetylhexosamines into sialic acids in human hepatoma cells was reduced. After intraperitoneal injection of *N*-propanoyl-D-hexosamines, *N*-propanoylneuraminic acid could be isolated from tissues at different relative amounts, showing that the enzymes for sialic acid biosynthesis and transfer can tolerate changes in the *N*-acetyl moiety of precursor acylhexosamines [580]. Other inhibitors specific for sialic acid enzymes will be mentioned as appropriate in the text.

8.2. Biosynthesis of CMP-sialic acids

The enzyme CMP-Neu5Ac synthase, which is also known as Neu5Ac cytidylyltransferase (EC 2.7.7.43), is ubiquitous in pro- and eukaryotic cells synthesizing sialic acids from glucose [5,33]. In contrast to other nucleotide-sugar-synthesizing enzymes, it is located

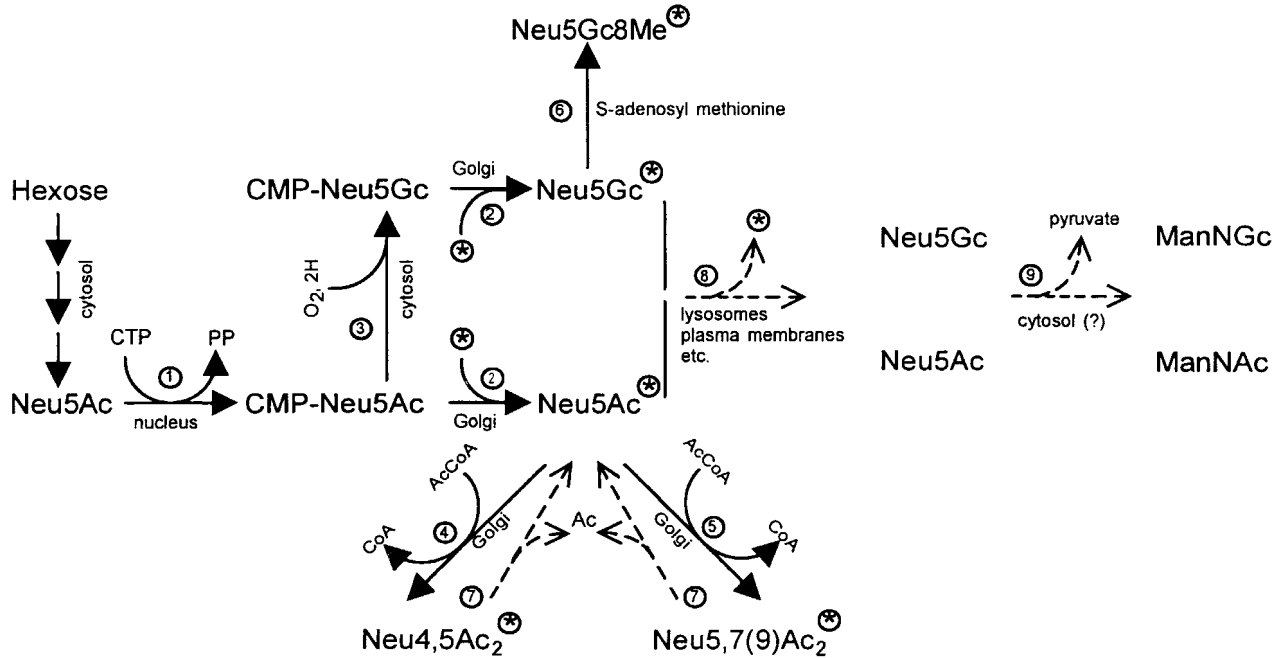


Fig. 13. Metabolism scheme of sialic acids. Anabolic (solid arrow) and catabolic (dashed arrow) reactions are indicated. For literature see the text. Enzymes: 1, CMP-sialate synthase (EC 2.7.7.43); 2, sialyltransferases (EC 2.4.99.1. ...); 3, CMP-Neu5Ac hydroxylase (EC 1.14.99.18); 4, acetyl-CoA:sialate 4-*O*-acetyltransferase (EC 2.3.1.44); 5, acetyl-CoA:sialate 7(9)-*O*-acetyltransferase (EC 2.3.1.45); 6, *S*-adenosyl-L-methionine:sialate 8-*O*-methyltransferase (proposed EC 2.1.1.78); 7, sialate 4- or 9-*O*-acetylesterases (EC 3.1.1.53); 8, sialidase (EC 3.2.1.18); 9, sialate-pyruvate lyase (aldolase; EC 4.1.3.3). Both Neu5Ac and Neu5Gc can be *O*-acetylated by the two *O*-acetyltransferases. There may also exist a sulfotransferase, since sulfated sialic acids have been found in e.g. echinoderms [13,577]. (*), Sialic-acid-accepting nascent glycoconjugate.

in the nucleus of mammalian cells, although it has not yet unequivocally been shown that CMP-Neu5Ac is really produced in this compartment [581]. It has been isolated from both animal and bacterial sources [5,33,582], and particularly large activities exist in frog [583] and trout liver [582], as well as in *Escherichia coli* [584,585]. The latter enzyme, which is expressed in *E. coli* K1 and K92 only at temperatures higher than 20°C, has been cloned and sequenced [586] and its molecular properties have been described. It exhibits 59% homology with the corresponding, cloned gene from *Neisseria meningitidis* [587, 588].

The availability of this enzyme is increasingly important, since CMP-Neu5Ac is required not only for sialylation of natural substances, but also for the combined chemical and enzymatic synthesis of sialylated complex carbohydrates in research and glycotechnology (see section 6.2 for details and ref. [589]). With the synthases from different sources it is possible to activate not only Neu5Ac, but also Neu5Gc, *O*-acetylated sialic acids and a variety of chemically prepared sialic acid derivatives (section 6.2). The transfer of non-natural sialic acids onto glycoconjugates may be useful for the study of sialic-acid-degrading enzymes and for various aspects of cell biology like cellular interactions and fluorescent labelling of cell surface glycoconjugates by CMP-9-fluoresceinyl-Neu5Ac [390,419]. The enzyme from trout also activates Kdn occurring as oligomers in this animal [107,122].

With regard to the reaction mechanism it has been shown with the *E. coli* synthase and ^{13}C-labelled Neu5Ac in NMR studies that CMP-β-Neu5Ac is probably formed by a direct transfer of the anomeric oxygen of β-Neu5Ac to the α-phosphate of CTP [584]. Activation of Neu5Ac is only possible, if the hydroxyl groups at C4 and C8 are in the correct, natural conformation [350]. Correspondingly, 4epi- and 8epi-Neu5Ac cannot be linked to CMP by the rat liver cytidylyltransferase. Furthermore, the enzyme requires the presence of a hydroxyl group at C8, since 8d-Neu5Ac is inactive. This is in contrast to 4d-Neu5Ac and 9d-Neu5Ac. Substituents at C4, such as in Neu5Ac4Me or Neu4,5Ac_2, are tolerated. The strong competitive inhibition effect of β-Neu5Ac methyl glycoside shows that the HO2 group interacts with the enzyme via the lone electron pair of the oxygen atom and not by a hydrogen bridge.

Little information is available on the active site of this enzyme. In the recombinant enzyme from *E. coli* two cysteine residues were recognized at the positions 129 and 329, one of which seems to be at or close to the active center, as was shown by inhibition with thiol-specific reagents partly in the presence of CTP. However, exchange of these cysteine residues for other amino acids by site-directed mutagenesis did not change the enzyme activity significantly [590].

Several cytidylyltransferases were shown to be inhibited by CMP and CDP [582]. The observation that 2′-CMP, 3′-CMP and 2′,3′-cyclic CMP do not inhibit shows that the phosphate residue at the 5′ position of ribose is required for inhibition. These studies also demonstrate that the ribose moiety must be intact for inhibition and that chemical modifications of the cytidine residue tested do not lead to inhibitors. An inhibitory effect of the methyl β-glycoside and the 4-oxo-derivative of Neu5Ac on the cytidylyltransferase from rat liver was described [408].

8.3. Transfer of sialic acids

Specific patterns of oligosaccharides as components of glycoproteins and glycolipids cover the surface of individual cell types and may change during variation of the function or differentiation of these cells. This "make up" of cells, which is formed by the expression of a set of glycosyltransferases [335,591–594] gains enormous diversity by the transfer of sialic acids into mostly terminal position of the oligosaccharide chains and in different linkages (see Tables 2–6). This great variety of structures may be even more enlarged by the modification of sialic acids, i.e. *N*-acetyl-hydroxylation, *O*-acetylation, *O*-lactylation, *O*-methylation or *O*-sulfation (see Table 1 for natural sialic acids).

Many studies have been carried out in the past few years on the transfer of sialic acids onto oligosaccharides, polysaccharides, glycoproteins and glycolipids mediated by sialyltransferases of animal cells and bacteria. These have given new insight into the wide occurrence and subcellular distribution of these enzymes comprising a family of over 10 members, their substrate specificities yielding α-2,3-, α-2,6-, α-2,8- and α-2,9-linkages, regulation of gene expression, and especially primary structures. Since these topics have been summarized in refs. [593,595] and are mentioned in several chapters of volume 29a of New Comprehensive Biochemistry series, here only a selection of some aspects of these enzymes mainly from the most recent literature will be reported and a list of cloned sialyltransferases (Table 14) is included. The isolation of CMP-Neu5Ac:Gal(β1–4)GlcNAc α-2,6- and Gal(β1–3/4)GlcNAc α-2,3-sialyltransferases from rat liver (ST6Gal I and ST3Gal III, respectively) by affinity chromatography has been described [624].

The sialyltransferase assay is usually carried out with radioactive CMP-Neu5Ac and the sialic acid acceptor, followed by identification of the radioactive product. As this product is often membrane-bound and the radioactive cosubstrate is expensive, a non-radioactive assay was developed [625], in which CMP released from CMP-Neu5Ac by the transfer of Neu5Ac is determined with HPLC (see section 5.3.2). Since CMP-Neu5Ac can be analyzed in the same run, this test is also suited for the activity of the CMP-Neu5Ac synthase described above.

With regard to acceptor specificity, the oligosaccharide sequences accepting sialic acids by the action of sialyltransferases are summarized in Tables 2–6. Although sialyltransferases exhibit strong linkage specificity, in some cases they seem to be involved in the sialylation of not one but several glycoconjugates [595]. In mitochondrial outer membranes, there exist two sialyltransferases with galactoside α-2,3- and α-2,6-specificities, which preferably sialylate O- and N-glycans, respectively, of glycoproteins [626]. CMP-Neu5Ac:Gal(β1–4)GlcNAc-R-α-2,6-sialyltransferase (ST6Gal) was found to sialylate the disaccharide unit GalNAc(β1–4)GlcNAc-R N-glycosidically linked to a variety of milk glycoproteins [627]. It is concluded from these studies that the same oligosaccharide moiety of a number of human glycoproteins is α-2,6-sialylated in a corresponding way by one sialyltransferase. With regard to tissue specificity, α-2,3- and α-2,6-sialyltransferase activities specific for Gal(β1–4)GlcNAc-R residues occur in both human liver and placenta [628].

In contrast to the acceptor specificity, the donor specificity of sialyltransferases is less pronounced, although differences exist. They can transfer not only Neu5Ac but

also other sialic acids from their CMP-glycosides, e.g. both the natural Neu5Gc and *O*-acetylated species as well as synthetic Neu5Ac derivatives, which have been mentioned in sections 6.2 and 8.2. This is of great importance for cell biological studies.

In order to gain more insight into the structural requirements of the acceptor for rat liver sialyltransferases, the residues in the acceptors Gal(β1–3)GlcNAc and Gal(β1–4)GlcNAc were modified mainly by deoxygenation of the pyranose rings to identify the key polar groups required for transfer [629]. Both the HO6 group of the β-Gal residue and the *N*-acetyl group of the GlcNAc unit are required for the activity of α-2,6-sialyltransferase (ST6Gal I). The α-2,3-sialyltransferase (ST3Gal III) requires the HO3, HO4 and HO6 groups of terminal β-Gal, and some influence from the subterminal sugars was noticed. Structural variants of the Gal residue can also be recognized by rat liver Gal(β1–4)GlcNAc α-2,6-sialyltransferase. Besides Gal(β1–4)GlcNAc(β1–O)Me and GalNAc(β1–4)GlcNAc(β1–O)Me, also Man(β1–4)GlcNAc(β1–O)Me, Glc(β1–4)GlcNAc(β1–O)Me and GlcNAc(β1–4)GlcNAc(β1–O)Me turned out to be acceptors, as proven by ^{1}H NMR spectroscopy [630]. In a more detailed systematic study the substrate specificity of α-2,3- and α-2,6-sialyltransferases was explored using a series of synthetic Hex(1–4)GlcNAc(β1–2)Man(α1–O)$(CH_2)_7CH_3$ acceptors with Hex = β-D-Gal, 4-deoxy-β-D-Gal, 4-*O*-methyl-β-D-Gal, 4-deoxy-4-fluoro-β-D-Gal, β-D-Glc, 3-*O*-methyl-β-D-Gal, 3-deoxy-β-D-Gal, 3-deoxy-3-fluoro-β-D-Gal, 3-amino-3-deoxy-β-D-Gal, β-D-Gul, α-L-Alt and β-L-Gal [630a]. These reactions are relevant to the finding of unusual sialic-acid-containing structures, namely Neu5Ac(α2–6)Man(β1–4)GlcNAc in the urine of patients with β-mannosidosis [153]. They now enable the synthesis of unusual sialic-acid-containing carbohydrate sequences.

Sialyltransferases may be glycoproteins and the carbohydrate chain can modify enzyme activity. Thus, deglycosylation of rat liver Gal(β1–4)GlcNAc α-2,6-sialyltransferase protein markedly decreases enzyme activity, showing that the trimannosyl-*N,N'*-diacetylchitobiose core with GlcNAc attached is particularly important for the expression of catalytic activity [631]. Glycosylation of α-2,6-sialyltransferase from rat hepatoma cells with *N*-acetyllactosamine- and oligomannose-type sugar chains was also reported [632].

The polypeptide part of an acceptor glycoprotein may also influence the activity of sialyltransferase, as was shown for desialylated human chorionic gonadotropin and bovine colostrum CMP-Neu5Ac:Gal(β1–4)GlcNAc-R α-2,6-sialyltransferase [633]. When compared with the heterodimeric form of this hormone, the glycans of the α-subunit exhibited a higher kinetic efficiency (V/K_M). In contrast, the branch specificity of this enzyme, i.e. the preference for the Gal(β1–4)GlcNAc(β1–2)Man(α1–3)Man branch for attachment of the first sialic acid molecule into the diantennary glycans of desialylated gonadotropin was not influenced.

Interestingly, evidence was obtained that α-2,3-sialyltransferase from cultured C6 glioma cells [634] must be phosphorylated for activity and it is postulated that enzyme activity is regulated by the action of Ca^{2+}/calmodulin and phosphatase, respectively.

We are only beginning to understand the regulation of the expression of sialyltransferase activity. The higher level of sialylation of many tumor cells and increased sialyltransferase activities are well known (see chapter 3 of Vol. 30 and section 10.5 below]. Transformation of FR3T3 cells with the c-Ha-ras oncogene resulted in a marked increase of the expression of β-galactoside α-2,6-sialyltransferase activity and,

Table 14
Sialyltransferases with known gene structures[a]

Abbreviations	Structures synthesized	Tissue	Ref.	Comments
Mammalian enzymes				
ST3Gal I	**Sia(α2–3)**Gal(β1–3)GalNAc-R	porcine submand. gland	[596]	acts on O-linked chains and glycolipids
		mouse brain	[597]	
		chick embryo	[598]	
ST3Gal II	**Sia(α2–3)**Gal(β1–3)GalNAc-R	mouse and rat brain	[599,600]	similar to ST3Gal I, but seems to act better on glycolipids than ST3Gal I; kinetics see ref. [600]
ST3Gal III	**Sia(α2–3)**Gal(β1–3/4)GlcNAc-R	rat liver	[601]	acts preferentially on Gal(β1–3)GlcNAc
		human placenta	[602]	
		mouse brain	[603]	
ST3Gal IV	**Sia(α2–3)**Gal(β1–3)GalNAc-R and **Sia(α2–3)**Gal(β1–4)GlcNAc-R	human melanoma, placenta, mouse brain	[603–605]	also called STZ or SAT-3, acts preferentially on O-glycans [Gal(β1–3)GalNAc] and on N-glycans [Gal(β1–4)GlcNAc], also on glycolipids
ST6Gal I	**Sia(α2–6)**Gal(β1–4)GlcNAc-R	rat liver	[606]	acts on N-glycans, possibly also on lacto-neo-glycolipids
		human placenta	[607]	
		mouse liver	[608]	
		chick embryo	[609]	
ST6GalNAc I	**Sia(α2–6)**GalNAc-R	chick embryo	[610]	
		mouse submand. gland	N. Kurosawa, pers.commun.	
ST6GalNAc II	**Sia(α2–6)**[Gal(β1–3)]GalNAc-R	chick testis	[611]	requires Gal(β1–3)GalNAc as substrate, does not act on GalNAc-R
		human epithelial cells	[612]	
		mouse	[612a]	
ST8Sia I	**Sia(α2–8)**Sia-R (glycolipid)	human melanoma	[613–615]	synthesizes GD3 ≫ GT1a and GQ1b
		mouse brain	[616]	

continued on next page

Table 14, *continued*

Abbreviations	Structures synthesized	Tissue	Ref.	Comments
ST8Sia II	**Sia(α2–8)**Sia-R	rat brain	[617]	also known as STX, specificity see ref. [618]
		human fetal brain	[619]	human homologue to STX [617], partial cDNA-clone
		mouse brain	[618]	murine homologue to STX [617], acts on N-glycans
ST8Sia III	**Sia(α2–8)**Sia-R	mouse brain	[616]	acts on N-glycans, synthesis of GD3 and GT3
ST8Sia IV	**Sia(α2–8)**Sia-R (NCAM)	Chinese hamster fibroblasts	[620]	synthesis of polysialic acid on NCAM
		mouse lung	[621]	acts on α-2,3-linked Sia of N-glycans
ST8Sia V	**Sia(α2–8)**Sia-R	mouse brain	[621a]	acts on glycoprotein
Bacterial enzymes				
PST	**Sia(α2–8)**Sia-R on Sia-polymers	*Neisseria meningitidis* group B	[622]	
PST	**Sia(α2–8)**Sia-R on Sia-polymers	*Escherichia coli* K1	[622]	
PST	**Sia(α2–8)**Sia-R and **Sia(α2–9)**Sia-R on Sia-polymers	*Escherichia coli* K92	[623]	synthesis of alternating α-2,8- and α-2,9-linkages

[a] With regard to nomenclature, ST stands for sialyltransferase, 3, 6 and 8 for the linkage formed, Gal or Sia for the monosaccharide carrying the acceptor hydroxyl group, and Roman numbers (I–V) for the chronological order of published primary structures. The species designation should be given as a single letter at the beginning (e.g. pSTGal I for the porcine, mSTGal I for the murine and cSTGal I for the chicken enzyme). This nomenclature follows suggestions by Tsuji et al. [623a]. PST stands for bacterial polysialyltransferases. In the structures synthesized, Sia is used, since the sialyltransferases may transfer not only Neu5Ac, but also other natural as well as synthetic sialic acids (see the text). As an example, the systematic name of ST3Gal I is CMP-Neu5Ac:Gal(β1–3)GalNAc α-2,3-sialyltransferase (EC 2.4.99.4). However, this name would also be used for ST3Gal II and ST3Gal IV.

Table 15
Natural and synthetic inhibitors of sialyltransferases[a]

Inhibitor	K_i values (mM)			Ref(s).
	ST6GalI[b]	ST3GalI[c]	Lactosyl-ceramide α-2,3-sialyl-transferase[d]	
5′-CMP	0.090	0.064	n.t.[e]	[642]
5′-CDP[f]	0.050	0.050	n.t.	[642]
5′-CTP	0.046	0.060	n.t.	[642]
Cytidine	0.13	22	n.t.	[642]
2-Thiocytidine	0.15	n.i.[g]	n.t.	[642]
CMP-dialdehyde[h]	3.3	5.7	0.085	[642,643]
UDP-dialdehyde	n.t.	n.t.	0.22	[643]
6′-deoxy-lactosaminide[i]	0.76			[644]
6′-thio-lactosaminide	3.78			[644]
6′-disulfide-lactosaminide	2.00			[644]
Protein (20 kDa; calf brain)	80%[j]			[642,645]
Protein (14.8 and 22.4 kDa; rat brain)	50%[k]			[642,646]

[a] For sialyltransferase nomenclature see Table 14.
[b] From rat liver.
[c] From porcine submandibular gland.
[d] From embryonic chicken brain.
[e] n.t., not tested.
[f] This compound inhibits β-galactoside α-2,6-sialyltransferase from bovine colostrum with a K_i of 0.025 mM [647].
[g] n.i., no significant inhibition.
[h] Obtained by periodate oxidation of the ribose moiety.
[i] Lactosaminide stands for the methyl glycoside of *N*-acetyllactosamine.
[j] This inhibition was observed at 20 μg/ml [642]; ovine submandibular gland CMP-Neu5Ac: GalNAc α-protein α-2,6-sialyltransferase was inhibited to a similar extent [645].
[k] Inhibitor concentration 20 μg/ml.

correspondingly, of the α-2,6-sialylation of cell surface glycoconjugates [635]. Sialylation may also be increased by hormones, e.g. interferon [636], although in this publication the activity of sialyltransferase was not described. However, in the rat intestine hydrocortisone leads to an increase of sialyltransferase activity during tissue maturation [637]. Similar observations were made with rat fibroblast FR3T3 cells [638]. Cytokines were found to induce the expression of β-galactoside α-2,6-sialyltransferase in human endothelial cells, which mediates sialylation of adhesion molecules and CD22 ligands [639]. Based on mRNA measurements using *in situ* hybridization histochemistry, it was found that the expression of β-galactoside α-2,6-sialyltransferase in rat retina increases in the early post-natal days but to different degrees in the different retinal cell types [640]. Five different sialyltransferases were described to be involved in the synthesis of specific terminal sialoside structures of human fetal and adult tissues, and each gene of these enzymes seems to be regulated independently [619]. During the development of trout eggs α-2,6-, α-2,8-sialyl- and α-2,8-polysialyltransferase activities required for the synthesis of polysialoglycoproteins were expressed at various levels [641].

Little is known about inhibitors of sialyltransferases, although such compounds are very interesting for the modulation of sialylation, e.g. to study tumor biology and cancer therapy. Various sialic acids and their derivatives, as well as other synthetic or natural substances were tested as inhibitors (refs. [642,642a], Table 15). The K_i values of periodate-treated UDP and CMP, resulting in two aldehyde groups from the ribose ring each, which inhibit CMP-Neu5Ac:lactosylceramide α-2,3-sialyltransferase in microsomes of embryonic chicken brain in an irreversible, competitive manner [643], are also presented. While the natural 5′-CMP nucleotide is a potent inhibitor of rat liver and porcine submandibular gland sialyltransferases, the 3′-CMP isomer has no inhibitory effect. Strikingly, cytidine itself and its 2-thio-derivative also markedly inhibit the α-2,6- and α-2,3-sialyltransferases tested. Other substituents on cytidine both on the ribose and base moiety abolish this inhibitory effect. Oxidative opening of the ribose group of CMP significantly reduces the inhibitory potency of CMP on the liver and submandibulary gland enzymes, but yields a good inhibitor for the chicken brain sialyltransferase. The interesting compound 5-fluoro-2′,3′-isopropylidene-5′-*O*-(4-*N*-acetyl-2,4-dideoxy-3,6,7,8-tetra-*O*-acetyl-1-methoxycarbonyl-D-*glycero*-α-D-*galacto*-octapyranosyl)uridine (KI-8110), a CMP-Neu5Ac analogue, has been synthesized and inhibits the sialylation of cells in culture and pulmonary metastasis of mouse colon adenocarcinoma [648,649]. Although this compound does not inhibit the pure sialyltransferases from rat liver and porcine submandibular gland, it seems to inhibit the translocation of CMP-Neu5Ac through the Golgi membrane and thus sialylation of nascent glycoconjugates, as was observed with mouse and trout microsomes [642] as well as with Golgi vesicles from human liver and colorectal cancer cells [650]. As consequence of reduced sialylation, the growth of Sindbis viruses in cell culture was much hindered (M. Odenthal-Schnittler et al., unpublished observations).

While these inhibitors are on the donor site of sialyltransferase substrates, the effect of inhibiting substances on the acceptor site of Neu5Ac has also been reported [644]. For example, the methyl glycosides of 6′-deoxy- and 6′-thio-*N*-acetyllactosamine, which inhibit rat liver α-2,6-sialyltransferase in the millimolar range, are shown in Table 15. In all these cases, the inhibition does not follow pure Michaelis–Menten kinetics, but is of mixed competitive/non-competitive type. Natural sialyltransferase inhibitors have also been found, which are considered to play a role in the regulation of the synthesis of sialoglycoproteins and gangliosides. Whereas heparin and other polyanions are weak inhibitors, proteins from rat and calf brain have been isolated which inhibit α-2,6-sialyltransferases from ovine submandibular gland and rat liver by about 80% at a concentration of 20 μg/mol [641,645]. Two proteins of molecular mass 14.8 and 22.4 kDa isolated from rat brain inhibit both CMP-Neu5Ac:lactosylceramide α-2,6-sialyltransferase [646] and rat liver α-2,6-sialyltransferase [642]. This inhibitor also occurs in chicken and bovine brain as well as in other tissues of these animals and of rat [646].

Many enzymatic and histochemical (see refs. [651,652] and chapter 5 of Vol. 29a, "Glycoproteins") studies have shown that sialylation occurs in the trans-Golgi network [653] as a terminal glycosylation step after the transfer of CMP-sialic acid from the cytosol through the Golgi membrane by a specific translocator (see also Fig. 15 below). This protein was shown to transfer both CMP-Neu5Ac and CMP-Neu5Gc equally well [654]. A detailed study on the glycosylation including sialylation of oligosaccharides

in rat liver Golgi preparations was reported in refs. [655,656]. In rat liver hepatoma cells, the glycosylation of α-2,6-sialyltransferase is involved in the maturation of the enzyme to a higher molecular mass form [632]. The enzyme follows the secretory pathway as a membrane protein and is retained at a late Golgi stage. Due to rapid degradation in a post-Golgi compartment, the enzyme, exhibiting a half-life time of 3 h, is secreted from the cell in only small amounts. For the retention of the sialyltransferase in the trans-Golgi network, signals in the enzyme's polypeptide chain were suggested to be responsible for the specific binding to Golgi membranes. It was shown with mutant and chimeric α-2,6-sialyltransferase that the cytoplasmatic tail and signal anchor region alone are not sufficient for Golgi retention [657]. However, when two lysine residues were placed next to the signal anchor on the luminal side of the enzyme, more efficient Golgi retention was observed. This suggests that the signal anchor region is not sufficient for Golgi retention and that it can be replaced by any transmembrane region which allows correct spacing and folding of sequences flanking the membrane.

So far, the acceptor specificity of nearly 20 eu- and prokaryotic sialyltransferases has been elucidated and 15 different cDNA clones of these enzymes have been obtained (Table 14 and references therein). This list demonstrates the rapid progress in this field and also includes the first cloning of mammalian polysialyltransferase (polysialyltransferase-1 from hamster ovary cells) [620]. The same group has recently published the molecular analysis of the biosynthetic pathway of the α-2,8-polysialic acid capsule by *Neisseria meningitidis* serogroup B [658].

The sialyltransferase clones contain a stretch of about 50 amino acids with significant homology and this so-called sialyl motif is possibly involved in substrate binding [601]. Using polymerase chain reactions with degenerate primers deduced from this motif, additional cDNA clones have been obtained [617], for which, however, no sialyltransferase activity can yet be assigned. Sialyltransferases, like other glycosyltransferases, are structurally highly related, consisting of a short NH_2-terminal cytoplasmic domain, a signal-membrane anchor domain, a proteolytically sensitive stem region, and a large COOH-terminal catalytic domain. For example, the deduced amino acid sequence of the mouse brain Gal(β1–3)GalNAcα-2,3-sialyltransferase shows 80% identity with that of the porcine submandibular gland Gal(β1–3)GalNAcα-2,3-sialyltransferase [597].

8.4. Enzymatic modification of sialic acid

8.4.1. Biosynthesis and functions of N-glycolylneuraminic acid

Neu5Gc is present in essentially all animal groups of the deuterostomate lineage, from the echinodermata up to the mammals, as component of all types of glycoconjugates [5,8,33, 659,660]. Remarkably, in healthy human tissues it is missing, but it is expressed in small quantities in some tumors [661]. Although Neu5Gc has never been found in bacteria, it is a component of membrane glycoconjugates of *Trypanosoma cruzi* [662].

The wide occurrence of this sialic acid and its tissue-specific and developmentally regulated expression suggest that it has important biological functions. However, very few established functions have yet been allocated to Neu5Gc. Our knowledge of this field is summarized in Table 16. It shows that in addition to structural aspects, the presence of Neu5Gc modulates the general functions of the main, precursor sialic acid, Neu5Ac.

Table 16
Biological significance of *N*-glycolylneuraminic acid

Phenomenon	Reference(s)
Influence on physicochemical properties of glycoconjugates, e.g., increasing hydrophilicity	[5]
Slower hydrolysis rate by sialidases when compared with Neu5Ac, thus possibly hindering spreading and virulence of bacteria	[5,33,245]
Slower degradation rate by sialate-pyruvate lyase	[5,33,245]
Slower rate of demasking of subterminal galactose residues, thus prolonging the biological effect of sialic acids	[663]
Modulation of sialic acid–receptor interactions, e.g., increase of the binding of murine B lymphocyte CD22 adhesin and of *Escherichia coli* K99 lectin; decrease of the binding of mouse macrophage sialoadhesin and Myelin-Associated Glycoprotein (MAG)	[664–667]
Specific antigenic epitopes, such as Hanganutziu–Deicher antigens	[668–670]
Dog and cat blood group determinants	[671–673]
Tumor-associated antigen in man and chicken	[661,670]
Differentiation marker	[307,674]
The *N*-glycolyl group can be an acceptor site of *O*-acetylation and *O*-methylation, yielding Neu5GcAc and Neu5GcMe (Table 1) in rat thrombocytes and starfish, respectively	[43,44]
The *N*-glycolyl group can be a site of glycosylation, leading to	
(a) Neu5Gc8Me(α2–O5)Neu5Gc8Me- chains in the starfish *Asterias rubens*	[675]
(b) Branching points of glycans in echinoderm glycoconjugates	[5,13,31,676]

This is best illustrated with sialidases, the action of which is suppressed by the presence of a *N*-glycolyl group in sialic acids. This may afford some protection for host cells from attack by pathogen-derived sialidases, since the removal of sialic acids facilitates the further degradation of cells by other glycosidases and proteases [245]. Neu5Gc may therefore effectively act against the virulence of certain infectious bacteria. It is also conceivable that Neu5Gc may mask subterminal galactose residues of oligosaccharide chains thus preventing their recognition and phagocytosis by macrophages more potently than does Neu5Ac [663]. Other cell recognition phenomena, such as binding of sialic acids to sialoadhesin of mouse macrophages [664] or to the enterotoxic *E. coli* strain K99, which infects young pigs [665], is modulated by Neu5Gc.

Since the Neu5Gc epitope on glycoconjugates is antigenic to man and leads to Hanganutziu–Deicher antibodies [668,677], the clinical application of recombinant glycoproteins, e.g. erythropoietin (EPO), containing small amounts of Neu5Gc due to

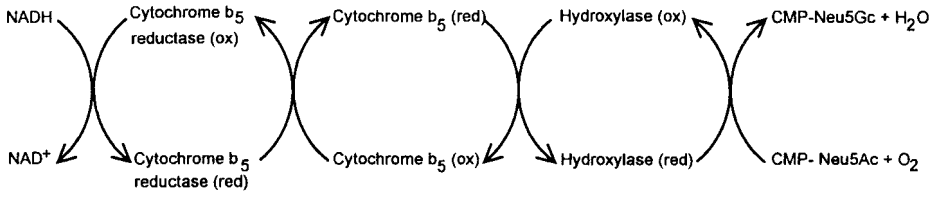


Fig. 14. Redox-protein compounds involved in the formation of Neu5Gc by CMP-Neu5Ac hydroxylase [188, 680,681].

their production in cells such as CHO cells, requires special attention [669]. Formation of such antibodies would lead to rapid removal of the drug from the blood stream and the immuno-complex accumulated in renal glomeruli may cause nephritis or other symptoms known as serum sickness. To the authors' knowledge, however, no such problems with recombinant glycoproteins have been reported.

While it was already detected in 1968/69 that Neu5Gc is derived from Neu5Ac by hydroxylation of the *N*-acetyl moiety [678,679], it was not until 1988 that CMP-Neu5Ac was recognized as the substrate for this hydroxylase [188]. This fact, together with the solubility of the enzyme following tissue homogenization stimulated purification and characterization of the enzyme from porcine submandibular gland [680] and mouse liver [681,682]. The enzyme from both sources is a monomer with a molecular mass of 65 kDa. The CMP-Neu5Ac hydroxylase was also studied in the gonads of the starfish *Asterias rubens,* which belongs to the phylogenetically oldest animals synthesizing Neu5Gc [683]. It has many characteristics in common with the mammalian enzymes. All hydroxylases studied so far show high affinity for CMP-Neu5Ac [660], i.e. apparent K_M values in the low micromolar range (e.g. 1.3 μM for the mouse liver enzyme [684]). They require a number of cofactors for activity including molecular oxygen, reduced pyridine nucleotides, and, as was shown for the mammalian enzymes, cytochrome b_5, and cytochrome b_5-reductase. These form an electron transport chain resulting in the incorporation of one oxygen into the methyl residue of the *N*-acetyl group, as shown in Fig. 14. Some experimental evidence was presented [685] that at the beginning of this concerted action the binding of CMP-Neu5Ac to the hydroxylase changes the conformation of the enzyme in a way leading to the recognition and binding of the enzyme by cytochrome b_5 and the formation of a ternary complex. After the transfer of electrons from NAD(P)H to the enzyme through cytochrome b_5, CMP-Neu5Ac is converted to CMP-Neu5Gc which was shown not to bind to the enzyme and is therefore released with concomitant dissociation of the ternary complex. The observation that the addition of iron salts can stimulate enzyme activity and iron-binding substances are potent inhibitors, together with electron-spin-resonance studies of the purified hydroxylase, strongly point to the presence of an iron-sulfur center in the active site, which is possibly of the Rieske type [686]. The CMP-Neu5Ac hydroxylases from mouse liver [687] and pig submandibular gland [688] have both been cloned. The gene structures show high homology.

In addition to the CMP-Neu5Ac-specific hydroxylase, pig submandibular glands were reported to contain a second hydroxylase, which is specific for free Neu5Ac and also

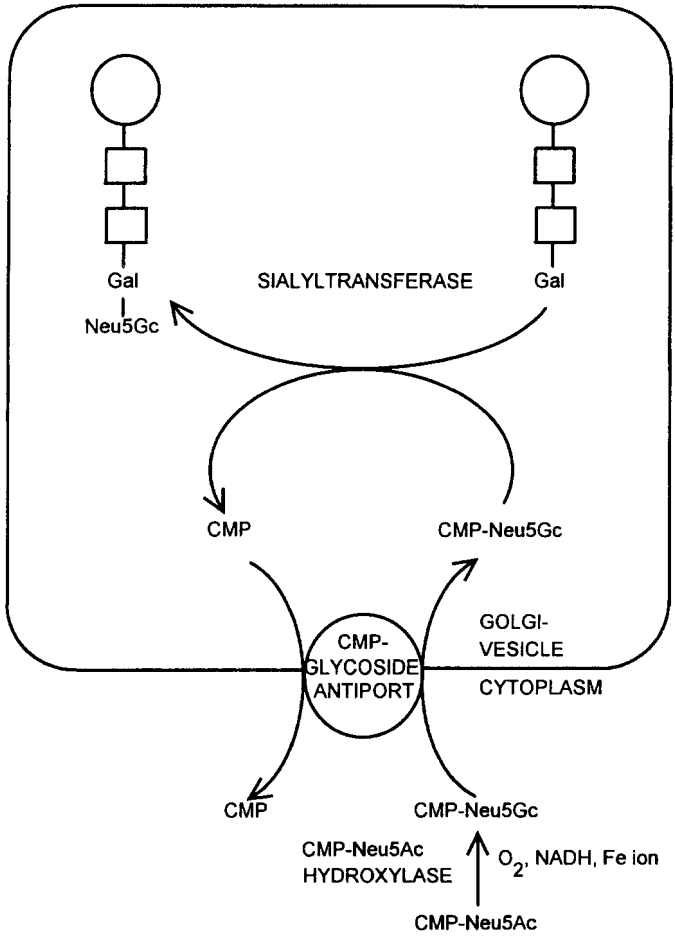


Fig. 15. Biosynthesis of CMP-*N*-glycolylneuraminic acid with the aid of CMP-*N*-acetylneuraminic acid monooxygenase in the cytosol, translocation of CMP-Neu5Gc into the Golgi vesicle and transfer of the Neu5Gc moiety onto nascent glycoconjugates [188,654,684]. From ref. [690] by permission of Oxford University Press, Oxford.

differs from the former hydroxylase with respect to its sensitivity to certain inhibitors [689].

The activity of CMP-Neu5Ac hydroxylase and accordingly the concentration of CMP-Neu5Gc in the cytosol probably play the most important role in regulating the level of sialylation with Neu5Gc. This is assumed because neither the Golgi CMP-sialic acid antiporter nor the sialyltransferases exhibit a pronounced preference for CMP-Neu5Ac or CMP-Neu5Gc (Fig. 15). The activity of the hydroxylase may thus be tuned so that the ratio of Neu5Gc and Neu5Ac required in the resulting glycoconjugates is generated in the form of CMP-glycosides in the cytosol. The multiplicity of functions and constitutive expression of the cytochrome b_5 system, together with the lack of effect of any metabolites

on the hydroxylase activity [681], suggest that the rate of production of CMP-Neu5Ac is regulated at the level of the expression of the monooxygenase. The tissue-specific and developmental factors affecting the production of this protein on the gene level as well as a possible influence of the oxygen pressure still remain to be elucidated. No experimental explanation has so far been obtained for the formation of Neu5Gc in human tumors, which may be caused by the anomalous expression of a CMP-Neu5Ac hydroxylase gene, which seems to be dormant or repressed in normal tissues. Significantly, in T-cell lymphomas of baboons the level of Neu5Gc, as component of gangliosides, was much higher than in those from the corresponding normal tissues [691]. The fusion of human B lymphocytes with mouse myeloma cells leads to high expression of Neu5Gc, showing activation of the enzymes involved in Neu5Gc biosynthesis due to the somatic cell fusion process [692]. It was furthermore reported that the insertion of retroviruses into Chinese hamster ovary cells almost completely replaces Neu5Ac by Neu5Gc [693].

8.4.2. Biosynthesis and functions of O-acetylated sialic acids

O-Acetylated sialic acids are found in all types of glycoconjugates and in oligo- and polysaccharides of many animal species from the echinoderms onwards and also in some bacterial species (refs. [5,7,8,11,13,27,690,694]; see also sections 2 and 3, and Table 1). This modification is either at position 4 of the pyranose ring of both Neu5Ac and Neu5Gc, as was found in horse, donkey, guinea pig and echidna (*Tachyglossus aculeatus*), or in the glycerol side chain of sialic acids. In the latter case, the *O*-acetyl group is most frequently located at C9, but it may be accompanied by acetylation at C8 and/or C7, leading to di- and tri-*O*-acetylated species. This side-chain *O*-acetylation is very frequent in the animal kingdom and is also found in tissues and fluids of man. However, in the expression of *O*-acetylated sialic acids ethnic differences do exist in colonic mucin of Sino–Japanese and non-Sino–Japanese races. *O*-Acetylation was more frequent in the latter ethnic groups studied (British, Icelanders, South African blacks and Bahrainis) than in Chinese and Japanese. It is speculated that loss of *O*-acetylation may be due to a single mutation of the gene regulating *O*-acetylation and that this may be related to selection pressure resulting from differential enteric colonization by bacterial flora and from resulting infectious diseases [695].

The occurrence of differently *O*-acetylated sialic acids suggests an important biological role of these modifications. We are only just beginning to understand these effects, which influence many biological and pathobiochemical systems, as listed in Table 17. As with Neu5Gc, *O*-acetylation may influence the physicochemical properties of sialylated glycoconjugates, which is especially important on cell surfaces. Correspondingly, an increased protective effect of the mucin containing a large quantity of *O*-acetylated sialic acids and lining the endothelia of human colon is discussed [245]. This protection against an aqueous environment containing a large quantity of different bacteria may be due firstly to the greater hydrophobicity imparted by the *O*-acetyl groups and secondly to a greater resistance of *O*-acetylated sialic acids to microbial sialidases when compared with Neu5Ac.

Sialic acid *O*-acetylation not only prevents (in the case of 4-*O*-acetylation) or much reduces (in the case of side-chain *O*-acetylation) the activity of viral, bacterial and animal (including trypanosomal) sialidases, but also impairs the activity of acylneuraminate-

Table 17
Effects of *O*-acetyl groups in sialic acids

Phenomenon	Reference(s)
Influence on physicochemical properties of glycoconjugates, e.g. increasing hydrophobicity	[5]
Hindering biologically active sialoglycoconjugates from degradation, by inhibition of sialidase, *trans*-sialidase, endoglycosidase and lyase action	[5,33,660,696]
Hindering cell degradation, e.g., of erythrocytes	[5,690,697]
Hindering binding of influenza A and B viruses, reovirus, *Plasmodium falciparum*, mouse macrophage sialoadhesin, B cell adhesion molecule CD22	[664,698–700]
Hindering activation of the alternate complement pathway, probably by preventing sialic acid binding to factor H	[701,702]
Hindering infection of cattle by *Trypanosoma brucei*	[703]
Decreasing antigenicity	[5,668,704]
Providing epitopes for the binding of influenza C, Corona and encephalomyelitis viruses	[705–707]
Increase of bacterial virulence	[708]
Providing epitopes for recognition by antibodies	[5,709]
Providing epitopes for recognition by lectins	[709–711]
Representing differentiation antigens and influencing morphogenesis	[5,668,712–716]
Representing tumor-associated antigens	[5,668,717–723]
Regulating environmental adaptation	[724]

pyruvate lyase, the next enzyme involved in the catabolism of sialic acids [5,33] (see Fig. 13). This effect may facilitate recycling of sialic acids released from glycoconjugates. *O*-Acetyl groups have a further hindering effect on the binding by several proteins, as was observed with the hemagglutinins of influenza A and B viruses, which only bind to non-*O*-acetylated Neu5Ac and Neu5Gc [699], with the malaria parasite *Plasmodium falciparum*, where the attachment of *O*-acetylated mouse erythrocytes was weaker than to non-*O*-acetylated cells [700], and with sialoadhesin from mouse macrophages [664]. In contrast to this masking of recognition sites, 9-*O*-acetylated sialic acids are specifically recognized by influenza C virus [705,725], as well as encephalomyelitis and corona viruses [706], leading to binding of these viruses to cells with concomitant infection. In the case of influenza C virus, this may occur in human nasal epithelia, which contain *O*-acetylated gangliosides and glycoproteins [J. Tack et al., unpublished]. In human nasal mucin 5–10% of the Neu5Ac residues were found to be *O*-acetylated [726].

A polyacrylamide glycopolymer containing Neu5,9Ac_2 was synthesized (see section 6.2) as inhibitor of influenza C virus hemagglutination, which is useful as a ligand for binding studies and as a potential anti-influenza C virus drug [489].

Influenza C viruses can be used for the detection of glycoproteins and gangliosides with 9-*O*-acetylated sialic acids on thin-layer chromatograms, and blots of SDS-polyacrylamide gels as well as in histochemical sections (see ref. [727] and unpublished data). This staining procedure, described in section 4, is possible with the aid of the hemagglutinin and the sialate-9-*O*-acetylesterase activities (section 9.1) of influenza C virus, which is part of the viral spike hemagglutinin-esterase-fusion (HEF) glycoprotein [234,728,729]. In Fig. 16 an example for the localization of Neu5,9Ac_2 in the mucin-producing Goblet cells of the mucosa of human colon is shown. With these techniques the frequent occurrence of Neu5,9Ac_2-containing glycoconjugates especially in epithelia and in endothelia of blood vessels of rat, cow and man was demonstrated [234,235,727,730,730a]. Sialic acids *O*-acetylated at C4 cannot be stained by influenza C virus.

In addition to these functions, *O*-acetylated sialic acids are involved in differentiation and tumorigenesis as evidenced by their differential expression during these processes (for some examples see Table 17). In the adenoma–carcinoma sequence in colorectum, analysis of sialic acid *O*-acetylation may assist in diagnosis of tumor disease [723]. 9-*O*-Acetyl-GD3 was found to be a surface marker for basal cell carcinomas, which may lead to novel, immunological therapeutic interventions [718], as is also expected for Neu5,9Ac_2-containing glycoconjugates of melanoma cells. The great importance of *O*-acetylation during ontogenesis was demonstrated by transfection of sialate-9-*O*-acetylesterase into mouse embryos, which led to developmental abnormalities in these transgenic mice [714]. There is also evidence that the expression of *O*-acetyltransferase activity in human T lymphocytes is regulated by maturation events taking place in the periphery [716].

Although both the sialate 4- and 9(7)-*O*-acetyltransferases (EC 2.3.1.44 and 45) were discovered 27 years ago in bovine and equine submandibular glands, respectively [731,732], the enzymes have not yet been purified and characterized in detail. Furthermore, nothing is known about the regulation of their expression at the gene level. In contrast to the CMP-Neu5Ac hydroxylase, the *O*-acetyltransferases are bound to subcellular membranes, as was studied with bovine and equine submandibular glands [733] and with rat liver [284,734]. Using detergents, the *O*-acetyltransferase from bovine submandibular glands was partially solubilized and enriched about 600-fold [7]. This labile enzyme exhibits a pH optimum of 7.2 and transfers acetyl groups from acetyl-CoA only onto α-glycosidically bound sialic acids of glycoconjugates. This corresponds with the finding that *O*-acetylation of the side chain at C7 and C9 of Golgi-bound sialic acids occurs after the translocation of acetyl-CoA into this compartment [8].

The complete pattern of sialic acid side-chain *O*-acetylation may require several sialate *O*-acetyltransferases each with a specificity for only one position. On the other hand, the primary insertion place for the *O*-acetyl function may be the HO7 group alone from where the ester group migrates even under physiological conditions to the 9 position, presumably via C8 [7,23], leaving HO7 ready for a new transfer. Thus, for complete side-

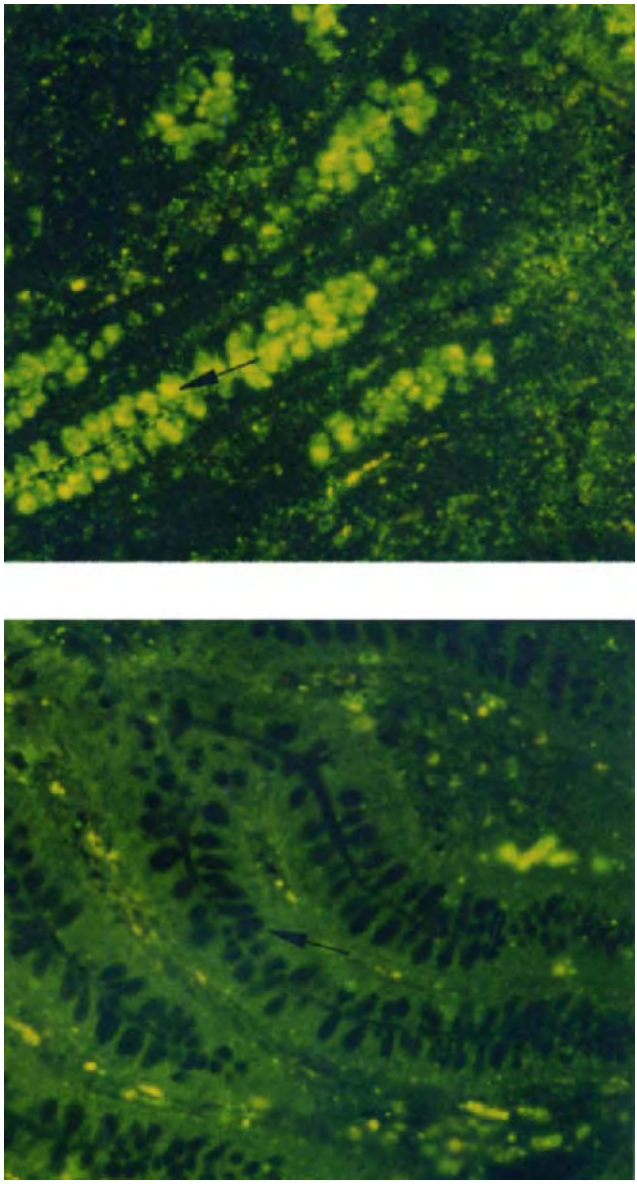


Fig. 16. Staining with influenza C virus of Neu5,9Ac_2-containing mucin in goblet cells (arrow) of the mucosa of human colon tissue. Thin-sections were overlayed with virus suspension and virus bound to *O*-acetylated sialic acids visualized using FITC-conjugated anti-influenza antibodies [730]. (top) Goblet cells containing native mucin are stained yellow-green. (bottom) After removal of Neu5,9Ac_2 by sialidase, no staining occurs and the cells appear dark. This effect can also be obtained by saponification of the ester groups. Magnification about 400-fold.

chain *O*-acetylation only one enzyme, acetyl-CoA:sialate 7-*O*-acetyltransferase, may be necessary together with non-enzymatic migration of this substituent.

It is also possible that several sialic acid *O*-acetyltransferases preferring glycoproteins or gangliosides or at least specific oligosaccharide chains exist. Enzymes were reported to be specific for Neu5Ac in N-glycans of rat liver [735] and for gangliosides in human melanoma cells [694,736], respectively. An *O*-acetyltransferase incorporating acetyl residues into C7 and C9 of polysialic acid was demonstrated to occur in K1-positive *Escherichia coli* [159]. From the results reported above, an *O*-acetyltransferase specific for the O-glycan of mucin in bovine submandibular glands could also be postulated.

8.4.3. Biosynthesis of 9-O-lactylated sialic acid

5-*N*-Acetyl-9-*O*-lactylneuraminic acid (Neu5Ac9Lt) has been found in man, cow and trout (see refs. [5,8,13,582,737], see also Table 1). Not much is known with regard to the origin of this compound. However, investigation with a particulate fraction from horse liver suggested that this modification occurs enzymatically, although the exact mechanism and type of lactyl donor is not known [29].

8.4.4. Biosynthesis of 8-O-methylated sialic acids

Sialic acid *O*-methylation is a modification that seems to be restricted to the echinodermata, especially in certain starfish [5,8,13]. *O*-Methyl groups have only been found in the 8 position of Neu5Gc and in much lower quantities on Neu5Ac [675]. 9-*O*-Acetylated derivatives of 8-*O*-methylated sialic acids were also detected in small amounts (Table 1). The existence of a sialate 8-*O*-methyltransferase required for the biosynthesis of this sialic acid was first demonstrated to occur in the starfish *Asterias rubens* [675,738]. The enzyme has now been further characterized after solubilization by detergents from a crude preparation of starfish gonads as a membrane-associated protein that transfers methyl groups from *S*-adenosyl-L-methionine preferably onto glycosidically linked Neu5Gc residues. Neither free Neu5Gc nor CMP-Neu5Gc are substrates for this enzyme. Horse erythrocytes or glycopeptides from pig submandibular gland mucin immobilized on Sepharose, containing almost exclusively Neu5Gc residues, are excellent substrates for the sialate *O*-methyltransferase [739]. The systematic name *S*-adenosyl-L-methionine:sialate-8-*O*-methyltransferase (EC 2.1.1.78) has been proposed.

8.4.5. Biosynthesis of 5-N-acetyl-2-deoxy-2,3-didehydro-neuraminic acid

This Neu5Ac derivative lacking a glycosidic hydroxyl group at C2 has been found as free sialic acid in small quantities in blood serum, saliva and urine of man [5,8,13] and in tissue extracts of starfish [36] (Table 1). It is unknown, whether this compound, which is a potent competitive inhibitor of sialidases (see below), has a biological function. Its formation under physiological conditions has been described in section 2.

8.4.6. Biosynthesis of 5-N-acetyl-2,7-anhydro-neuraminic acid

The occurrence of this neuraminic acid derivative (Table 1) has first been described in cerumen of men [37], although its origin in this material remained unknown. Some hints to the possible enzymatic formation (by microorganisms?) come from the discovery

that Neu2,7an5Ac is a reaction product of a leech (*Macrobdella decora*) sialidase ("sialidase L") with sialoglycoconjugates [61] (see also section 2).

8.4.7. Occurrence of 2-keto-3-deoxynononic acid (Kdn)

This substance (for the chemistry see section 3, Tables 1 and 3) was found in the eggs of rainbow trout [740], and the egg jelly coat from the amphibians *Pleurodeles waltlii* [48], *Axolotl mexicanum* [105], and *Xenopus laevis* [741]. In trout, it is part of gangliosides [122] and glycoproteins, where it can terminate long sialic acid chains or form poly-Kdn chains [267]. To our knowledge, nothing is known about the biosynthesis of this sugar, including the possibility of deamination of neuraminic acid at C5. However, the linkage of Kdn to CMP by the cytidylyltransferase from the testis of rainbow trout has been reported [107,122]. This enzyme can also activate Neu5Ac and Neu5Gc. A Golgi-membrane-bound Kdn-transferase from the ovaries of the rainbow trout *Oncorhynchus mykiss* has been characterized [545]. This enzyme terminates the growth of α-2,8-polysialyl chains in a series of actions of four glycosyltransferases involved in the glycosylation of ovary glycoproteins. Three of these transferases are sialyltransferases, the first transferring Neu5Gc to C6 of serine- or threonine-bound GalNAc, the second linking another Neu5Gc residue to C8 of this first Neu5Gc and the third enzyme being an α-2,8-polysialyltransferase for chain elongation, before termination of its growth by the 2,8-Kdn-transferase [545]. Remarkably, a developmentally regulated α-2,8-poly Kdn was also discovered in various tissues of mammals [J. Roth, personal communication].

9. Catabolism of sialic acids

A well-balanced system of enzymes involved in the degradation of sialoglycoconjugates is required for the normal functions of cells and tissues. A lack of catabolic enzymes, often due to genetic errors, may lead to severe diseases, as may a surplus of such enzymes, e.g. due to infections by microorganisms. These problems will be addressed in section 10.5.

Three enzyme systems are required for the catabolism of sialic acids: *O*-acetylesterases, sialidases and lyases. Furthermore, a sialic acid transporter has been described (section 10.5), which carries liberated sialic acids from lysosomes into the cytosol, where they are either degraded by the lyase or recycled after activation with CTP (section 8.3). Sialic acid permeases provide bacteria with sialic acids for nutritional purposes (section 9.4).

9.1. Sialate-O-acetylesterases

One of the main functions of sialic acid *O*-acetyl groups is their inhibitory effect on the action of both sialidases and sialic acid-specific lyases [5,33]. While a 4-*O*-acetyl group completely hinders the action of these enzymes (with the exception of a slow release by viral sialidases [252]), such ester groups at the sialic acid side chain appreciably hinder hydrolysis of the glycosidic bond of these sugars and their further breakdown by lyases. The existence of esterases acting on *O*-acetylated sialic acids prior to sialidase is therefore a prerequisite or at least supports the rapid turnover of *O*-acetylated sialoglycoconjugates. The possibility of the existence of such enzymes was raised by the observation that

horse glycoproteins, which are heavily 4-*O*-acetylated, seem to have a normal turnover, and sialidase studied in horse liver cannot act on 4-*O*-acetylated sialic acids [742]. Consequently, the existence of such esterases in horse liver was assumed and two enzymes were found, one hydrolyzing only 9-*O*-acetyl groups and the other mainly 4-*O*-acetyl residues from sialic acids (ref. [266], and unpublished work). Furthermore, sialate-9-*O*-acetylesterases (EC 3.1.1.53) have been isolated and characterized from influenza C virus [705,743], bovine brain [744] and rat liver [745,746]. They also occur in human erythrocytes [747] as well as in enteric bacteria [748]. These enzymes specifically release 9-*O*-acetyl groups from free and glycosidically bound sialic acids. 9-*O*-Lactyl groups, *O*-acetyl groups of Neu5,7Ac_2 and methyl-esterified carboxyl groups of sialic acid are not hydrolyzed. Although they also attack *O*-acetyl groups of non-physiological substances, e.g. naphthyl acetate [266,749], they belong to the few esterases which have been recognized to have a physiological function [266]. Amide groups including the synthetically added 9-*N*-acetyl moiety of 9NAc-Neu5Ac (Table 13) are resistant to the action of these esterases. However, the latter Neu5Ac derivative binds to influenza C virus hemagglutinin and it does this in a stable manner, as it cannot be removed by the viral esterase [750]. The staining of glycoconjugates containing *O*-acetylated sialic acids with influenza C virus and the problems involved due to esterase activity were discussed in sections 4 and 8.4.2.

The mammalian sialate esterases are considered to be involved in the turnover processes of glycoconjugates in lysosomes, thus facilitating the action of sialidases as was discussed for the rat liver enzyme [735,745]. Tissue esterases may also participate in the recycling of sialoglycoconjugates, as the *O*-acetyl groups of the ganglioside GD3 in human melanoma cells turn over faster than the underlying sialic acids of this molecule [751]. It should be noted that the de-*N*-acetylated sialic acid (Table 1), as a component of GD3 ganglioside, can also occur in the same malignant cells [14], as was shown by immunological means. Thus, the unstable neuraminic acid molecule can exist in nature, however, only in glycosidic linkage (section 1). The viral enzyme may be of pathophysiological significance and be involved in the binding to mucins and to endothelial cell surfaces prior to endocytosis of the virus particles [266,728]. With regard to the epidemiology of viral infections, the viral esterase might contribute to the unmasking of new receptor sites to facilitate superinfection by type A and B influenza viruses [752]. Sialate esterase from human enteric bacteria acts on *O*-acetylated sialic acids of colon mucin and thus facilitates the further degradation of the mucin barrier by bacterial sialidase and other glycosidases [748].

The activity of sialate-*O*-acetylesterase can easily be followed using free Neu5,9Ac_2 either isolated from bovine submandibular gland mucin [5,13] or *O*-acetylated chemically (see section 6) and HPLC determination (see section 5.3.2). Alternatively, 5-*N*-acetyl-9-*O*-acetyl-2-[4-(dansylamino)phenylthio]-α-neuraminic acid [285] acts as a specific and highly sensitive, fluorescent esterase substrate (see sections 5.3.2 and 6.2). With the corresponding 7,8,9-tri-*O*-acetylated derivative it was shown that influenza C virus esterase can remove all three *O*-acetyl groups [469]. Since this esterase acts only on the *O*-acetyl residue at C9, de-*O*-acetylation of the whole sialic acid side chain can occur only after migration of the other *O*-acetyl groups to the primary alcohol group at C9. These experiments provide further evidence for the existence of the cascade of

alternative enzymatic de-*O*-acetylation and non-enzymatic *O*-acetyl migration proposed earlier (refs. [5,7,33], and sections 2 and 5.3.5).

9.2. Sialidases

These enzymes (EC 3.2.1.18) are essential tools in sialic acid metabolism, mainly in catabolic reactions, usually hydrolyzing the O-glycosidic linkages between the terminal sialic acids and the subterminal monosaccharides of free and glycoconjugate-bound oligosaccharides. Sialidase, as well as its substrate, is common in metazoan animals of the deuterostomate lineage from echinoderms to mammals [33,244,246,753]. Diverse viruses and microorganisms, such as fungi, protozoa and bacteria also produce sialidases [33,244,246,753–756], although they mostly lack sialic acids. The most recent review on the biochemistry and function of the large sialidase family discusses great variety in cellular location, molecular mass and substrate specificity [246]. Therefore, only a few, more general aspects of sialidases will be summarized, such as the type of sialidase reactions, including *trans*-sialidases, the molecular biology, the pathophysiological significance and inhibitors.

9.2.1. Types of sialidases

Sialidases are exo- or endo-sialidases, hydrolyzing either terminal sialic acid residues of complex carbohydrates or internal sialic acid glycosidic bonds of oligo- or polysialyl chains. Most microbial and animal sialidases belong to the first group [33,244,246,753–755], in the following simply referred to as "sialidases", while an endo-sialidase has been found in the coliphage E hydrolyzing the α-2,8-linkages of colominic acid [757].

Based on the nature of the chemical reaction, sialidases can tentatively be grouped into eight types, irrespective of the nature of the complex carbohydrate attacked and the subcellular location of the enzyme.

(1) Most microbial and animal sialidases readily hydrolyze terminal α-2,3-, α-2,6- and α-2,8-glycosidic linkages, although at different rates. In the majority of cases, the α-2,3-linkage is cleaved most rapidly [244,246,753–755]. Due to steric hindrance the side-positioned (branched) terminal Neu5Ac as in GM1, however, is resistant to the action of most sialidases with the exception of the *Arthrobacter ureafaciens* sialidase. The sialidases from *C. perfringens* and *V. cholerae* cleave the glycosidic bond of their substrates between C2 of Neu5Ac and the glycosidic oxygen, as demonstrated by release experiments carried out in $H_2[^{18}O]$ buffer solutions and monitoring by GLC [758]. Sialic acids are released in the α-anomeric form after which they slowly mutarotate to the more stable β-form (see section 5.3.5). Although Neu2en5Ac is a competitive inhibitor for most members of this sialidase group, it has not been detected to be an intermediate of enzyme reaction, as was the case for some viral sialidases (see below).

(2) With regard to the primary reaction product, *Salmonella typhimurium* sialidase behaves quite differently, since it was reported not to retain the configuration of sialic acid during the hydrolysis of Neu5Ac *p*-nitrophenyl α-glycoside. As primary reaction product the β-isomer was detected by optical rotation measurements [759,760].

(3) Close investigation of the reaction mechanism of influenza virus sialidases [60,320] has shown that Neu2en5Ac appears as a transition intermediate, resulting in the

appearance of small amounts of this substance in free form during prolonged incubation of sialic acids with influenza B virus sialidase and sialyllactose, as was identified by GLC/MS analysis [60]. Thus, this sialidase can "synthesize its own inhibitor". During the reaction, the chair conformation of Neu5Ac is distorted to the boat conformation by the concerted action of mainly aspartate, arginine, glutamate and tyrosine residues of the active center. The aspartate serves as proton donor for the release of the glycosidic partner of sialic acid. After the formation of the oxocarbonium intermediate, the addition of a hydroxyl group leads to the formation of α-Neu5Ac, or, in a side reaction by deprotonation, to Neu2en5Ac. The site of enzyme catalysis was shown by X-ray crystallographic studies of the inhibitor–influenza A N9-sialidase complex [761]. Here, twelve amino acid residues directly interact with Neu2en5Ac. Additionally, a further six conserved amino acids exist lining the active site pocket. On the basis of these studies, a mechanism of influenza virus sialidase action was suggested [762].

(4) As mentioned in sections 2 and 8.4.5, a sialidase from the leech *Macrobdella decora* yields Neu2,7an5Ac as reaction product [61]. The purified enzyme interacts only with the α-2,3-glycosidically bound sialic acid of sialyllactose and various sialoglycoconjugates. This marked linkage specificity is unique among the sialidases. The origin of Neu2,7an5Ac in cerumen [37] is unknown, but it is hypothesized that it results from the action of a similar, probably microbial sialidase. The small amounts of this sialic acid found in human urine [763] may have the same origin.

(5) The bacteriophage E which infects strains of *E. coli* displaying the α-2,8-linked polysialic acid colominic acid possesses an endo-sialidase, which was first described in ref. [764]. The reports on such endo-sialidases associated with K1 coli phages have been summarized [765]. These enzymes recognizing α-2,8-linkages of both Neu5Ac and Neu5Gc are believed to be involved in the attachment of the phages to the bacteria.

(6) Shortly after the discovery of Kdn, now also included in the sialic acid family (sections 1–3, Tables 1–3), an enzyme activity was discovered in the loach *Misgurnus fossilis* hydrolyzing the terminal α-glycosidic linkage of this monosaccharide [39,766]. Although conventional sialidases cannot hydrolyze Kdn bonds, the *Misgurnus* Kdn-ase can also act on Neu5Ac bound to 4-methylumbelliferone (MU) or GM3 ganglioside. Kdn-ase found in several tissues, particularly in the ovary of rainbow trout, also has sialidase activity [254]. In contrast, a Kdn-ase from *Sphingobacterium multivorum* only hydrolyzes α-2,3-, α-2,6- and α-2,8-bonds of Kdn glycosides, but does not cleave Neu5Ac or Neu5Gc from a variety of sialoglycoconjugates tested [255]. The use of these hydrolases for analytical purposes is mentioned in section 5.1.

(7) All these rather different sialidase types specifically recognize α-glycosidic bonds. However, a hydrolase acting on the β-glycosidic bond of CMP-Neu5Ac is CMP-sialate hydrolase (EC 3.1.4.40) which occurs in plasma membranes [767]. It is assumed to be involved in the regulation of the cellular level of CMP-sialic acids. Evidence for the existence of a Golgi-associated CMP-sialate hydrolase was also obtained in rat and mouse liver [654].

(8) While all these enzymes hydrolytically cleave α-glycosidic bonds of sialic acids, another type of sialidase has been discovered, which can also hydrolyze these linkages but preferably forms sialic acid linkages under physiological conditions in the presence of suitable acceptors. These are the *trans*-sialidases, occurring in parasites, and a

special section (9.2.3) is devoted to these enzymes. Since in principle all enzymatic reactions are reversible, transglycosidation of a Neu5Ac unit is also possible by the use of conventional, bacterial sialidases under appropriate conditions. For examples of corresponding glycoside syntheses see section 6.3.

9.2.2. Primary structures of sialidases

Comparison of the enzymatic properties of sialidases from a variety of microbial and animal sources revealed that these enzymes are highly diverse with respect to subcellular location, molecular mass, number of subunits, isoelectric point, temperature optimum, influence of Ca^{2+} on activity, substrate specificity and specific activity [244,246,753,754]. The only common properties are the α-anomeric configuration of the sialic acid glycosidic linkage and the acidic pH optimum (pH 5.0–6.1), though the trypanosomal *trans*-sialidases exhibit maximum activity at neutral pH [755]. As these properties apparently do not reveal much relationship between the different microbial sialidases, the primary structures of the enzymes were further investigated at the DNA level, from which it became clear that, although their enzymatic properties show many differences, the pro- and eukaryotic sialidases so far investigated belong to one superfamily [660,768].

In the first primary structures of microbial sialidases, obtained by cloning and sequencing of the respective genes from *Clostridium perfringens* [769], *Vibrio cholerae* [770], *Clostridium sordellii* [771] and *Salmonella typhimurium* [772], an amino acid sequence motif was detected, which is repeated four-fold in each protein: S–X–D–X–G–X–T–W [773]. This motif, named the Asp-box, was found in all 16 sialidases of animals, trypanosomes, and bacteria, which have so far been sequenced (see refs. [660,768] and Table 18). In viral sialidases, however, the motif was rarely detectable (e.g. only the sialidase from N9 influenza A virus strain exhibits the complete motif [786] and has probably undergone mutational alterations).

Further identical motifs, or single amino acids at certain positions, became evident by an alignment of the pro- and eukaryotic sialidase sequences known [660]. Gaps had to be introduced as a consequence of the differences in protein size. The central regions of these proteins are especially homologous and exhibit most of the conserved amino acids. A further motif, the F–R–I–P region, which is located N-terminally from the first Asp-box, is highly conserved in clostridial sialidases, but was found to be degenerated to X–R–X–P, when further bacterial and animal sialidase sequences were included in the alignment. Altogether, sixteen amino acids are found to be conserved.

The function of the conserved and repeated Asp-box is not yet fully understood. In the N9 influenza A virus sialidase [786], the single corresponding motif is located as part of a β-pleated sheet polypeptide at the connections between the four protein subunits. Immunological studies revealed that the Asp-box I of *Trypanosoma cruzi trans*-sialidase (see below) is inaccessible to antibodies, which has been taken to indicate that it is not part of the external catalytic domain [788]. Site-directed mutagenesis experiments of the "small"(43 kDa) sialidase isoenzyme of *C. perfringens* resulted in only small alterations of enzyme activity by changing some of the Asp-box amino acids [789], while the exchange of other highly conserved amino acids drastically reduced enzyme activity and increased the K_M value, e.g. by replacement of the N-terminally located, conserved arginine-37, probably belonging to the active center, with lysine.

Table 18
Cloned sialidases and *trans*-sialidases with Asp-boxes[a]

Organism	Reference(s)
Rattus rattus (rat)	[774]
Cricetulus griseus (hamster)	[775]
Macrobdella decora (leech)[b]	[61]
Trypanosoma cruzi	[776]
Trypanosoma rangeli[c]	[777]
Actinomyces viscosus	[778,779]
Bacteroides fragilis	[780]
Clostridium perfringens ("small" isoenzyme)[d]	[769]
Clostridium perfringens ("large" isoenzyme)	[782]
Clostridium septicum	[781]
Clostridium sordellii	[771]
Micromonospora viridifaciens	[783]
Salmonella typhimurium	[772]
Streptococcus pneumoniae	[784]
Vibrio cholerae	[770,785]
Influenza virus (A/whale/Maine/1/84; H13n9)[e]	[786]
Bacteriophage E[f]	[787]

[a] The gene structures can be found in the EMBL Data Library.
[b] The reaction product is Neu2,7an5Ac.
[c] Sialidase-like protein.
[d] This enzyme has also been cloned [769a].
[e] No Asp-boxes were found so far in the other viral sialidase sequences of the EMBL Data Library.
[f] Endo-sialidase.

More insight into the possible role and spatial location within the protein molecule of the Asp-boxes and the other conserved amino acids came from the crystal structure of the sialidase from *S. typhimurium* [790]. The enzyme has the shape of a six-bladed propeller, each blade consisting of four-stranded antiparallel β-sheets (Fig. 17). The axis of this propeller passes through the active site. The Asp-boxes are located at topologically equivalent positions on the outside of the blades which is not in favor of a role of these sequence motifs in enzyme catalysis. Cocrystallization of the enzyme with the inhibitor Neu2en5Ac enabled the identification of the amino acids involved in sialic acid binding and catalysis. Accordingly, the active site consists of an arginine triad, a hydrophobic pocket and key tyrosine and glutamic acid residues. The interaction of these residues with different parts of the Neu5Ac molecule is shown in Fig. 18 for three bacterial sialidases. Identical amino acids are assumed to be involved in catalysis by the low-molecular-mass sialidase from *C. perfringens*, as was shown by the finding of conserved amino acids at positions identical or similar to the *S. typhimurium* enzyme, site-directed mutagenesis

Fig. 17. Three-dimensional structure of *Salmonella typhimurium* LT2 sialidase with bound inhibitor Neu2en5Ac obtained from X-ray crystallography. From ref. [790] by permission of National Academy of Sciences, New York.

and hydropathy studies of the amino acid sequence [Kleineidam et al., unpublished]. The same is valid for the *V. cholerae* sialidase, although the positions of the catalytic amino acids are different, due to the large size of this enzyme [791].

Since the tertiary structure of bacterial sialidases is similar to that found in viral sialidases (e.g. refs. [756,792–794], for a review see ref. [246]) and residues of the Asp-box have also been found, viral and bacterial sialidases can be considered to belong to one enzyme family. The few mammalian sialidases sequenced so far, i.e. from rat muscle [774] or hamster ovary cells [775], as well as trypanosomal sialidases (see in section 9.2.3), also show many structural features in common with viral and bacterial sialidases and are correspondingly members of this family. A DNA sequence of human origin showing features similar to microbial sialidase genes is also known [795]. However,

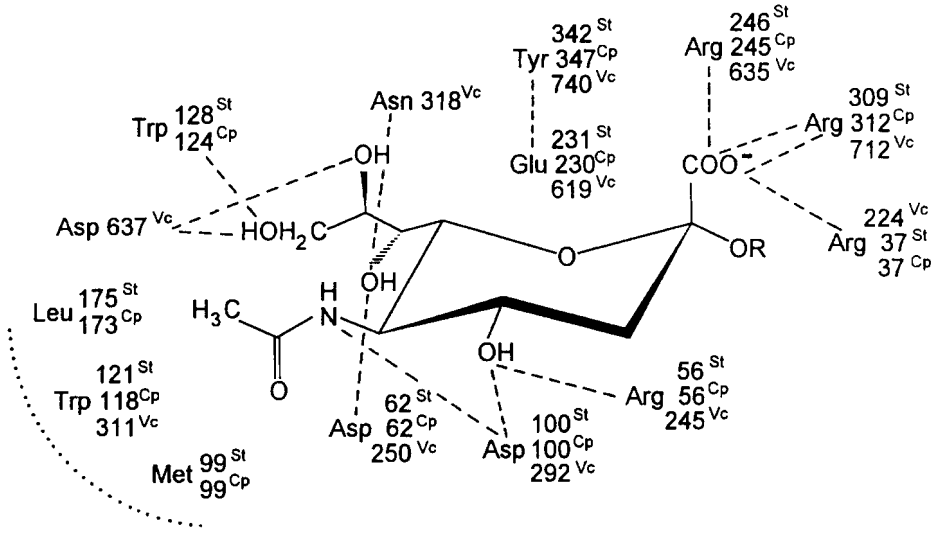


Fig. 18. Amino acids essential for bacterial sialidase action. The positions of the amino acid residues interacting with different parts of the Neu5Ac molecule or forming a hydrophobic pocket (by Leu, Trp and Met; indicated by a dotted line at the left side of the sialic acid molecule) are shown. Vc, *Vibrio cholerae* sialidase [791]; St, *Salmonella typhimurium* sialidase [790]; and Cp, *Clostridium perfringens* sialidase [R.G. Kleineidam, personal communication].

the expression of an active enzyme from this nucleotide sequence has not yet been reported. The human sialidase gene is located on chromosome 10 [796].

Interestingly, the leech sialidase resulting in Neu2,7an5Ac also belongs to this family, since the consensus repeat S–X–D–X–G–X–T–W was found in tryptic peptides of this enzyme [61].

The following are considerations with regard to the evolutionary origin of sialidases. Those enzymes whose gene structures are known, have been found in mammals or in microorganisms frequently living in a symbiotic or pathogenic association. This fact and the similarity of the structures and properties of mammalian and microbial sialidases together with the observation that the acquisition of sialidase activity may be of benefit for the microorganisms (see below) has led to the assumption that the sialidase gene may have been acquired by microorganisms from the host [797]. This may have occurred during infections by the uptake of DNA from decomposed host cells or during the life of microorganisms in intestine. It is therefore necessary to investigate the sialidase genes from lower animals, particularly of echinodermata, which are considered to be the "inventors" of both the sialidase and sialic acids. The sialidase from the starfish *Asterias rubens* was isolated to apparent homogeneity [798] and experiments for the elucidation of its gene structure are intended.

Evidence for a horizontal sialidase gene transfer between bacteria has been obtained by comparison of the similarities of bacterial sialidases so far sequenced [246,660,768,799]. It was found that some of the sialidases are related in accordance with the phylogenetic distances of their producers, e.g. *Micromonospora viridifaciens* and *Actinomyces viscosus*

sialidase, or the "large" (72 kDa) isoenzyme of *C. perfringens* and the *C. septicum* sialidase. On the other hand, the three "small" sialidases (42–44 kDa) produced by *S. typhimurium*, *C. perfringens* and *C. sordellii*, exhibit a higher similarity than is expected from the phylogenetic relationship of the bacterial species. These Gram-positive (*Clostridia*) or Gram-negative (*Salmonella*) bacteria are quite distinct from an evolutionary point of view, but are found at the same ecological location, e.g. in wounds or intestine of vertebrates. Here, an exchange of genes even between unrelated microorganisms might be possible via phages, transposons or plasmids by mechanisms of transfection or conjugation. The participation of phages is indicated by typical sequence motifs up- and downstream from sialidase genes of *S. typhimurium* and *M. viridifaciens* [772,783], and from the observation that the "small" sialidase gene is located near a phage attachment site on the chromosome of *C. perfringens* [800].

Further evidence for such a horizontal gene transfer comes from the investigation of the percentages of the bases G + C, which in the sialidase genes are frequently atypical for the chromosomal DNA of the respective bacterial species. From these data the hypothesis of a gene transfer, from mammals to microorganisms, is supported, since on basis of the mol% G + C, the origin of bacterial sialidase is expected in an organism exhibiting genes which contain about 45 mol% G + C, a value not uncommon in higher animals [660].

9.2.3. Trans-sialidases

As all chemical reactions, the sialidase reaction is theoretically reversible. This has been exploited, by choosing suitable conditions and bacterial or viral sialidases, for the α-2,3- or α-2,6-sialylation of oligosaccharides as final step in the course of chemical synthesis (see section 6.3). During the last years, several sialidases have been discovered in some protozoa which behave like normal sialidases if only water is present, but preferably transfer sialyl residues from one glycan chain to the terminal galactose of another non-sialylated oligosaccharide or glycoconjugate forming α-2,3-linkages.

The first hint for the existence of an unusual sialic acid transfer reaction was given in 1983, when sialic acids (Neu5Ac and Neu5Gc) were detected in *Trypanosoma cruzi* [662,801], which were found not to be synthesized by the parasites themselves. Since the molar ratio of these trypanosomal sialic acids corresponded to that of the incubation medium, their acquisition from this source, or in the case of an infection, from the host glycoconjugates, was assumed. Furthermore, an involvement of the sialidase activity found in these trypanosomes [802] and later localized on their cell surface, was suspected. These observations finally led to the discovery of enzymes called "*trans*-sialidases", first in the American species *T. cruzi* [803–805] and later in the African species *T. brucei* [755] and *T. congolense* [806]. A closer investigation revealed the presence of this enzyme in the whole *T. brucei* group, i.e. several strains of *T. brucei brucei*, *T. brucei rhodesiense* and *T. brucei gambiense* [806]. It also occurs in *Endotrypanum* promastigotes, an intraerythrocytic flagellate distantly related to trypanosomes [807]. In contrast, many other members of the kinetoplastida lack both sialidase and *trans*-sialidase activities, or express only sialidase, such as *T. vivax* [808] and *T. rangeli* [809]. This rapidly expanding field has been reviewed in refs. [246,810]. It is possible to discriminate between morphologically indistinguishable trypanosomatids by measurement of *trans*-sialidase and sialidase activities [811]. Furthermore, the occurrence

of sialic acids on major cell-surface epitopes correlates with the expression of *trans*-sialidase [812].

With regard to substrate specificity, *trans*-sialidases exhibit a preference for sialyl α-2,3-linkages in common with most of the usual sialidases. While for the *T. cruzi* enzyme only α-2,3- but not α-2,6-linked sialic acids are donors in the transfer reaction [550], the *T. brucei trans*-sialidase can also transfer α-2,6-linked sialic acids from sialyllactose to other glycans, however at an eight times lower rate than from sialyl(α2–3)lactose [755]. While both Neu5Ac and Neu5Gc are transferred equally well, Neu5,9Ac_2 from the corresponding sialyllactose derivative serves as sialyl donor only at a much reduced rate [696]. This may be the reason why N'dama cattle exhibiting a high degree of *O*-acetylation of erythrocyte sialic acids are more trypanotolerant than Zebu cattle, whose erythrocyte sialic acids are much less *O*-acetylated [703]. Only β-galactose residues of a variety of oligosaccharides and glycoconjugates are acceptors of these transferases. Sialyl(α2–3)lactose is the best donor for the *trans*-sialidase from *T. brucei* and lactose the best acceptor when compared with serum glycoproteins, mucins and gangliosides [755]. MUNeu5Ac also serves as a sialyl donor. A novel substrate for the assay of *trans*-sialidase activities is MUβ-D-galactopyranoside as sialic acid acceptor which was tested with the *T. brucei* enzyme [806]. The main advantage of this accurate and sensitive assay is the avoidance of radioactive lactose as substrate.

Due to its wide substrate specificity but very limited linkage specificity, the *trans*-sialidase from *T. cruzi* has been used for the sialylation of oligosaccharides, yielding Neu5Ac(α2–3)Gal sequences, in glycotechnological experiments [554], discussed in detail in section 6.3.

Remarkably, the *trans*-sialidases from *T. cruzi* and *T. brucei* are not inhibited by Neu2en5Ac [550,809,813]. As this is in contrast to most other sialidases described in the groups 1–3 of the previous section, Neu2en5Ac is probably not an intermediate of the *trans*-sialidase reaction. Furthermore, *N*-(4-nitrophenyl)oxamic acid and related *N*-acylanilines do not inhibit *T. cruzi* or *T. brucei trans*-sialidases, which is also in contrast to the influence of this substance on the activity of sialidases from *V. cholerae* and other bacteria [813].

The term "*trans*-sialidase" used for the transglycosidase described in this section is not an adequate denomination of this enzyme, which also can react as a conventional sialidase, thus representing a hybrid glycosidase/glycosyltransferase. The reactions are shown in Fig. 12 of section 6.3. The product of the *trans*-sialidase reaction corresponds to that formed by sialyltransferases, however, instead of CMP-Neu5Ac, α-sialyl residues bound to β-galactose of glycan chains or to aglycones such as 4-methylumbelliferone (MU) are the sialyl donors. Therefore, the systematic name for the glycosyltransferase function of this enzyme, acylneuraminosyl-(or sialyl-):β-D-galactoside-α-2,3-sialyltransferase (EC 2.4.99.7?), has been proposed. This name takes into account that both Neu5Ac and Neu5Gc can be transferred.

Presently, several laboratories are intensively studying the gene structures of trypanosomal *trans*-sialidases, in order to understand the differences between this unique enzyme and conventional sialidases. In accordance with the latter sialidases, the *trans*-sialidase genes of *T. cruzi* [768,776,814–818] also contain several Asp-boxes, first found in bacterial sialidases (see section 9.2.2 and Table 18). In addition, the *T. cruzi trans*-

sialidase contains conserved amino acids, found to be involved in the catalysis of *S. typhimurium* sialidase [790] at similar positions [768,819]. It was shown at least for one of these amino acids, namely tyrosine-342, that it is involved in enzymatic catalysis [819]. Enzyme activity was lost after exchange of this amino acid by a histidine residue. Remarkably, the latter, inactive mutant is a member of a group of proteins with similar structures, expressed by several homologous genes, of which only some exhibit *trans*-sialidase activity [815,816]. The others are enzymatically inactive and their function is as yet unknown. However, these studies show that all animal, microbial and probably viral exo-sialidases and *trans*-sialidases studied belong to one gene family [660,768].

As first observed by Pereira et al. [776], the *T. cruzi trans*-sialidase consists of an N-terminal half, containing the enzymatic function and a C-terminal portion, composed mainly of a tandem series of twelve amino acid repeats. Four Asp-boxes exist within the N-terminus of the enzyme. However, only three of these conserved motifs occur in positions of the amino acid sequence similar to those found in bacterial sialidases, while the distance between the third and the fourth box is much larger in the parasite enzyme than in the bacterial proteins. It may be assumed that this extended sequence together with some other structural features is a domain functioning in the *trans*-sialidase mechanism [768,815]. The carboxy-terminal repetitive motif is most probably not involved in catalytic activity [815,820] and a recombinant *T. cruzi trans*-sialidase lacking the amino acid repeats was shown to retain the enzymatic activity [821]. Remarkably, the American parasite *T. rangeli* secretes a conventional sialidase [809] lacking *trans*-sialidase activity [822], the primary structure of which is related (70% identity of the amino acid sequences) to that of the *trans*-sialidase of *T. cruzi* [777]. Sequences encoding the tandemly repeated motif characteristic of the members of the *trans*-sialidase family were not detected in the *T. rangeli* genomic DNA.

T. cruzi trans-sialidase first named SAPA ("Shed-Acute-Phase-Antigen") and found in human serum in Chagas disease, is also unique from an immunological aspect, since it contains two immunologically distinct domains and seems to be a naturally chimeric protein having functionally independent enzymatic and antigenic domains [823]. (It is thus reminiscent of *V. cholerae* sialidase which consists of a catalytic domain and a non-catalytic sequence similar to those of legume lectins [768,791].)

Antibodies against both regions have been raised, and the one recognizing the catalytic region inhibits enzyme activity [788,823,824]. The SAPA epitopes were shown to be located on the tandemly repeated 12-amino-acid units and to induce an early and strong antibody response in acute and congenital human infections, as well as in mouse infections. In patients, antibodies against the immunodominant, non-enzymatic domain are formed much earlier (8 days) after infection than against the enzymatic domain (about 30 days) [823,824]. The availability of antibodies may be suitable for diagnosis and therapy of Chagas disease which affects several million people in Central and South America. The structural and functional properties of *Trypanosoma trans*-sialidases have been reviewed [825,826].

9.2.4. Pathophysiological significance of sialidases and trans-sialidases

9.2.4.1. Eukaryotic (trans-)sialidases. It is well known that sialidases are involved in the breakdown of sialoglycoconjugates, which may be of physiological or pathological

significance. These enzymes are physiologically important in the lysosomes, their main localization in animal cells, where they release sialic acids from glycoconjugates, thus initiating the degradation of these molecules by other glycosidases or proteases [5,8,33, 244]. The significance of mammalian sialidases is indicated by diseases, called sialidosis [827]. In these genetic disorders, sialidase activity is reduced or lacking, resulting in reduced turnover of sialylated glycoconjugates, excretion of sialo-oligosaccharides in the urine as well as mental and physical impairments (see below).

Since sialidases not only occur in lysosomes, but also in other compartments such as the cytosol or plasma membranes [33,244,246,660], other cellular functions of these enzymes are assumed. This is easily understandable, since sialic acids are not only structural but also functional constituents of many biologically active molecules of the cell, for example receptors, ion channels and growth-regulating molecules (refs. [246,660] and section 10). Sialidases may regulate the functional activity of these and other molecules. Plasma-membrane-bound sialidase for instance is involved in the degradation of cell membrane gangliosides, which may not only represent a catabolic function, but also a regulative effect on the biological functioning of these molecules [828]. A striking example of such a role is the myelin-associated sialidase of rat oligodendroglial cells which was found to adhere to immobilized GM1 and to be inhibited by this ganglioside [829]. This interaction can also be inhibited by the sialidase inhibitor Neu2en5Ac. It is concluded from these experiments that the sialidase–GM1 interaction plays a role in the formation and stabilization of the multilamellar structure of the myelin sheath.

Another system of eukaryotic sialidases, whose significance we are just beginning to understand, is the trypanosomal *trans*-sialidase described above. It is evident that the main function of this enzyme is the acquisition of sialic acids by the parasites at expense of the host which is presumably of benefit for the trypanosomes. In the case of *T. brucei* the accepting glycoprotein is the GPI-anchored "procyclic acidic repetitive protein" (PARP/procyclin) [755] and in the case of *T. cruzi* the Ssp-3 epitope of trypomastigotes [823]. In epimastigote forms glycoproteins with molecular masses of 38–43 kDa were described to be the main sialic acid acceptor, and the structures of the oligosaccharides of these glycoproteins, linked O-glycosidically via GlcNAc to serine or threonine, were elucidated [830].

Some understanding of this chemical modification of cell surface molecules may come from consideration of the period of sialylation during the life cycles of the African and American trypanosomes. In *T. brucei,* sialylation only occurs in the insect stage, where sialic acids are probably derived from mammalian blood sucked in by the tsetse fly [806]. In contrast, in *T. cruzi* sialic acid is acquired by the parasite not in the insect (triatomine bugs) stage, but only during its life in the mammalian host. Since sialylation may be of advantage to the trypanosomes, it is hypothesized that the dense, charged glycocalix formed by sialylation of *T. brucei* may protect *T. brucei* from the attack of proteases, glycosidases, antibodies or complement from the fly's digestive tract or from the ingested blood meal [806]. It is also conceivable that the parasites require sialic acids for maturation and adhesion to the fly's salivary glands before transfer to the mammalian hosts. Furthermore, after infection, sialylation may be of benefit for the survival of the parasites in the primary infection site ("chancre") of the host's skin, before the non-sialylated variable surface antigenic glycoproteins (VSG) are expressed. These

antigens then represent another potential site for the immunological protection of the parasites when sialic acids are no longer available.

Since *T. cruzi* does not express such variable antigens on the cell surface, it is assumed that it starts to express *trans*-sialidase in the mammalian host to capture sialic acids for defense against the host's immune and complement systems and against phagocytosis [818,831,832]. The role of sialic acid in the resistance of *T. cruzi* trypomastigotes to complement has clearly been shown [833]. Other experiments demonstrated that the acquisition of sialic acids increases the infectivity of *T. cruzi* and is involved in invasion of host cells by these parasites [823,831,834], and accordingly the action of sialidase reduces their virulence. In mice the virulence-promoting activity of *trans*-sialidase was achieved with tiny doses of 1–2 μg enzyme/kg animal [835]. (An increase of pathogenicity, i.e. evasion of host defence systems, is also achieved by sialylation of some pathogenic bacteria, such as group B *Streptococcus* [836] and *Neisseria gonorrhea* [837] (see also Table 3 and sections 3 and 10.2). Correspondingly, monoclonal antibodies against the Ssp-3 epitope, the target of *trans*-sialidase activity and required for invasion after sialylation inhibit infection of the host cell [823,838]. Such antibodies or other *trans*-sialidase inhibitors (see below), as well as prevention of the expression of this enzyme on the gene level, may become potent therapeutic tools in Chagas and Nagana (sleeping) diseases.

The role of conventional sialidases secreted by non-sialylated trypanosomes lacking an insect vector (see above), such as *T. rangeli* [809,822] or *T. vivax* [806], is unknown. It is likely that they hydrolyze sialic acids from host glycoconjugates for nutritional purposes as do bacteria (see below). It is, however, also possible that these trypanosomes demask recognition sites on host cells, e.g. subterminal galactose residues, for their attachment mediated by lectins or other receptors on the parasite's surface. This hypothesis clearly warrants further investigation.

Infection of host cells by *T. cruzi* parasites is a complicated mechanism which is not yet fully understood. However, sialylation of both cell types seems to be necessary for this process. For the attachment and penetration of *T. cruzi* into host cells the latter must be sialylated, as was shown with CHO cell mutants expressing no or varying amounts of sialic acids. O-Linked sialylated glycans of host cells were shown to be particularly important for infection [838]. Various trypanosomal surface glycoproteins are considered to be involved in adhesion, i.e. the developmentally regulated MTS-gp82 [839], a small mucin-like sialoglycoprotein [840], or enzymatically inactive members of the (*trans*-)sialidase family, which may be capable of binding sialic acid without cleaving it, and in this way may function as mammalian-stage-specific surface receptors [841]. On the parasite surface, the relative balance between exposed sialic acid and galactose/*N*-acetylgalactosamine residues may determine the parasite's capacity to invade host cells including macrophages [842]. A stage-specific 82 kDa adhesion molecule of *T. cruzi* metacyclic trypomastigotes may play a role in host cell invasion [843].

After binding, *T. cruzi* enters host cells via an acidic vacuole, which fuses with lysosomes [818,844–846]. The parasitophorous vacuole formed in this way is later disrupted in order to release the trypanosomes into the cytosol. *Trans*-sialidase activity seems to be required for the latter step, as it desialylates the highly sialylated lysosomal

membrane, which then becomes more susceptible to a parasite hemolysin [844,846]. The crucial role of sialic acid in host-cell–parasite interaction and the reversible sialylation of cell surface molecules mediated by sialidase and *trans*-sialidase activities, which in some respects resembles the infection of mammalian cells by influenza viruses, has also been pointed out in other studies [826,847].

In a species of *Acanthamoeba,* another protozoon, the sialidase of the trophozoites and cysts also seems to play a pathophysiological role, as it was shown to be involved in colonization and damage of the sialic-acid-rich corneal epithelium [848]. Spreading of the microorganisms may have been facilitated by a decrease in the viscosity of the protective mucus layer occurring after desialylation.

9.2.4.2. Bacterial sialidases. In bacteria, sialidases also seem to be of great significance in pathogenesis, although only few well-defined effects are known, in contrast to the wide occurrence of this enzyme. Strikingly, bacterial species pathogenic for higher animals and man often produce sialidases so that it is suspected that they enhance virulence. It was also discussed above that bacteria may have acquired the sialidase gene from the mammalian host, which may have been of evolutionary advantage.

Certainly, not only one function can be attributed to bacterial sialidases, since their cellular localization varies much with the species. They may be intracellular, periplasmic, membrane-bound or excreted, and they vary in their properties such as complexity, molecular mass and substrate specificity (refs. [33,244,246,660,768], and section 9.2). However, they belong to one gene family.

One of the main functions of bacterial sialidases is nutritive, providing an energy source for the microorganisms [245,246]. This has long been assumed and a model first proposed for the *C. perfringens* enzyme shows that sialic acids released from host tissues by secreted sialidase are taken up by the bacteria with the aid of a permease, intracellularly cleaved by acylneuraminate-pyruvate lyase to pyruvate and *N*-acylmannosamines (section 9.3) and then further metabolized [5,33,245]. After the loss of sialic acids, host cell membrane glycoconjugates are more vulnerable to the action of other glycosidases and proteases secreted by bacteria, since the presence of terminal sialic acids on glycoconjugates is known to hinder the action of these enzymes [5]. Thus, sialidase can be considered as pacemaker in the breakdown of host cells by a lytic cocktail secreted by many infectious bacteria.

Suitable models are required to verify this assumption. For example, the advantage of the availability of free sialic acid for bacterial survival has been demonstrated in two model systems [849]. The growth of two isogenic strains of *Bacteroides fragilis* in CHO cell culture monolayers *in vitro* or in rat granuloma pouches *in vivo* was compared. While one strain expressed sialidase, the activity of this enzyme was deleted in the other group. At the beginning of the culture, both strains grew equally well, however, after 48 to 72 h, the sialidase-producing strain was enriched. The latter bacteria grew more rapidly than those lacking sialidase, because after the consumption of glucose some 16 to 24 h after infection, other carbon sources, including liberated sialic acid, were required for further bacterial growth. This experiment supports the assumption that sialidase may facilitate spreading and thus virulence of bacteria in infected tissues. Similar conclusions were drawn from studies on arteritis caused by *Erysipelothrix*

rhusiopathiae [850], in which a close correlation between bacterial invasion, desialylation and cell infiltration in the common iliac artery was observed.

Several clostridial species secrete large amounts of this enzyme into their environment, which, together with other lytic enzymes, is believed to facilitate their rapid and life-threatening spreading and desialylation of vital structures in the whole organism [851, 852]. The sialidase production of *C. perfringens* is so great that it can be detected not only in wound fluids, but also in blood serum. Antibodies were raised against sialidases from *C. perfringens, C. septicum* and *C. sordellii,* the main causative agents for clostridial myonecrosis or gas oedema, which allow an early, sensitive and specific diagnosis of this infection in wound fluids or blood serum. This enables a prompt, specific and therefore life-saving treatment of this severe disease.

The action of sialidase also contributes to the liquification of mucin barriers lining endothelia, as mentioned above for *Acanthamoeba* infection of the eye. This property is most relevant in tracheobronchial, intestinal and urogenital tracts [245,853]. Often other hydrolases, "mucinases", support the viscosity-decreasing effect initiated by mere desialylation of mucins, as was observed with intestinal [854,855], vaginal [856] and other [857] bacteria, as well as with the flagellates *Trichomonas vaginalis* and *T. foetus* [858]. Microbial sialidase is considered a risk factor for intra-uterine infection and preterm birth [858].

O-Acetylation of mucin-bound and membrane-bound sialic acids makes them more resistant towards sialidase action [8,33,245,853,854]. This may be one of the reasons why intestinal mucins, especially of the colon, are often *O*-acetylated (ref. [245], and section 8.4.2). The high level of *O*-acetylation of sialic acids observed in the endothelia of blood vessels, e.g. in liver, detected by histochemical methods using influenza C virus hemagglutinin, is assumed to have a similar function [234,235,730].

A pathogenic role facilitating colonization and invasion of the host during the development of pneumonia is also attributed to the sialidase from *Streptococcus pneumoniae* [859]. This enzyme has also been sequenced and found to contain four copies of the sequence S–X–D–X–G–X–T–W typical for sialidases [784]. The antigenic potential of this enzyme as well as other pneumococcal proteins makes them potentially suitable candidates for the vaccination against lung inflammation (refs. [784,860], and [K. Fischer, personal communication]). During the inflammation of bovine lung, sialidase from *Pasteurella haemolytica* may play a supportive role [861].

In all these cases, the production of sialidase may be physiologically important for the microorganisms causing an enhancement of their virulence. A further, well-defined example for such a role is the *Vibrio cholerae* sialidase, which is an accessory virulence factor, causing a partial desialylation of higher sialylated gangliosides in intestinal endothelia, resulting in GM1 [770]. Cholera toxin subsequently binds to this ganglioside and is taken up by intestinal cells, which are stimulated to secrete cell fluid required for growth of the vibrions in the intestinal lumen. The efficiency of the sialidase in this process is increased by the capability of the enzyme to attach to cell surfaces via lectin-like domains [768,791].

Since sialic acids are involved in so many important physiological processes in animals and man (section 10), the flooding of a tissue or the whole organism with bacterial sialidases is potentially disastrous, leading to acute and chronic diseases.

As a consequence of sialidase production by microorganisms and viruses, chronic inflammatory or immunological diseases such as glomerulonephritis or asthma may result [245,795]. Sialidase-producing bacteria in disease and phenomena resulting from the action of sialidases in bacterial infections have been summarized [245].

9.2.4.3. Viral sialidases. Sialidase is a surface-bound enzyme of ortho- and paramyxoviruses as reviewed [246,862]. The enzymes of influenza A and B viruses have been intensively studied with regard to their structure, substrate specificity, immunological properties and function during viral infection. These sialidases play a crucial role during penetration of the virus into cells and probably also in their release from infected cells, especially of respiratory endothelia, after the attachment to these cells via the sialic-acid-specific receptor hemagglutinin [246,863,864]. The first step of this process is the binding of viruses to cell surfaces, which is followed by loosening of these linkages as a prerequisite for endocytosis of virus particles. Thus, the host sialic acid is exploited by the infecting virus. Sialidase may also be required by the virus to reach the endothelial surface and not to be bound to the highly sialylated, sticky, protective mucus layer, which would lead to elimination of the virus from the respiratory tract.

9.2.5. Sialidase and trans-sialidase inhibitors

The involvement of sialidases in the severe pathogenesis of many viruses and other microorganisms and the aggravation of these infectious diseases including late and often irreversible, e.g. immunological effects prompted pharmaceutical strategies for the inhibition of these enzymes. Some sialidase inhibitors will therefore be described in the following. For the terminology and abbreviations of natural and synthetic sialic acids see Tables 1 and 13 and section 6.1.

A survey of sialidase inhibitors was given earlier [5,33,865] and some recent inhibitors of higher efficiency ($K_i < 10^{-4}$ M) are listed in Table 19. Neu2en5Ac is a competitive inhibitor which is still widely used, since it inhibits most viral, bacterial and mammalian sialidases with a K_i of around 5 μM. This substance is therefore very useful to prove the specificity of sialidase action when studying the role of sialic acids in biological experiments. The replacement of the *N*-acetyl moiety in Neu2en5Ac by an *N*-trifluoroacetyl residue leads to an about 2-fold better inhibitor of *V. cholerae* sialidase with a K_i of $1.3–1.8 \times 10^{-6}$ M [389,866]. In the case of 9d-Neu5Ac a K_i of 1×10^{-5} M was measured [869]. Using *V. cholerae* sialidase, it was shown, with various Neu2en5Ac derivatives structurally varied at C4, that the highest inhibitory effect was obtained with compounds having an axial hydroxyl residue, as in the parent substance Neu2en5Ac [430], whereas the 4epi- and 4d-derivatives were less inhibitory. These studies also revealed that 4amino-Neu2en5Ac inhibits *V. cholerae* sialidase only slightly, which is in contrast to influenza virus sialidases. The transition-state analogue 4d-Neu2en5Ac inhibits influenza virus sialidase with a K_i of 8×10^{-5} M) [441].

Most interest is currently being focussed on the inhibition of viral sialidases, since their role in the infection mechanism has long been known. The fact that Neu2en5Ac is a transition-state analogue for the sialosyl residue during the viral enzyme reaction described in section 9.2.1 [60,320], served as a point of reference in the design of new inhibitors. The observation that the 4-*O*-acetyl group in the ring of Neu4,5Ac$_2$

Table 19
Sialidase inhibitors[a]

Name	Inhibition (M)	Sialidases tested	Reference(s)
Neu2en5Ac	$K_i = 1\times10^{-6}$–2×10^{-5}	viral, bacterial, mammalian	[428,865]
5-Trifluoroacetyl-Neu2en	$K_i = 8\times10^{-7}$–2×10^{-6}	viral, bacterial	[389,866]
4amino-Neu2en5Ac	$K_i = 4\times10^{-8}$–7×10^{-4}	viral, bacterial, mammalian	[428,867,868]
4guanidino-Neu2en5Ac	$K_i = 1\times10^{-9}$–1×10^{-2}	viral, bacterial, mammalian	[428,867,868]
$3F_{eq}$-Neu5Ac	$K_i = 2\times10^{-6}$–8×10^{-6}	viral, bacterial, mammalian	[442]
2d-6amino-$2H_{ax}$-Neu5Ac	$K_i = 5.4\times10^{-5}$	bacterial	[438,452]
2d-4epi-6amino-$2H_{ax}$-Neu5Ac	$K_i = 2.9\times10^{-5}$	bacterial	[438,452]
Phosphonate analogue of Neu5Ac	$K_i = 5.5\times10^{-5}$	bacterial	[449]
7d-Neu2en5Ac	$K_i = 9.0\times10^{-5}$	bacterial	[869]
8d-Neu2en5Ac	$K_i = 5.0\times10^{-5}$	bacterial	[869]
9d-Neu2en5Ac	$K_i = 1.1\times10^{-5}$	bacterial	[869]
8epi-Neu2en5Ac	$K_i = 3.8\times10^{-5}$	bacterial	[870]
Neu5Acα(2-S-6)Glc(β1–1)ceramide	$K_i = 2.8\times10^{-6}$–4.0×10^{-4}	viral, bacterial	[871]
Neu5Acα(2-S-6)Gal(β1–4)Glc(β1–1)ceramide	$K_i = 1.5\times10^{-5}$	viral	[871]
Siastatin A and B	$K_i = 1.7\times10^{-5}$–4.3×10^{-5}	bacterial	[865,872]
N-(1,2Dihydroxypropyl)-siastatin B	$IC_{50} = 1.0\times10^{-5}$–$2.7\times10^{-5}$	bacterial	[873]
N-(1,2Dihydroxypropyl)-4-deoxy-siastatin B	$IC_{50} = 5\times10^{-6}$–5×10^{-5}	bacterial	[873]
3epi-Siastatin B	$IC_{50} = 1\times10^{-5}$–7.4×10^{-5}	viral	[874]
5,7,8,4′-Tetrahydroxyflavone (Isoscutellarein)	$K_i = 4.1\times10^{-5}$	viral	[875]
5,7,4′-Trihydroxy-8-methoxy-flavone	$IC_{50} = 5.5\times10^{-5}$	viral	[875]

[a] For the abbreviation of natural and synthetic sialic acids see Tables 1 and 13, respectively.

completely hinders the action of mammalian and bacterial sialidases [5,33] but allows slow hydrolysis of the glycosidic bond by viral enzymes [252,876], as well as the finding by X-ray crystallography studies that in the active center of viral sialidases only acidic amino acids (i.e. aspartic acid) interact with HO4 of sialic acid, led to the synthesis of much more potent inhibitors of influenza viruses. These are 4amino-Neu2en5Ac and 4guanidino-Neu2en5Ac [428,429,867,868,877], which exhibit K_i values in the nanomolar range, corresponding to 2–4 orders of magnitude lower than Neu2en5Ac. The guanidino derivative inhibits more effectively than the amino compound. 4-Guanidino-Neu2en5Ac was shown to inhibit influenza virus A sialidase in a slow-binding fashion, and a K_i value of 3×10^{-11} M was observed for the tightly bound form under steady-state conditions [878]. This strong binding to viral sialidases and not to those from bacteria or mammals, is due to the existence of a pocket in the binding site of viral sialidases near to the HO4 group of sialic acid [867,879,880]. This pocket is considered to be the reason why Neu4,5Ac$_2$ [252] and Neu5Ac4Me [407] can be released by viral sialidase, but not by the bacterial enzyme. Since the other sialidases do not seem to have this pocket (for bacterial sialidases see refs. [790,881]) and are therefore inhibited to a much lesser extent, the new Neu2en5Ac derivatives appear suitable for the prophylaxis and/or therapy of influenza A and B virus infections [867]. It has been shown for 4guanidino-Neu2en5Ac that it inhibits the growth of a wide range of influenza A and B viruses in *in vitro* systems, including human respiratory epithelium, at lower concentrations than classical inhibitors of virus replication such as amantadine or ribavirin [868,882–884]. The same effect was observed in mice and ferrets when the new compounds were administered as a nasal spray [867,880,882], thus raising hopes that these non-toxic substances can be used therapeutically during epidemics of influenza in man. These viral sialidase inhibitors are an excellent example of how drugs may be designed on the basis of a crystal structure of a target protein and on other biochemical parameters [867]. Potent inhibitors of bacterial sialidases or trypanosomal *trans*-sialidases are also desirable as pharmaceuticals to assist the therapy of inflammatory diseases caused by these microorganisms.

Some natural substances exist which inhibit viral sialidases in the 10 μM range, such as siastatin A and B and panosialin [865] as well as flavonoids [875] (Table 19). Two epimers of siastatin B, 3-epi-siastatin B and 3,4-diepi-siastatin B, have been synthesized which are good inhibitors of sialidases from various influenza virus strains, as well as from Sendai and Newcastle disease viruses [874]. They effectively inhibit the growth of these viruses in cell cultures. Sialidases from *Streptococcus* sp. and *C. perfringens* are inhibited to an extent comparable to Neu2en5Ac by the *N*-(1,2-dihydroxypropyl) derivative of siastatin B and its 4-deoxy analogue [873]. Isoscutellarein (5,7,8,4′-tetrahydroxyflavone) isolated from the leafs of *Scutellaria baicalensis* is a good inhibitor of influenza virus sialidases ($K_i = 4 \times 10^{-5}$ M). It is not toxic to mice and inhibits replication of influenza viruses in Madin–Darby bovine kidney cells. It was shown to be a better inhibitor than amantadine. The 8-methoxy derivative of isoscutellarein is also a potent inhibitor [885].

Thioglycoside-analogues of gangliosides [871] (for structures see Table 19) are good inhibitors of viral sialidases, but hinder the bacterial enzymes to a considerably lower degree ($K_i = 0.1$ mM). Various sialyl(α2–6)galactosides in which one of the hydrogen atoms at C6 of Gal has been replaced by a methyl group, yielding *R* and *S* isomers, and in some of which thioglycosidic bonds occur, act on both viral and bacterial

sialidases [886]. Although their inhibition constants are only in the millimolar range, these compounds are expected to have important implications in the rational design of sialidase inhibitors. The finding that these glycosides are the first common inhibitors of both hemagglutinin and sialidase of influenza viruses may prove to be of great advantage. The *o*-(difluoromethyl)phenyl-glycoside of α-Neu5Ac irreversibly inhibits *C. perfringens* sialidase [887]. This mechanism-based inhibitor acts by a fluoromethylene-quinone formed spontaneously from the liberated difluoromethylphenol. Analogues of free Neu5Ac, such as HO4 epimers of 6-amino-6-deoxy-sialic acid are competitive inhibitors of medium potency for both viral and bacterial sialidases [438]. 3F-Neu5Ac strongly and competitively inhibits bacterial, viral and mouse spleen sialidases [442]. Other strategies for the production of sialidase inhibitors are the Neu5Ac isomer with a 6-acetylamino group occurring in the furanose ring form, which could be interpreted as a 6-acetylamino derivative of Kdn or 4epi-Kdn [888]. Promising inhibitors also represent 4azido-Neu5Ac-, 4amino-Neu5Ac- and 4NAc-Neu5Ac-containing sialosides and thiosialosides [889].

Another principle for tailor-made sialidase inhibitors is the design of low-molecular-mass compounds that mimic the binding function of a macromolecular antibody. Such studies are based on the crystal structure of an influenza virus N9-sialidase (antigen)–NC41 (antibody) complex, showing the direct contact of four amino acid residues on the antibody binding surface with the active-site loop 368–370 of the antigen [890]. Correspondingly, a constrained cyclic peptide composed of 5 amino acids was synthesized, which mimics the receptor-bound conformation of these amino acids and inhibits the sialidase with a K_i of 0.1 mM. Although this is about 3 orders of magnitude less than the parent protein, because the antibody forms contacts with about 17 amino acid residues of the antigen sialidase, it nevertheless opens a possible new approach for the design of more potent inhibitors of this important viral enzyme.

The rapid development of research on trypanosomal *trans*-sialidases, their involvement in the pathogenic mechanism and the morbidity of men and agricultural animals caused by these parasites, has evoked considerable interest in inhibitors of these enzymes. One potential hope is the availability of antibodies, mentioned in sections 9.2.3 and 9.2.4.1. There it was also outlined that classical sialidase inhibitors like Neu2en5Ac and *N*-(4-nitrophenyl)oxamic acid are not effective on the *trans*-sialidases studied. A non-immunological, non-competitive inhibitor of *T. cruzi* sialidase activity, named cruzin, was first found in blood plasma from patients with Chagas' disease and later identified as high density lipoprotein (HDL) [834,891]. This lipoprotein does not inhibit sialidases from *T. rangeli*, various bacteria and influenza viruses. Remarkably, in high concentrations it enhances infection of culture cells by *T. cruzi* trypomastigotes, while *V. cholerae* sialidase reduces it, which points to the significance of sialylated structures on the parasites during infection. All these effects could be explained by the fact that HDL is not an inhibitor of *T. cruzi* sialidase, but as a glycoprotein provided sialic acids for the *trans*-sialidase action of this enzyme, thus mimicking an inhibition of enzymatic sialic acid release in the test system used [815].

9.3. Sialate-pyruvate lyase

As shown in Fig. 13, sialic acids liberated by sialidase are degraded by the aldolase systematically named sialate (acylneuraminate)-pyruvate lyase (EC 4.1.3.3), resulting in

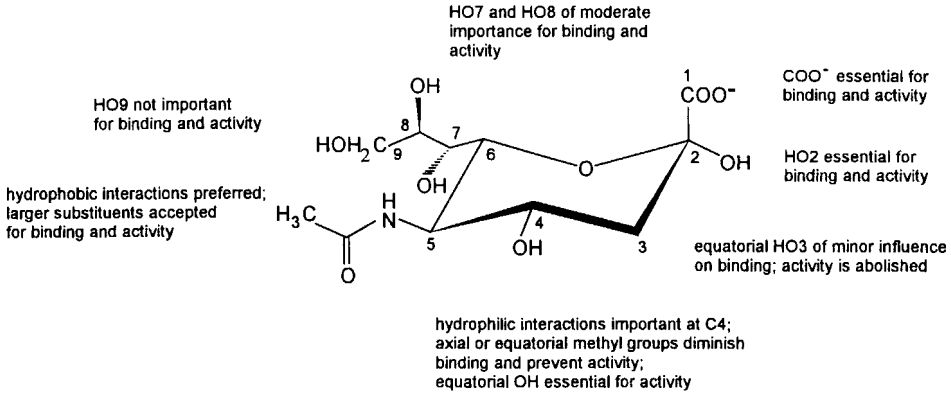


Fig. 19. Model of sialic acid structural features required for the interaction with sialate lyase and binding of inhibitors. Based on data from ref. [892].

acylmannosamines and pyruvate. This reaction and the occurrence of the soluble enzyme in both bacteria and higher animals as well as the influence of sialic acid substituents and enzyme inhibitors has been reviewed [5,8,33]. The biological role of this lyase in bacteria is believed to be nutritive, by splitting the sialic acid 9-carbon chain as prerequisite for further consumption in the energy-producing metabolism. In animals, the main function of the cytosolic enzyme seems to be in the degradation of sialic acids in order to regulate sialic acid metabolism by prevention of recycling of this sugar.

With regard to substrate specificity, the lyase from pig kidney, the first mammalian sialic-acid-specific aldolase purified [891a], shows similar behavior to the enzyme from *Clostridium perfringens* studied earlier [5,33]. The relative cleavage rates for Neu5Ac, Neu5Gc and Neu5,9Ac$_2$ are 100%, 70% and 33%, respectively. Neu4,5Ac$_2$ and Neu2en5Ac are inactive. An investigation into the kinetic behavior of various epi- and deoxy-analogues of Neu5Ac with the lyase from *C. perfringens* revealed that modifications at C8 and C4 strongly reduce cleavage rates and affinity of the substrates to the enzyme, or are even inhibitory [409,892,893]. 4Epi- and 4d-Neu5Ac were most inhibitory, with K_i values in the millimolar range. Computer-assisted drawings of these sialic acids (CPK models) indicate that the region most important for the binding of sialic acids to the enzyme is an equatorial zone stretching from C8 via the ring oxygen atom to C4 of the monosaccharide. The substituents at C5 and C9 may be varied to a greater extent without significantly disturbing enzyme action. The structural features of sialic acid required for the interaction with the lyase [892] are depicted in Fig. 19.

With regard to enzyme catalysis, for the *C. perfringens* lyase a Schiff-base mechanism involving a lysine residue was elucidated to be part of the catalysis of this enzyme [5, 33]. The participation of a histidine residue in the cleavage reaction was also shown to be likely. The same amino acids probably are also parts of the active center of the aldolase purified from porcine kidney [891a], as was shown by borohydride reduction in the presence of Neu5Ac and the influence of reagents such as Rose Bengal which interacts with histidine. Heavy metal ions and other substances were also inhibitory, pointing to the essential role of cysteine residues.

^{1}H NMR spectroscopic investigations have shown that only the α-anomer of Neu5Ac is consumed by the *C. perfringens* lyase, yielding α-ManNAc, as described in detail in section 5.3.5. From this observation, the chemical modification experiments, and models drawn in refs. [33,892], a reaction scheme for the heterolytic fragmentation of cyclic sialic acids into pyruvic acid and *N*-acylmannosamines is delineated (Fig. 20, overleaf).

Since the enzyme reaction is reversible, conditions for the synthesis of Neu5Ac and natural or synthetic derivatives in high yield as well as of Kdn with the aid of bacterial lyase were elaborated (see section 6.1). The recombinant and overexpressed sialate–pyruvate lyase from *E. coli* is now in wide use as a specific chiral catalyst which mediates highly enantioselective aldol condensation reactions leading to a variety of sialic acids.

The enzyme first purified from *E. coli* [894] was cloned, the nucleotide sequence of its gene elucidated [895–897] and later crystallized [898]. The lyase gene encodes a polypeptide of 297 amino acids [897]. The three-dimensional structure of this aldolase from *E. coli* has been investigated by X-ray crystallography and shown to be a tetramer, each subunit representing an eight-stranded α/β-barrel [899] (Fig. 21). The active center was tentatively identified as a pocket located at the carboxy-terminal end of this barrel. Lysine-165 lies within this pocket and is probably the reactive residue forming the Schiff-base intermediate with the substrate described above (see Fig. 20). Several additional amino acids were recognized to line this pocket.

It is tempting to speculate that, as with sialidases, an exchange of the gene for the sialate lyase between mammals and microorganisms may have occurred. This assumption is based on the similarity of many properties of both bacterial and mammalian lyases studied so far.

It has been mentioned in section 5.3.2 and refs. [11,12] that the commercially available sialate lyase is an excellent tool for the analysis, e.g. by HPLC or GLC, of a substance suspected to be a sialic acid. The enzyme has also proved to be useful in clinical determinations of these monosaccharides [900,901]. For the analysis of glycoconjugate-bound sialic acids, e.g. in the bloodstream, a combination with sialidase treatment is necessary.

9.4. Sialic acid permease

Although only few experiments have been carried out, sialic acids do not seem to be able to penetrate bacterial and animal cell membranes at significant amounts. Only minimal amounts of radioactivity from Neu5Ac are taken up by surviving tissue slices from porcine submandibular gland [679]. Furthermore, Neu5Gc and Neu2en5Ac administered orally and intravenously into mouse and rat are excreted rapidly, mainly in the urine [902]. While the unsaturated sialic acid was excreted completely, less than 10% of the radioactivity from Neu5Gc was found in the tissues. It is assumed that most of this portion was cleaved by the sialate-pyruvate lyase before its uptake by cells.

Since sialic acids released from host glycoconjugates by sialidases are of advantage for the growth of bacteria, as described above, it is conceivable that these microorganisms developed a mechanism for the uptake of these anionic sugars [245]. First hints for the existence of a sialate permease were obtained with *C. perfringens* when studying the induction of both sialidase and sialate lyase with free or glycopeptide-bound

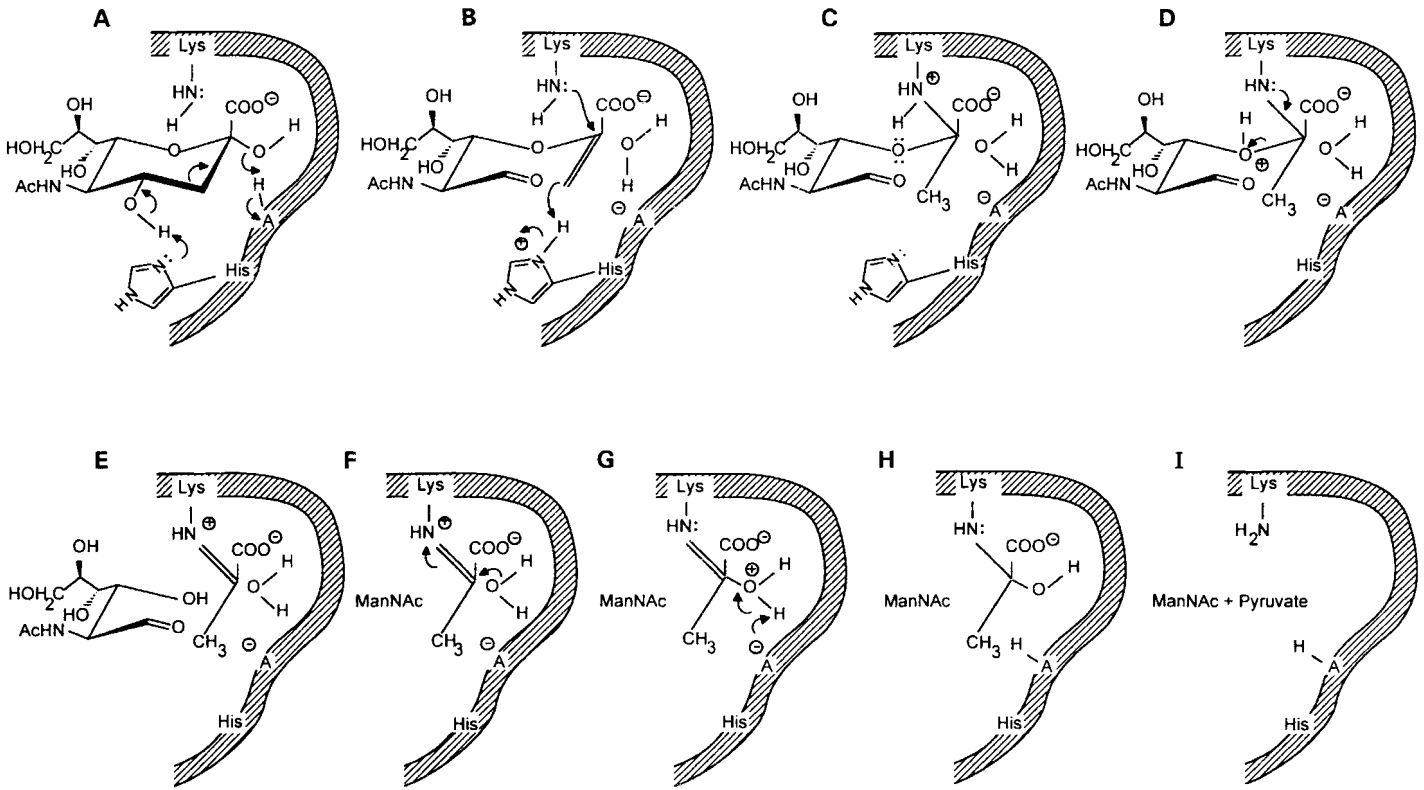


Fig. 20. Proposed reaction scheme of sialate-pyruvate lyase from *Clostridium perfringens*. Based on data from refs. [33,892].

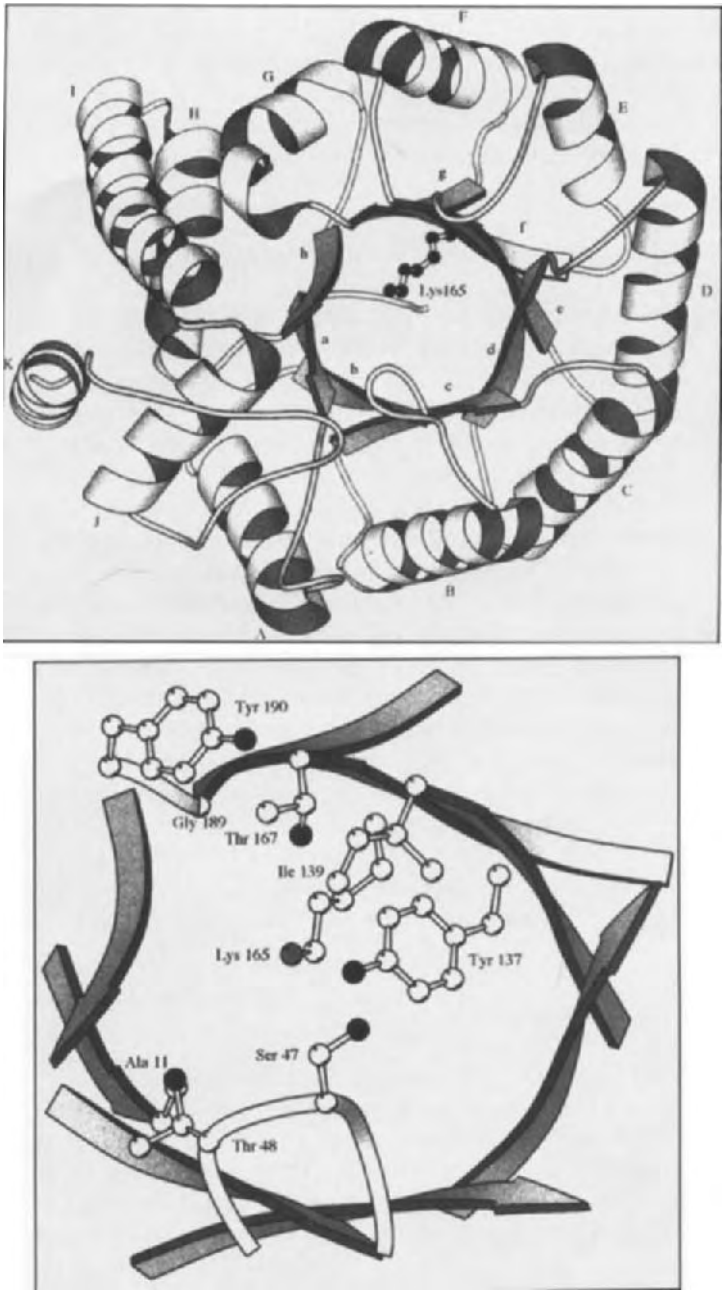


Fig. 21. Three-dimensional structure of the sialate-pyruvate lyase from *Escherichia coli*. (top) Viewed down the β-barrel axis from the carboxy-terminal end of this aldolase. The putative catalytic residue Lys-165 is shown in ball-and-stick representation. (bottom) Putative active site of Neu5Ac lyase showing the side chains of nine of the residues forming the surface of the pocket. Carbon atoms are white, oxygens black and nitrogens grey. From ref. [899] by permission of Current Biology Ltd., London.

Neu5Ac [903]. A K_M value of 0.3 mM for Neu5Ac was measured for this hypothetical transporter protein. This question has not yet been solved in *C. perfringens,* although recent experiments have also shown that sialic acid uptake is not due to mere diffusion (C. Traving et al., unpublished). However, in *E. coli* evidence for a sialic acid permease was obtained, based on molecular biological studies [585,904].

10. Physiological and pathobiochemical significance of sialic acids

The growing understanding of the involvement of sialic acids in many biological and pathological processes has considerably enhanced interest and research in this field. It started in 1946 when McFarlane Burnet and colleagues [905] recognized the reversible binding of influenza viruses to mammalian erythrocytes via cell surface sialic acids. Several reviews on the biological aspects of sialic acid biochemistry have appeared [5,8,245,660,663,690,853,906–909].

From these articles it is clear that there are general functions of sialic acids resulting from their physico-chemical properties such as electronegativity, hydrophilicity and relatively large size, coupled with their exposed position on cells and secreted macromolecules. However, there is a growing number of examples where sialic acids exert specific functions, mostly in molecular and cellular adhesion phenomena. The influence of natural and artificial modifications of the structure of Neu5Ac on enzymatic and some biological phenomena has been mentioned at relevant places throughout this article, showing that the configuration of Neu5Ac and related molecules is of great importance for the metabolism and biology of these monosaccharides, including those of the whole glycoconjugate molecules, cells and tissues, to which sialic acids are bound.

When trying to summarize the numerous roles of sialic acids, three groups of main effects can be described. Although there is considerable overlap, this classification has proved to be very useful and has been supported by many recent examples. Due to lack of space, only a few characteristic examples will be given here and new trends will be emphasized.

10.1. General physico-chemical effects

Firstly, due to their high acidity (pK value around pH 2), sialic acids are fully dissociated under physiological conditions and act as anions for the binding of inorganic and organic cations, e.g. on cell surfaces, thus facilitating the cellular uptake of substances with physiological or pharmacological significance. Cell aggregation may be prevented by the repulsive effects of the negatively charged sialic acids, as was observed for example when studying the adhesion of cultured cells to their substratum [910]. These cells adhered better to a layer of desialylated gangliosides than to the native, negatively charged, species, or after desialylation of the cells. The prevention of erythrocyte aggregation by surface sialic acids is a further, excellent example of their repulsive effect [911,912]. After the removal of sialic acids by sialidase, erythrocytes were found to aggregate readily, either without the influence of other compounds or under the influence of immunoglobulins and fibrinogen, respectively. Correspondingly, erythrocytes which are undersialylated in

diabetes, as was analyzed with their glycophorin components, tend to increase aggregation and adhesion to blood vessel walls. This may be of importance in the development of vascular disease during diabetes [913]. The high degree of sialylation existing in the epithelial sialomucin "episialin", which is expressed not only on several secretory epithelial cell types but also in many carcinoma cell lines, is also believed to have anti-adhesion function. This effect may facilitate metastasis of tumor cells [914]. Such a role can also be attributed to the sialic acids found on the surface of Streptococci of the serological group B. In contrast to the wild-type strain, an asialo mutant obtained by the transposon method showed stronger cell adherence, probably due to the more hydrophobic surface resulting from the lack of sialic acid [915].

In contrast to this repulsive effect of sialic acids, cell adhesion may be facilitated via positively charged substances or Ca^{2+} bridges. Furthermore, binding of Ca^{2+} to ganglioside sialic acids is of great importance in the function of nervous tissues [916]. These glycoconjugates are localized in clusters on neuronal and especially synaptic membranes in the vicinity of a membrane-bound calcium pump, thus facilitating the supply of these ions for neuronal cells. These calcium–ganglioside interactions may modulate neuronal functions, not only for the short-term process of synaptic transmission of information, but also for long-term events of neuronal adaptations including storage of information. The highly charged anionic sialic acid residues, α-2,3- and α-2,6-glycosidically bound to the trabecular meshwork of the human eye, are considered to be involved in the regulation of the aqueous outflow and the control of intraocular pressure [917].

Secondly, sialic acids may modulate the conformation of glycoproteins and gangliosides, thus influencing their arrangement, physical properties and biological functions in cell membranes, as well as the solubility, thermal stability and other properties of soluble glycoproteins including sialylated enzymes and hormones [5,8,907]. This is, indeed, a very large field and we still are only beginning to understand the influences of glycoconjugate conformations in cell biology. To obtain such information on individual molecules existing on living cells is particularly difficult to obtain with the techniques available. However, with α-dodecyl-Neu5Ac anchored to the surface of a phospholipid-based membrane fragment, it was shown by ^{1}H and ^{13}C NMR studies that the sialic acid pyranose ring is extended into the aqueous phase with the carboxyl group remaining close to the membrane surface, where it may interact with a hydrogen acceptor [918]. For glycoproteins it has been postulated that the carbohydrate moiety mimics the effect of a molecular chaperon [919].

The anti-proteolytic effect, long known [906] to be exerted by glycan chains on glycoproteins in general and sialic acids in particular, should also be mentioned here. This may be due to conformational changes mediated by sialic acids, to their negative charge, or to mere shielding of e.g. peptide bonds from proteases by the bulky carbohydrate moiety. The high grade of sialylation of lysosomal cell membranes [920] may be a protective barrier against proteases in this cellular compartment. Glycosylation of influenza virus hemagglutinin governs hydrolysis of certain peptide bonds by host proteases in order to enhance the virulence of these viruses [863,921]. It is suggested that the removal of sialic acid from the carbohydrate moiety of the hemagglutinin of influenza B virus by sialidase is essential for the cleavage of this glycoprotein

by cellular protease [922]. Mucins, which are highly glycosylated and often heavily sialylated [5,13,659,906] are excellent anti-proteolytic substances of high viscosity, protecting endothelia particularly in the intestine or respiratory tract from the attack by proteases and pathogenic substances [5,8,245,660,853]. The high viscosity of mucins is due, to an extent, to their sialic acid and often also sulfate content, or, in lower animals, to the presence of other anionic components such as uronic acids, sulfate or phosphate residues [5,853,906,923]. This property enables mucins to act as very efficient biological lubricants, thus for example facilitating the transport of foodstuffs through the intestine, assisting in the movement of the eye bulbus, facilitating the mechanical processes of reproduction, or serving as protection for fish and many lower animals, the body surfaces of which must be lubricated for movement in aqueous or muddy environments. The structure, biosynthesis and molecular biology of mucins expressing cell- and tissue specificity have been reviewed [722,924].

The next two large groups of sialic acid functions are two-fold: sialic acids are either masks of biological recognition systems or they are directly recognized during the interaction of molecules and cells [663,925]. Since the masking functions of these sugars were recognized earlier, they will be described first.

10.2. Sialic acids masking biological recognition sites

It is well established that the main reason why sialic acids occur chiefly on membrane surfaces as well as on circulating and secreted molecules is because of the protective role these highly acidic and relatively bulky sugar molecules play. In this way, cells and molecules are protected against damaging influences from the environment, such as proteases and immunological or phagocytotic agents. This may be the reason why so many microorganisms such as trypanosomes (see section 9.2.4.1), bacteria (see below) and even pathogenic fungi [926–928] also developed mechanisms to sialylate cell surface components. Remarkably, in the fungi *Sporothrix schenkii* [926] and *Fonsecaea pedrosi* [928], both of which are pathogenic to man, Neu5Ac and Neu5Gc were found. The protective role of sialic acids was shown in the case of *S. schenkii,* whose phagocytosis by macrophages was increased after sialidase treatment [929].

In addition to this more general function, sialylation can mask specific biological recognition sites and, by reversible sialylation, can control biological events such as the action of hormones, ion channels, growth and ageing, some examples of which will be discussed in the following.

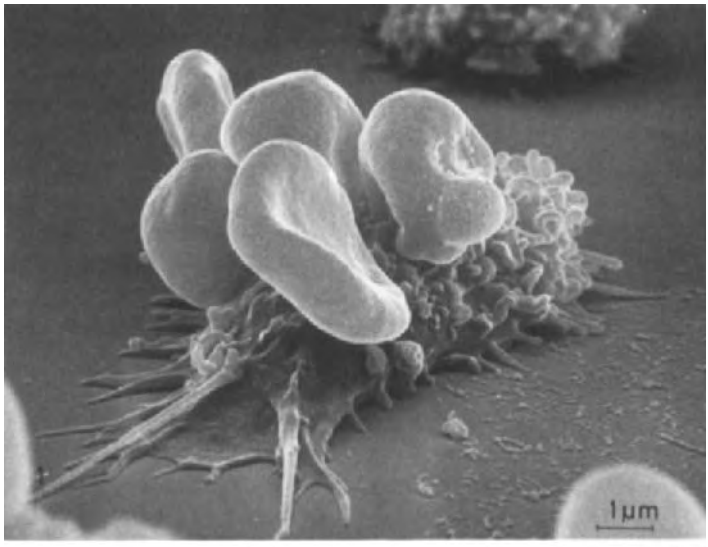
One of the most frequent biological recognition sites of glycoconjugates, which are often masked by sialic acids, are galactose residues. These monosaccharides, after their exposure by sialidase or reduced sialyltransferase activity, interact with a variety of galactose-specific receptors and are thus involved in many physiological and pathological events (for reviews see refs. [5,8,663,925,930–934]). The galactose-specific lectin of animals was found to be a phylogenetically very old protein, with homologues occurring in organisms ranging from the sponge *Geodia cydonium* to man [935,936]. One of the best known galactose-specific receptors is the hepatic asialoglycoprotein receptor, which was the first well-characterized animal lectin [933,937]. The properties, molecular biology and biosynthesis of this Ca^{2+}-dependent receptor have been intensively studied (e.g.

ref. [938]). It is proposed to function as a "vacuum cleaner" for the removal of serum glycoconjugates, which had become "non-self" and have exposed galactose residues, resulting from the loss of sialic acids. However, this lectin also mediates the hepatic binding and uptake of hepatitis B virus particles by the same mechanism [939]. This clearance of asialoglycoconjugates is also a problem during the therapeutic administration of recombinant glycoproteins, including those with hormonal or enzymatic activity, or the follicle-stimulating hormone isolated from urine [940–945]. Only the fully sialylated glycoproteins exhibit a long life in serum and thus extended biological activity, in contrast to those possessing terminal mannose or galactose residues. In the latter cases, rapid removal mediated by carbohydrate-specific receptors takes place.

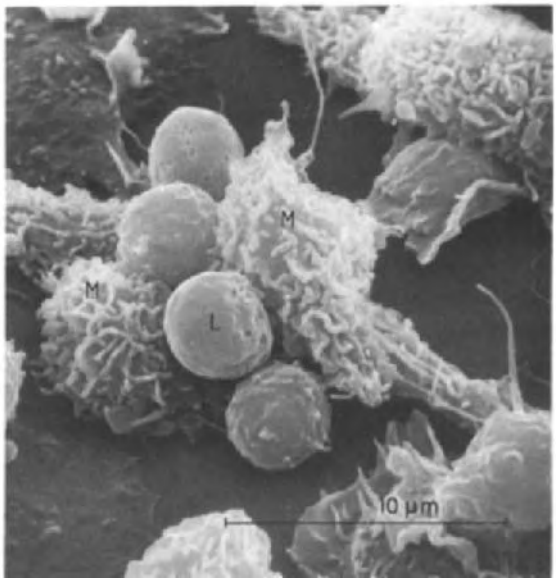
In contrast to this positive effect of sialic acids on the circulation time and the biological activity of these glycoprotein hormones, the same sugar negatively influences the hormonal effect of e.g. thyrotropin [940], erythropoietin [944] and prolactin [946] *in vitro*. It was shown in these studies that asialo-erythropoietin binds better to the hormone receptor than the sialylated form. Deglycosylated human lutropin and chorionic gonadotropin also bind better to the receptors than the native form, but their hormonal activities, such as cAMP formation and steroidogenesis are lost [947]. The presence of sialic acid residues is a functional requirement of the hormone molecules and may modulate the efficiency of signal transduction. It was shown in these studies that a sialic acid residue from a chorionic gonadotropin glycan either binds to the hormone receptor directly or to a neighboring lectin-like molecule. This interaction is essential for signal transduction mediated by the hormone. A comparable receptor which is responsible for the sequestration of sialic-acid-depleted blood cells, such as erythrocytes [948], lymphocytes [949] and thrombocytes [43] exists on the surface of liver and spleen macrophages, and also on the peritoneal phagocytes of some animals [925,950,951]. This receptor was isolated from rat peritoneal macrophages [952]. It mediates binding of desialylated blood cells via demasked galactose and *N*-acetylgalactosamine residues by macrophages and requires Ca^{2+} for this function. Immunoglobulin or complement factors do not influence this process. Although sialidase-treated erythrocytes and thrombocytes are phagocytozed after adhesion [43,948], lymphocytes are released from the macrophages within 24 h after binding [949], probably due to the resynthesis of their sialic acid moieties. The binding of the three cell types to phagocytes is shown in Fig. 22.

This lectinophagocytosis not only acts on blood cells damaged by sialidase, which may have resulted from viral or bacterial infections (see above), but also seems to be responsible for the removal of aged human erythrocytes [953]. It was shown with lectins and by resialylation with 9-fluoresceinyl-Neu5Ac that old blood cells expose more terminal galactose residues compared with young erythrocytes. Loss of sialic acid residues from glycophorin during the life-time of human erythrocytes seems to be responsible for the clearance of senescent red blood cells from circulation [954]. Injection of sialidase into mice induces lectin-mediated thrombocytopenia, which stimulates thrombopoiesis [955].

As a consequence of programmed cell death ("apoptosis"), changes of surface carbohydrates including a loss of sialic acids and exposure of galactose residues occurs leading to phagocytosis of apoptotic cells [956]. In neonatal rat liver cell cultures, the asialoglycoprotein receptor has been implicated in the ingestion by healthy hepatocytes

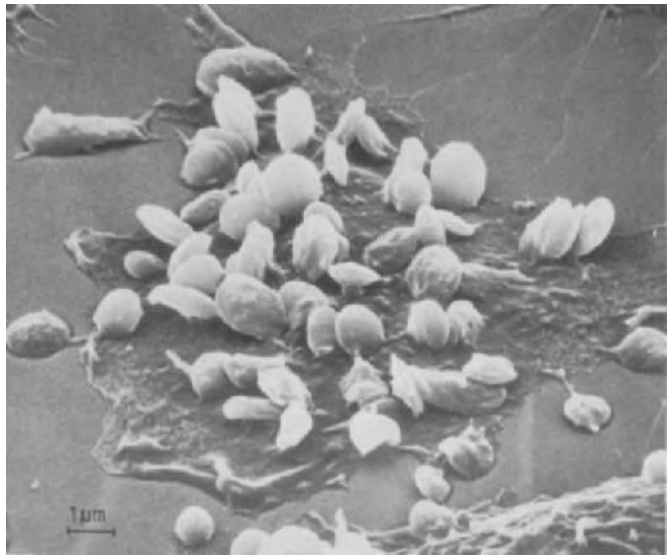


(a)



(b)

Fig. 22. Binding of sialidase-treated rat erythrocytes, lymphocytes and thrombocytes to homologous peritoneal macrophages by the galactose-specific receptor. (a) Scanning electron microscopy of erythrocyte–macrophage interaction (from ref. [925] by permission of Kodansha Publishers, Tokyo). After prolonged incubation, the erythrocytes were ingested. (b) Scanning electron microscopy of cultured, sialidase-treated lymphocytes (L) bound by two macrophages (M). Lymphocytes were not phagocytozed but separated from the macrophages within one day [949]. (c) Scanning electron microscopy of sialidase-treated thrombocytes to a macrophage adherent to a Petri dish. From ref. [925] by permission of Kodansha Publishers, Tokyo.



(c)

Fig. 22c.

of neighboring cells undergoing apoptosis [957]. After desialylation of rooster sperm glycocalyx, the fertility of the spermatozoa was reduced due to an increased rate of sequestration in the hen's reproductive tract when compared with untreated cells [958]. This effect is believed to be due to demasking of antigenic or other recognition sites on the spermatozoa. However, treatment of human motile spermatozoa with sialidase results in better attachment of these cells to the zona pellucida of oocytes [959]. It is concluded from this observation that the release of sialic acid from the sperm plasma membrane could be one of the capacitation events necessary for unmasking certain sperm surface antigens or carbohydrate ligands implicated in zona pellucida recognition. The surface of human spermatozoa is highly sialylated, containing predominantly Neu5Ac(α2–6)Gal/GalNAc-glycoconjugate sequences [959,960]. It was also shown with a sialylated wild-type and a mutant *Streptococcus* strain that sialic acids hinder phagocytosis of the bacteria and thus increase their virulence [915].

The galactose receptor also enables rat peritoneal macrophages to take up either free [961] or gold-particle-bound asialoglycoproteins [925,950,962]. Interestingly, for activity this receptor requires the presence of sialic acids on the macrophages to which it is bound, as was shown by sialidase treatment [925]. No explanation for this phenomenon is available so far. It may be due to the requirement of sialic acid for stabilization of the conformation of the receptor, to prevent self-aggregation, if it is a glycoprotein, or to hinder interaction with neighboring desialylated molecules in the macrophage membrane. The interaction of galactose-exposing particles with rat Kupffer cells has also been characterized [963].

From the reversible binding of lymphocytes mentioned above, as well as from the inhibition of binding of erythrocytes by enzymatic resialylation [964], a model has been proposed for the reversible binding of cells via sialic acid and galactose, which is

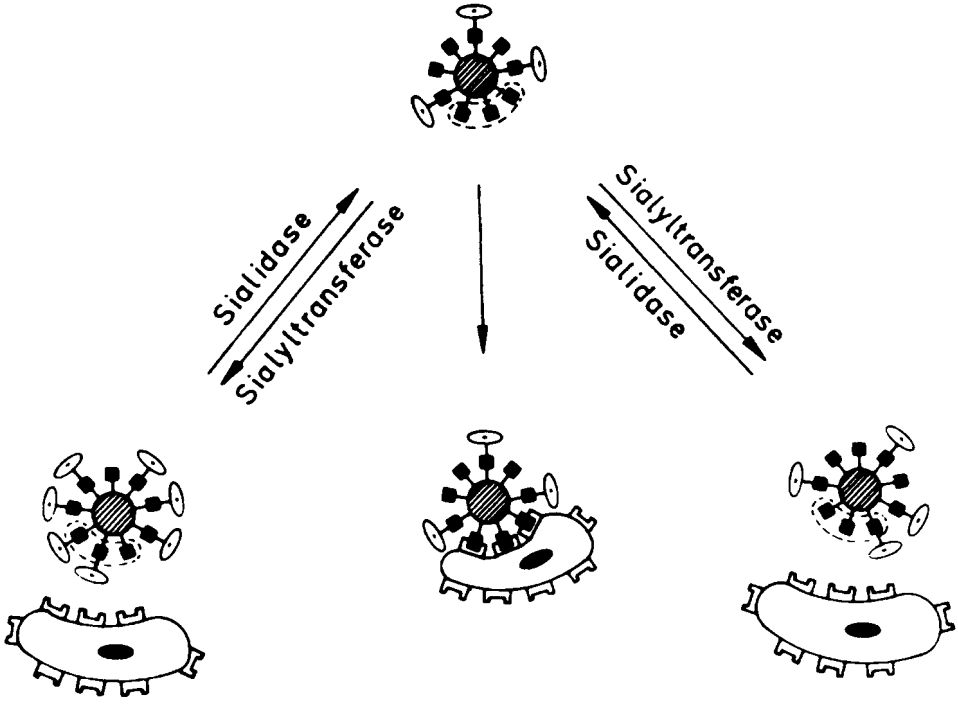


Fig. 23. Model showing how sialidase and sialyltransferase may regulate cellular interactions, i.e. association and dissociation mediated by a galactose-recognizing system. Symbols: solid square, galactose; open oval, sialic acid; open castle, galactose-recognizing receptor; dashed sausage, hypothetical recognition site (cluster of three galactose residues) by the lectin. From ref. [965] by permission of Gustav Fischer Verlag, Jena.

regulated by the activity of sialidase and sialyltransferase [925,950] (Fig. 23). This may generally operate in cell communication, especially in growth, differentiation, ageing, malignant transformation and metastasis.

The protection of host cells by sialic acids from the interaction resulting in colonization and possibly infection with bacteria is illustrated by the adhesion of *Bacteroides intermedius* to sialidase-treated human buccal epithelial cells and erythrocytes [966]. A corresponding phenomenon was described above in the sialidase section 9.2.4 for another oral bacterium, *Actinomyces viscosus,* which is also capable, by sialidase secretion, to demask its own attachment sites on host cells. *Pseudomonas aeruginosa,* a dangerous, infectious agent in patients suffering from cystic fibrosis or other diseases, employs pili to adhere to asialo-GM1 [967,968]. As sialic acid interferes with binding, GM1 itself is not a ligand for this bacterium.

Sialic acid residues on cell surfaces are important as modulators of immune and complement reactivity. Most importantly, animal cells and some microorganisms are shielded from the influence of these defence systems, since sialic acids can mask corresponding recognition sites. This has many physiological and pathological implications, as it may prevent autoimmune diseases, but can also increase the virulence of microbial infections or of cancer.

A classical example for this protective effect is the interaction of the non-immune channel fish *Ictalurus punctatus* [969] with various Gram-negative bacteria. Very little bactericidal activity is exerted by the alternative complement pathway against the fish pathogens containing sialic acid, in contrast to a very strong bactericidal response against the non-pathogens lacking sialic acid. To show that only the sialic acid coat protected the bacteria against the complement system and thus rendered them virulent, the bacteria were treated with sialidase. This destroyed their virulence, due to an enhancement of the bactericidal response of the fish.

The virulence of Gonococci (*Neisseria gonorrhea*) is also much increased by sialylation of the bacteria [184,185,970]. Terminal Gal(β1–4)GlcNAc structures of the bacterial 4.5 kDa lipopolysaccharide are sialylated during the life-time of the bacteria in the host organism. In this way, Gonococci in patients become resistant to complement-mediated killing by serum and to phagocytosis [971]. This resistance is lost in subcultures of the bacteria, but can be acquired again in the host. It is also absent in mutants lacking the necessary lipopolysaccharides. However, after infection with such Gonococci, variants appeared in the patient again expressing the acceptor for sialic acids, which shows a fascinating capability of the bacteria to adapt to the life in the host [970]. Closer investigation has revealed that sialic acids also mask porin epitopes on the Gonococci, which results in the reduction of the binding and bactericidal activity of antibodies recognizing this peptide moiety [972]. Furthermore, binding to human neutrophils and killing of these bacteria is prevented after sialylation [973].

Curiously, this chemical modification of Gonococci living in the host is possible by a sialyltransferase expressed by the bacterium itself and the sialic acid donor substrate, CMP-Neu5Ac, produced by the host. The properties of the sialyltransferase extracted from the bacteria have been reported [974]. It would be very interesting to compare the primary structure of this enzyme with those of host sialyltransferases. This mechanism is different from that used by trypanosomes for sialylation, although in both cases sialic acids are acquired from the host.

One of the major functions of sialic acids on mammalian cells is their masking effect on immunological recognition sites, as has been reviewed [5,8,663,795]. Loss of sialic acids, which may occur by exogenous sialidases from infections by microorganisms and viruses (see section 9.2.4), can thus lead to autoimmune diseases. One example is glomerulonephritis of human kidney, probably resulting from streptococcal infections. We are only at the beginning to understand such pathobiochemical mechanisms. It was also shown that cultured epithelial cells from guinea pig after treatment with bacterial sialidase bind more IgM than the controls [795,975]. These cells and those from lung epithelium taken from influenza-virus-infected animals were found to be hyposialylated. In serum, the level of IgG autoantibodies binding to the sialidase-treated cells increased [975]. It is assumed that these autoantibodies participate in cellular disfunctions and modify bronchoreactivity occurring during the infection of the respiratory tract. Asthma and chronic pulmonary disease may be the consequence of such desialylation. In these processes, the parasympathetic tone of the airways and the affinity of muscarinic agonists is impaired and the interaction of acetylcholine and histamine with their receptors also seems to be disturbed [795,976].

10.3. Sialic acids representing biological recognition sites

Whereas for a long time the main function of terminal sialic acid residues was considered as a mask for recognition sites on cell surfaces, e.g. galactose residues or antigens as outlined above, it is only in the last few years that receptors have been described which connect the structural diversity of sialylated glycoconjugates to specific functions in cellular interactions.

10.3.1. Sialic acid receptors of microorganisms, plants and lower animals

Many microorganisms, plants and animals express proteins which bind to sialic acids as components of glycoconjugates. Such sialic-acid-recognizing proteins or lectins from microorganisms, plants and invertebrates (mainly snails, scorpions and crabs) have been summarized in Tables 20 and 21, and have been discussed elsewhere in this series. They show more or less specific binding to different sialic acids or even specific sialic acid linkages and oligosaccharide sequences, as reviewed [709]. A careful investigation of the binding determinants of the sialic-acid-specific lectin from e.g. the slug *Limax flavus* has revealed that the α-anomer, the *N*-acetyl group, and an intact side chain of sialic acid are necessary for high affinity interaction. An axial position of the hydroxyl group at C4, hydroxylation of the *N*-acetyl group or substitution by a formyl residue, as well as 9-*O*-acetylation or periodate cleavage of the glycerol chain of Neu5Ac considerably reduced binding to the lectin [224]. Since these organisms do not express sialic acids themselves, it is unlikely that these lectins play a role as sialic-acid-binding proteins in cell recognition. However, they may be involved in defence against sialic-acid-containing microorganisms and animals. Some of the sialic-acid-binding lectins have proved to be useful tools in the analysis and histochemistry of glycoconjugates. Most frequently used are the agglutinins from wheat germ (WGA), *Limax flavus* (LFA), *Sambucus nigra* (SNA) and *Maackia amurensis* (MAA) (see section 4). Initial insight into the putative sialic-acid-binding sites of these unique lectins can be gained from a comparison of the primary structure of MAA with other plant lectins [1001]. A conserved asparagine residue was found to be exchanged by aspartate (Asp-135), which is proposed to be involved in the interaction between MAA and sialo-oligosaccharides.

It has long been known that pathogens, i.e. viruses, mycoplasma, bacteria and protozoa, take advantage of cell surface sialic acids to adhere to their respective host cells (for summaries see refs. [660,1002,1003] and chapter 13 of the present volume). Of greatest importance in this connection is the role of sialic acids in the attachment to and infection of mammalian cells by influenza viruses (see section 9.2.4.3).

From 500 MHz ^{1}H NMR spectroscopic [1004] and from X-ray crystallographic studies [1005,1006], models for the position of sialic acid in the binding pocket and the interaction with certain amino acids of influenza A virus hemagglutinins were delineated (Fig. 24) [394]. The use of sialic acid analogues modified in the glycerol side chain and in the *N*-acetyl and in the carboxyl groups of Neu5Ac, gave support to these models and revealed that the hydroxyl group at C9 does not interact with the hemagglutinin [394]. The *N*-acetyl moiety is critical for the interaction of sialic acids via hydrophobic bonds. These studies were extended by investigation of the crystal structures of this influenza virus hemagglutinin with different high-affinity analogues

Table 20
Pathogenic microorganisms and toxins binding to sialic acids on host cells

Pathogen	Specificity	Ref(s).
Viruses		
Influenza A and B	Neu5Ac (some strains prefer Neu5Ac(α2–3)Gal or Neu5Ac(α2–6)Gal, dependent on host specificity)	[977]
Influenza C	Neu5,9Ac_2	[978]
Corona virus	Neu5,9Ac_2	[979]
Sendai virus	Neu5Ac	[980]
Polyoma virus	Neu5Ac(α2–3)Gal(β1–3)GalNAc	[981]
Rotavirus group C	Neu5Ac	[982]
Mycoplasma		
Mycoplasma pneumoniae	Neu5Ac(α2–3)Gal on polylactosamine chains	[983]
Bacteria		
Streptococcus sanguis	O-linked sialylated tetrasaccharides	[984]
Escherichia coli K99	Neu5Gc-containing glycolipids	[667,985]
Escherichia coli, S-fimbriae	Neu5Ac(α2–3)Gal(β1–3)GalNAc	[986]
(newborn human meningitis)	Neu5Gc-GM3, GD3, GD1b	[987–990]
Bordetella bronchiseptica	Neu5Ac	[991]
Pseudomonas aeruginosa	Neu5Ac	[992]
Helicobacter (Campylobacter) pylori	Neu5Ac(α2–3)Lactose > Neu5Ac(α2–6)Lactose	[993]
Streptococcus suis	Neu5Ac(α2–3)Gal(β1–4)GlcNAc(β1–3)Gal	[994]
Protozoa		
Plasmodium falciparum (Malaria, MSA-1)	Neu5Ac	[995]
Trypanosoma cruzi (Chagas disease)	Neu5Ac	[831]
Tritrichomonas species	Neu5Ac (α-2,3- and α-2,6-linkages)	[226,996]
Toxins		
Vibrio cholerae toxin	GM1	[997]
Petussis toxin	Neu5Ac	[998,999]
Tetanus toxin	sialoglycolipids	[1000]

of sialic acid in order to design possibly therapeutic inhibitors of viral attachment to host cells, which may prevent membrane fusion and circumvent evasion of inhibition by antigenic variation of the viruses [1006]. A series of poly(acrylic acid-co-acrylamides) and dextrans bearing pendant glycine-4-amidobenzyl α-Neu5Ac groups were synthesized for anti-influenza chemotherapy. Some of these compounds suppress virus replication in embryonated eggs [1007]. X-ray crystallographic studies of influenza virus sialidase–antibody complexes gave interesting insight into the structure of such complexes between two cross-reacting antibodies both recognizing sialidase [1008].

Table 21
Sialic-acid-binding lectins from plants and invertebrates (see ref. [709] and references therein)

Source	Specificity
A: Plants	
Wheat germ *Triticum vulgare*	Neu5Ac < GlcNAc
Elderberry *Sambucus nigra*	Neu5Ac(α2–6)Gal/GalNAc
Maackia amurensis	Neu5Ac(α2–3)Gal(β1–4)GlcNAc
B: Invertebrates	
Snail *Dolabella*	Neu5Ac
Slug *Limax flavus*	Neu5Ac > Neu5Gc
Snail *Cepaea hortensis*	Neu5Ac > Neu5Gc
Snail *Achatina fulica*	Neu5Ac(α2–3)Gal > Neu5Ac(α2–6)Gal
Snail *Pila globosa*	Neu5Gc
Oyster *Crassostrea gigas*	Neu5Ac
Horseshoe crab *Limulus polyphemus*	Neu5Ac
Lobster *Homarus americanus*	Neu5Ac, Neu5Gc
Horseshoe crab *Tachypleus tridentatus*	Neu5Ac, Neu5Gc
Scorpion *Androctonus australis*	sialyllactose
Horseshoe crab *Carcinoscorpius rotunda*	Neu5Ac(α2–6)Gal > Neu5Ac(α2–3)Gal
Scorpion *Centruroides sculpturatus*	Neu5Ac, Neu5Gc
Prawn *Macrobrachium rosenbergii*	Neu5Ac
Scorpion *Masticoproctus giganteus*	Neu5Ac
Spider *Aphonopelma cepaeahortensis*	sialoglycoproteins
Scorpion *Heterometrus granulomanus*	Neu5Ac(α2–3)Lactose
Prawn *Peneaus monodon*	Neu5Ac
Scorpion *Paruroctonus mesaenis*	sialoglycoproteins

The interest in sialic-acid-specific adhesion of bacteria is increasing, since it often is a critical step in infectious diseases. Examples are the inflammation of gastric mucosa by *Helicobacter pylori* after adhesion to sialoglycoproteins of the cell surface [993,1009] and meningitis of infants as well as urinary tract infections by *E. coli* [985–989]. The binding of the latter bacteria via their S fimbriae to epithelial, e.g. buccal cells can be inhibited by sialylated mucins from human milk. Correspondingly, in human newborn babies sialylated substances have an anti-infective effect. Colostrum was shown to be more effective in this respect than mature milk [1010]. The S fimbrial adhesin of *E. coli* represents a protein complex and the genes encoding its subunits have been sequenced [988]. The introduction of specific mutations into the subunit gene *sfa*S revealed that a region of six amino acids of the adhesin which includes two lysine and one arginine residues is involved in the interaction of S fimbriae with sialic acid [990].

It should also be mentioned that several bacterial toxins are known which bind to gangliosides in a sialic-acid-dependent manner, e.g. cholera, pertussis and tetanus toxins

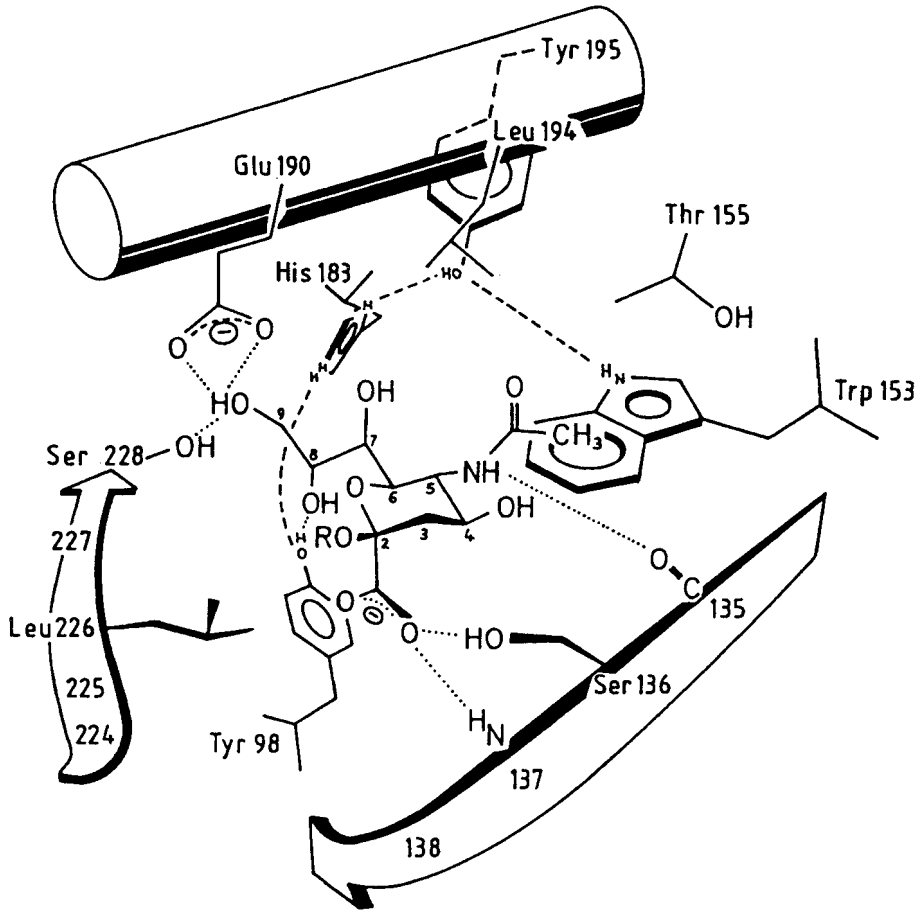


Fig. 24. Model for the position of sialic acid in the binding pocket of the hemagglutinin of influenza virus, based on X-ray crystallographic studies. Some of the hydrogen bonds proposed in this model are shown by dashed lines. From ref. [394] by permission of European Journal of Biochemistry, Zürich.

(ref. [660] and Table 20). The role of host sialic acids in the binding of protozoa is also of growing interest, and the possible involvement of this monosaccharide in the attachment of trypanosomes to host cells during infection has already been mentioned (section 9.2.4). The role of sialic acid in the invasion of erythrocytes by malaria parasites has been demonstrated and a 175 kDa protein of *Plasmodium falciparum* has been identified as an erythrocyte-binding protein with sialic acid specificity [1011]. It preferentially binds to Neu5Ac(α2–3)Gal sequences on the O-linked tetrasaccharide of glycophorin. Tritrichomonads (*Tritrichomonas suis, foetus* and *mobiliensis*) also exhibit a sialic-acid-dependent adhesion to host cells and mucus [226,996]. A corresponding lectin consisting of three subunits was isolated from *Tritrichomonas mobiliensis*, which recognizes free as well as α-2,3- and α-2,6-linked Neu5Ac [226]. A model of binding was delineated from the observation showing that optimal interaction with the receptor requires a free

carboxylic group, the *N*-acetyl moiety and a free HO8 group of sialic acid. Remarkably, *O*-acetylation at C9 or C4 does not influence adhesion.

10.3.2. Sialic acid receptors of vertebrates

Sialic-acid-recognizing receptors have been discovered in a variety of vertebrates, mostly mammalian tissues, summarized in Tables 22–24. This line of research was much stimulated by the discovery of a family of cell adhesion molecules now generally called selectins [1033]. Due to its great importance, this topic has been reviewed frequently [660,828,1034–1039]. It will, therefore, not be discussed in detail here.

The selectin family consists of three members, L-selectin, found on lymphocytes and other white blood cells, E-selectin expressed on endothelial cells activated by cytokines, and P-selectin occurring on activated blood platelets and endothelia (Table 23). They possess a similar primary structure, representing type I trans-membrane glycoproteins containing an amino-terminal carbohydrate recognition domain, a single epidermal growth-factor-like domain, a variable number of short consensus repeats and a relatively short carboxy-terminal cytoplasmic domain. The selectins also share common aspects in their function. They all play crucial roles in the initial event of white blood cell adhesion to specific endothelia, so-called rolling. Before firm adhesion, cells floating in the blood stream begin to slow down by rolling along the endothelial lining of the vessel. This is mediated by selectins interacting with sialic-acid-containing ligands. The specificity of this interaction is accomplished by the expression pattern of the receptors and their appropriate ligands. The selectins bind with low affinity to many small, sialylated, fucosylated and in some cases sulfated carbohydrates, of which the prototypes are the tetrasaccharides sialyl-Lewis^x {sLe^x;Neu5Ac(α2–3)Gal(β1–4)[Fuc(α1–3)]GlcNAcβ-R} and the isomeric sialyl-Lewis^a {sLe^a;Neu5Ac(α2–3)Gal(β1–3)[Fuc(α1–4)]GlcNAcβ-R}. At least two of the selectins (Table 23) recognize sLe^x and/or sLe^a, which are expressed at high levels on leukocytes [1040] and some tumor cells, particularly of the colon [1041–1044] as components of glycoproteins or gangliosides. Sialyl-Lewis^x has also been shown to be a marker of dysplasia in the colonic adenoma–carcinoma sequence [1045]. It has therefore been proposed that selectins are involved in malignant growth and metastatic events. Remarkably, 6′-sulfated sLe^x (Table 23) has been identified as a major ligand (GlyCAM-1) involved in the initial attachment of lymphocytes via L-selectin to high endothelial venules of lymph nodes [1046]. The synthesis of this "addressin", as L-selectin ligands are called now, *O*-sulfated at C6 of galactose, has been reported [1047]. Using an L-selectin chimera, it could be shown that ligands for L-selectin also occur on the myelin sheaths of neurons of the central nervous system [1048]. Progress has been made in understanding how these molecules function at the atomic level, by determination of the three-dimensional structure of a portion of the E-selectin molecule [1049,1050]. A spin label study has shown that the interaction of selectin on lymphocytes with sialic acid severely restricts the rotational mobility of the cell surface proteins and lipids. Additionally, the cytoplasmic viscosity increases appreciably [1051].

Presently, much interest is focussed on the role of the selectins during inflammation and in transplantation medicine, since it was recognized that selectins are involved in the rolling and adhesion of granulocytes to endothelia under the influence of inflammatory cytokines [1034,1037,1039,1052]. This is a multistep process in which other adhesion

Table 22
Vertebrate sialic-acid-binding proteins[a]

Source	Specificity	Reference(s)
Frog egg	sialylated glycoproteins	[1012]
Rat uterus	Neu5Ac	[1013]
Rat brain	Neu5Ac, Neu5Gc	[1014]
Rat brain myelin	gangliosides, preferentially GT1b, GQ1b, GD1b	[1015]
Human endometrium	Neu5Ac, Neu5Gc	[1016]
Human placenta (IgG)	*O*-acetylated sialic acids	[1017]
Blood (factor H of alternate complement pathway)	sialylated glycoconjugates, other polyanionic molecules	[1018]
Murine macrophages (sialoadhesin)	Neu5Ac(α2–3)Gal(β1–3)GalNAc on glycoproteins and glycolipids	[1019]
Bovine heart (calcyclin)	Neu5Ac, Neu5Gc	[1020]
Human placenta (sarcolectin)	Neu5Ac, Neu5Gc	[1021]
B lymphocytes (CD22)	Neu5Ac(α2–6)Gal(β1–4)GlcNAc	[1022,1023]

[a] For selectins and sialoadhesins see Tables 23 and 24.

Table 23
Distribution and binding specificity of selectins (for references see the text)

Selectin	Cell type	Ligand determinant
E-selectin	activated endothelia	sialyl-Le^x, Neu5Ac(α2–3)Gal(β1–4)[Fuc(α1–3)]GlcNAc sialyl-Le^a, Neu5Ac(α2–3)Gal(β1–3)[Fuc(α1–4)]GlcNAc
L-selectin	leukocytes	sialylated, sulfated and fucosylated O-glycans like Neu5Ac(α2–3)(SO_4-6)Gal(β1–4)[Fuc(α1–3)]GlcNAc
P-selectin	activated platelets and endothelia	sialyl-Le^x and sialyl-Le^a

molecules of the integrin superfamily [1053] are also involved. Such reactions may occur under the influence of microbial infections [1054] or other inflammatory events, such as in skin and neuronal tissue [1055] or in lung injury [1056]. The application of sLe^x oligosaccharide has thus been shown to have a protective effect in such inflammatory reactions, e.g. lung inflammation due to oxygen radical formation by cobra venom. From this research, a therapeutic effect of sLe^x analogues in the treatment of many diseases including reperfusion injury observed after organ transplantation, heart attack and stroke is expected, as shown in animal models (ref. [1057] and J.C. Paulson, personal communication). Consequently, much work is dedicated to the synthesis of selectin ligands on a commercial scale (ref. [1058] and section 6.3). Another strategy to prevent

Table 24
Sialoadhesins, a family of immunoglobulin-like adhesion molecules binding to sialylated glycans

Adhesion molecule	Occurrence	Number of Ig-domains	Ligand determinant	Target cells	References
Sialoadhesin	macrophage subpopulations	17	Sia(α2–3)Gal(β1–3)GalNAc Sia(α2–3)Gal(β1–3/4)GlcNAc	myeloid cells	[1019] [664,1024,1025]
Myelin-associated glycoprotein (MAG)	myelin of oligodendrocytes and Schwann cells	5	Sia(α2–3)Gal(β1–3)GalNAc	neurons, oligodendrocytes	[664,1026]
CD22	B cells	7	Sia(α2–6)Gal(β1–4)GlcNAc	lymphocytes	[1023,1027–1030]
CD33	myelomonocytic cells	2	Sia(α2–3)Gal(β1–3)GalNAc Sia(α2–3)Gal(β1–3/4)GlcNAc	myelomonocytic cells	[1031] [1032]

selectin-mediated adhesion phenomena in pathological states is the neutralization of the biological activity of these receptors by antibodies [1059,1060].

Other well defined sialic-acid-dependent adhesion receptors are sialoadhesin [660,664, 1019,1024] found on specific subsets of macrophages in bone marrow and lymphatic tissues, such as lymph nodes and spleen, as well as CD22 [660,1025,1061,1062], a B-cell-specific protein, CD33, occurring on myelomonocytic cells [1031,1032] and MAG, the myelin-associated glycoprotein of neuronal tissues [1025,1063] (Table 24). All these receptors belong to the immunoglobulin superfamily [1025]. The murine macrophage sialoadhesin has a molecular mass of 185 kDa as well as 17 immunoglobulin-like domains [1024] and binds to the sequence Neu5Ac(α2–3)Gal(β1–3)GalNAc or Neu5Ac(α2–3)Gal(β1–3/4)GlcNAc, i.e. both N- and O-glycans on glycoproteins and glycolipids of cell surfaces, preferentially of the granulocytic lineage [1064]. Remarkably, Neu5Gc and $Neu5,9Ac_2$ are not recognized by the receptor [664]. With regard to its function, it may play a role in hematopoiesis, since it was found to be enriched on bone marrow macrophages at the contact sites with developing myeloid cells. A role in the trafficking of leucocytes in lymphatic organs is also assumed. The receptor preferentially binds to inflammatory and circulating neutrophils.

CD22 occurs on B cells, where it mediates their binding to T cells and to neutrophils, monocytes and erythrocytes [1065]. The interaction with T cells is assumed to be involved in early B cell activation and may also modulate signalling through the surface IgM–cell receptor complex. In contrast to sialoadhesin, CD22 binds specifically to sialyl(α2–6)Gal(β1–4)GlcNAc structures of N-glycans [1023,1025]. Again, Neu5Gc and $Neu5,9Ac_2$ modulate binding of this ligand. Whereas Neu5Gc increases binding to murine CD22, 9-*O*-acetylation of Neu5Ac is inhibitory [664]. It can only be assumed so far that these sialic acid modifications, both occurring in mice, have a biological role in the activity of the two receptors. Recognition of Neu5Ac by human CD22 is also masked by 9-*O*-acetylation [235]. A further regulation of CD22 function is α-2,6-sialylation of the glycan chains of the receptor molecule itself [1066], which inhibits receptor activity. In this way, the ligand binding sites of CD22 may be blocked and the interaction of the receptor with ligands on adjacent cells prevented. In resting B cells, which only express low levels of α-2,6-sialyltransferase activity, CD22 binding sites may be free, thus enabling interaction with neighboring cells. Lymphocyte activation, however, results in an increase in the activity of this sialyltransferase, causing sialylation of the receptor and a decrease in cellular interaction. Regulation of the sialylation of CD22 may therefore mediate adhesion and trafficking at discrete stages of B cell differentiation. In this regulatory system the expression of sialylated ligands in non-lymphoid cells may be important. It was shown that inflammatory cytokines stimulate the expression of both ligands for CD22 and β-galactoside α-2,6-sialyltransferase in endothelial cells of human umbilical vein [1028,1029]. Thus, CD22 could direct interactions between mature B cells and endothelial cells during inflammatory stages. These events can be influenced, i.e. lymphocyte binding inhibited, by α-2,6-sialylated glycoproteins of the blood serum. Interestingly, the two serum molecules much involved in immune and inflammatory responses, haptoglobin and IgM, are selectively recognized by lymphocyte CD22 [1029]. Using a soluble CD22–immunoglobulin fusion product or expression of CD22 on the surface of Chinese hamster ovary cells, it was shown that this lectin has a higher

apparent affinity for multiply sialylated substances over monosialylated, unbranched glycans [1030]. This observation provides a mechanism for strong CD22-dependent cell adhesion despite the relatively low K_d for protein–carbohydrate binding.

The myelin-associated glycoprotein (MAG), involved in the proper myelination of axons [1063], has recently been shown to be a sialic-acid-dependent receptor, recognizing α-2,3-glycosidically bound Neu5Ac, preferably on O-glycans [1025]. It shares sequence similarity with both sialoadhesin and CD22, showing that it belongs to the same immunoglobulin superfamily. The term "sialoadhesin family" was proposed for this group of related sialic-acid-binding proteins [1025]. Since MAG can activate the MAG-associated tyrosine kinase [1067], it is possible that sialylated glycoconjugate ligands play a role in signal transduction during myelination. Furthermore, myelin-associated sialidase adhering to the ganglioside GM1 may be involved in the formation and stabilization of the multilamellar structure of the myelin sheath [829].

Other sialic-acid-binding activities have been found in mammalian tissues (Table 22), these being reviewed in ref. [660] and in chapter 14 of the present volume. Recently, a sialic-acid-binding protein was purified from human endometrium, which can bind to human spermatozoa, unless they have been sialidase-treated [1016]. Glycophorin A of mouse erythrocytes is recognized by a homologous peritoneal macrophage receptor specific for sialylated carbohydrates [1068]. It should, furthermore, be mentioned that various antibodies have been described, which recognize epitopes containing mono- or oligomeric sialic acids (glycoproteins, gangliosides and bacterial capsular polysaccharides) on a variety of cells (refs. [289,709,1011,1069–1071], and various chapters of this series). Monoclonal antibodies specific for α-2,8-linked Kdn sequences suitable for histochemistry are available now [1072]. The specificity of this interaction is higher than is generally the case for lectins, since the total carbohydrate structures bearing the sialic acids have a greater influence on the binding of antibodies. Interest is, therefore, increasing in the expression of sialyltransferase activity, this being a critical regulatory step in the formation of cell-surface differentiation antigens, which may be necessary for the function of these cells, so far most dramatically shown in lymphocytes [1070,1073,1074]. In the immune-reactivity of sialic acid epitopes, *O*-acetylation and *N*-acetyl hydroxylation have a strong modulatory role [668,669,1075].

10.4. Do sialic acids have "specific" functions?

The question arises as to whether these monosaccharides indeed have specific functions. Certainly, sialic acids are involved in numerous specific biological events, and many of them have been discussed in this and other articles. However, on closer inspection, it seems that sialylation exerts its influence in the diverse biological systems by the factors discussed, i.e. charge, conformation, masking and interaction with receptors. With regard to conformation, sialic acids may contribute to the specific structure of a given glycoconjugate molecule and thus may influence its biological activity. The highest degree of specificity can be observed in sialic-acid–receptor interactions, which require structurally well-defined features on both sites, not only related to the sialyl group but also to the penultimate sugars, thus resembling the formation of an enzyme–substrate complex. The interaction of influenza C virus with 9-*O*-acetylated sialic acid may be such an

example. The specificity in these phenomena can be related mostly to the non-sialylated part of the molecule or the cell, for example to the biological specificity of a glycoprotein hormone, a bacterial toxin, a neuronal reaction, the activity of an ion channel, or the nature of the receptor and the kind of signal it transfers into the cell after ligand binding. In these systems, the sialic acid moiety plays an essential role, since its presence is a prerequisite for the receipt and transfer of biological messages. Furthermore, sialic acid can control and modulate these biological events. The exposed position of this highly negatively charged, hydrophilic molecule on extracellular glycoconjugates makes it suitable for this purpose.

The physiological role of sialic acids could therefore be grouped on the basis of the long list of biological phenomena influenced by these carbohydrates. This will not be made here, since another grouping, into three main topics, was done in sections 10.1–10.3. However, a few interesting and unusual systems, influenced by sialic acids, as well as open questions for future research, will be mentioned.

In reproductive biology, sialic acid polymers are receiving more and more attention. Polysialic acid with α(2–8)-linkages was found to be expressed on mouse embryos before and after implantation and the neuronal cell adhesion molecule (NCAM), which bears a polysialic acid chain, seems to be involved in cellular interactions in the early mammalian embryo [101,1076]. A similar function is attributed to polysialic acid observed during a short period of the larval stage of *Drosophila melanogaster* [1077]. It has to be noted that this is the first report on the occurrence of sialic acid in insects.

The participation of sialic acids in different linkages and grade of polymerization during differentiation of cells and tissues was excellently demonstrated in a histochemical study [1078]. It was also reported there that polysialic acids typical for developing tissues are expressed in some malignant tumors. Sialic acid is involved in changes of the glycoconjugate patterns during the development of murine molar tooth germs [1079]. Corresponding to the variable expression of sialic acids during ontogenesis, the expression of sialyltransferases varies with the state of development, e.g. during the maturation of human myeloid cells [1080] or of the epithelium of small intestine upon weaning [1081]. Sialic acid modification is also involved in differentiation. In several rat and cow organs, the expression of Neu5Gc was found to be developmentally regulated [307,674]. Similar observations have been made for the appearance of $Neu5,9Ac_2$ in chicken erythrocytes (ref. [712]; see also section 8.4.2).

Many studies have revealed that sialic acids are of significance in the growth and functioning of neuronal cells. This can be mainly attributed to gangliosides, the most important carriers of sialic acids in these tissues [1082,1083]. How these molecules function is the object of intensive research, however, they are mainly involved in transmembrane signalling and the regulation of mediators. Interestingly, the synthetic glycoconjugate sialyl cholesterol also promotes neurite outgrowth in a mouse neuroblastoma cell line [1084]. Since this substance is transported to the nucleus, its localization may play an as yet unknown key role in neuritogenesis. Polysialic acid of NCAM affects cellular interactions not only during the development of the nervous system and skeletal muscle but also during the development of the ventricular conduction system in embryonic hearts of man and various animals [1085]. In adult rat brain, polysialic acid was found to be associated with sodium channels, and was shown to be

required for the conductivity of the channel and thus for the function of this nervous tissue [1086].

Interestingly, polysialic acid is a rather labile substance, which easily degrades spontaneously even under physiological conditions, due to an anomalously high pK value of the internal sialic acid residues [258]. This intramolecular self-cleavage may influence neuronal adhesion, embryogenesis and bacterial virulence.

10.5. Medical significance of sialic acids

Since sialic acids are involved in so many biological phenomena, it is easily understandable that disturbances in the metabolism of these sugars, either due to genetic errors or at the post-translational level, may impair the physiological functions of an organism and thus lead to disease. Such pathophysiological influences of sialic acids and sialidases as e.g. virulence factor of microbial diseases have been mentioned throughout this chapter. Four diseases in which metabolic errors of sialic acids have been clearly recognized will be briefly discussed: galactosialidosis, Salla disease, Alzheimer disease and malignant tumors. (Sialidosis and sialuria are described by Michalski in chapter 1b of *Glycoproteins and Disease,* Vol. 30 of this series).

Galactosialidosis, first described as sialidosis [1087], is a rare genetic disorder and occurs in at least three clinical phenotypes: an early infantile form where abnormalities (sialidase deficiency and an increased amount of sialo-oligosaccharides) can be detected in utero; a late infantile form characterized by skeletal abnormalities, macular cherry-red spot and mild mental retardation; and a juvenile–adult form with variable age of onset of skeletal abnormalities, corneal clouding, macular cherry-red spot, neurological manifestations and mental retardation [1088–1091]. All three forms are characterized by accumulation of sialo- and galactoglycoconjugates and the excretion of sialyloligosaccharides. The primary defect of this genetically well-studied disease is in the capability of a protective protein, a serine carboxypeptidase which has also deamidase activity (see chapter 1a by Jourdian of *Glycoproteins and Disease,* Vol. 30 of this series), to associate with β-galactosidase and sialidase to protect them from intralysosomal proteolysis. Fibroblasts from these patients show a marked reduction in β-galactosidase and virtually no sialidase activity. The genes encoding β-galactosidase, sialidase and the protective protein, are located on the human chromosomes 3, 10 and 20, respectively [1092,1093]. With regard to the function of the protease normally stabilizing the ternary enzyme complex, various mutations have been found which affect the stability of the carboxypeptidase/deamidase central sheet [1094]. The stoichiometry of this complex from human lysosomes has been described [1095].

Lysosomal accumulation of free sialic acid occurs in two phenotypically distinct inherited metabolic disorders, Salla disease and infantile sialic acid storage disease [1096]. Salla disease is an autosomal recessive lysosomal storage disorder and was first observed in patients of Finnish ancestry, but also occurs outside Finland. The clinical symptoms are a slow progressive psychomotor retardation, impaired speech, ataxia and a prolonged course. Sialic acid accumulates in the lysosomes due to a defective efflux into the cytosol. The genetic defect affects the function of the specific transport protein for sialic acid and other acidic monosaccharides in the lysosomal membrane [1097]. The Salla disease locus

was found on the long arm of chromosome 6, and the critical region for this locus is in the range of 190 kb [1098].

Both in Alzheimer's disease and older Down's syndrome subjects, a decrease in serum sialyltransferase activity was observed [1099,1100]. This was found to affect only the α-2,3-sialyltransferase, also leading to a decrease of α-2,3-linked sialic acid in serum glycoproteins. It can only be assumed at present that this observation mirrors reduced activity of sialyltransferase and decreased sialoglycoconjugate biosynthesis in neuronal tissue subjected to these degenerative diseases. Serum sialyltransferase may thus be an early biochemical marker of neurodegeneration.

Cell surface carbohydrates are recognized to play crucial roles in malignant growth and metastasis, as is reported in detail in volume 30 of this series, "Glycoproteins and Disease". Here, some recent observations on the special influence of sialic acids in these processes will be presented to complete the main aspects of sialic acid biology. There are several reports in the literature showing that the amount and type of sialylation of membrane components influence the metastatic potential of tumor cells. A higher amount of membrane-bound sialic acid as component of glycoproteins and gangliosides promotes invasion and interferes with intercellular adhesion. Sialic acid is also involved in tumor cell attachment to endothelial cells via sLe^x- or sLe^a-binding selectins (section 10.3.2), and in adhesion of tumor cells to substances of the extracellular matrix [1044,1101]. These oligosaccharides are therefore considered as markers of some tumors, e.g. of human colon [1102] or thyroid gland carcinomas [1103]. In the latter case, clinicopathological studies indicate that sLe^a antigens are related to biologically aggressive thyroid tumors. In human colon, sLe^x was found to be expressed only in cultivated cells from premalignant polyposis, but not in intermediate or carcinoma cells [1102]. The higher degree of sialylation in human colorectal tumor tissue seems to be due mainly to an increase of α-2,6-sialyltransferase activity sialylating N-glycan and *N*-acetyllactosamine sequences [1104–1106]. When studying the activity of several glycosyltransferases in human colorectal adenoma cells during progression to cancer, α-2,3-sialyltransferase activity exhibited a peak in the intermediate premalignant stage, while α-2,6-sialyltransferase appeared to be turned off in the final stage, i.e. the adenomacarcinoma cells [1102]. An increase of serum sialic acids was shown to be relevant for the diagnosis and prognosis of tumor diseases and leukemia [1101,1104,1107].

The role of sialic acids in the formation of metastases was also shown with nude mice and human colorectal cancer cell lines [1108]. The better differentiated cells had higher levels of sialyltransferase activity and sialic acids which correlated with their enhanced ability to form liver metastases compared to the poorly differentiated, less sialylated cell lines. The role of sialic acid in metastasis was further demonstrated by inhibition of enzymatic sialyltransfer with a CMP-sialic acid derivative (refs. [648,649] and section 8.3), which resulted in reduced liver metastasis. An increase of α-2,6-sialyltransferase activity was also observed in cultured Ehrlich ascites tumor cells [1109] and in human gastric epithelium carcinoma [1110]. The increase of sialylation of an antigenic epitope of the latter tumor cells enhanced their metastatic potential. The expression of *ras* oncogenes in NIH 3T3 fibroblasts increases the sialic acid density and concomitantly the invasiveness of these cells and reduces cell surface galactosylation [1111]. Correspondingly, α-1,3-galactosyltransferase activity was decreased and

those of α-2,3- and α-2,6-sialyltransferase activities were increased. Furthermore, the number and/or length of polylactosaminoglycan chains and the branching of N-glycans was also enhanced. This is another example, in which multiple changes in the expression of glycoconjugates occur on malignant transformation. It was shown with rat 3Y1 cell lines that oncogenesis can be accompanied not only by increased sialylation but simultaneously by decreased sialidase activity, thus supporting hypersialylation and metastatic potential of the cells [1112]. There was an inverse correlation between lysosomal-type sialidase activity and invasion. How the oncogenes mentioned operate in these processes is presently being studied. All these studies and experiments with murine lymphoid tumor cells [1113] suggest that changes either in the amount, the type or linkage of sialic acid in tumor cell glycoconjugates can affect tumor growth and metastasis.

11. Conclusion

In the field of the primary structural analysis and synthesis of sialic acids and sialic-acid-containing glycans, the methods developed have reached a high level of sophistication. The conformational analysis of sialylated carbohydrate chains of glycoconjugates in their natural environment needs specific attention in order to better understand the physical and biological functions of these glycosylated molecules. Although deep insight into the metabolism of the different sialic acids has been obtained, it is challenging to further unravel the secrets of expression and transfer of these monosaccharides on the enzyme and gene levels, as well as receiving information on their regulation including hormonal control. This is particularly valid for the new group in the sialic acid family, Kdn and its derivatives. The biological roles of sialic acids are least understood, although solving these open questions seems to gain momentum. Thus, sialobiology, sialopathology and also sialopharmacology will remain most fascinating topics of future research on sialic acids.

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