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MINI REVIEW

Diversity in the sialic acids

Ajit Varki

UCSD Cancer Center and Department of Medicine, University of California, San Diego, La Jolla, CA 92093-0063, USA

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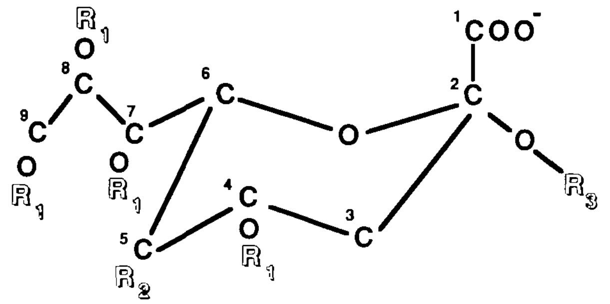

R_1 = H, ACETYL(4,7,8,9), LACTYL(9) METHYL(8),SULFATE(8), PHOSPHATE(9), ANHYDRO(4,8 or 2,7), SIALIC ACID (8,9), FUCOSE (4), GLUCOSE(8), OR GALACTOSE(4)

R_2 = N-ACETYL, N-GLYCOLYL, AMINO, HYDROXYL

R_3 = Gal(3/4/6), GalNAc(6), GlcNAc(4/6) or Sialic Acid (8/9) (Absent in 2,6 and 2,7 anhydro compounds)

Fig. 1. The sialic acids. The 9-carbon backbone common to all sialic acids is shown in chair conformation Natural substitutions described to date (at R_4, R_5, R_7, R_8 and R_9) are indicated. Additional diversity is generated by various types of glycosidic linkage (at R_2), by generation of lactones (at R_1), by dehydro forms (eliminating R_2) and anhydro forms

Historical background

It is now more than 50 years since *N*-acetyl-neuraminic acid was first discovered and subsequently characterized by several groups (reviewed in Roseman, 1970; Gottschalk, 1972; Rosenberg and Schengrund, 1976; Schauer, 1982; Faillard, 1989). Relatively soon after its discovery, it became apparent that this molecule was actually the major member of a family of compounds related to neuraminic acid that were christened the 'sialic acids' (Blix *et al.*, 1957). Early studies paid close attention to the different types of sialic acids and the interrelationships between them. However, interest in these complexities subsequently waned and unpublished 'folk-lore' had it that modified sialic acids were species-specific curiosities found only in a few tissues, such as erythrocytes and submaxillary glands. In fact, many investigators in the 1970s and 80s used the terms '*N*-acetyl-neuraminic acid' and 'sialic acid' synonymously. Thus, for example, when structural or biological changes were noted following treatments with a sialidase (neuraminidase), it was often assumed that the sialic acid released was *N*-acetyl-neuraminic acid. It is now clear that the different types of sialic acids are much more widely distributed than previously thought. This review attempts to briefly summarize current knowledge concerning the occurrence, structure, biochemistry and biological significance of this diversity in the sialic acids. Particular attention is given to the two most common and better studied modifications: the addition of *O*-acetyl esters to the hydroxyl groups at the 4-, 7-, 8- and 9-positions, and the conversion of the *N*-acetyl group to an *N*-glycolyl group. Given the breadth of the review, the bibliography is only representative, and tends to emphasize more recent studies.

Structural basis for diversity in the sialic acids

The sialic acids are a family of 9-carbon carboxylated sugars usually found as terminal monosaccharides of animal oligosaccharides (see Figure 1). The most common is *N*-acetyl-neuraminic acid (2-keto-5-acetamido-3,5-dideoxy-D-glycero-D-galactononulopyranos-1-onic acid) (Neu5Ac), which is believed to be the biosynthetic precursor for all other members of the family (reviewed in Roseman, 1970; Rosenberg and Schengrund, 1976; Schauer, 1982). Hydroxylation of the *N*-acetyl group gives *N*-glycolyl-neuraminic acid (Neu5Gc) (Jourdian and Roseman, 1962; Schoop *et al.*, 1969; Roseman, 1970). The 5-amino group can be replaced by a hydroxyl group, giving 2-keto-3-deoxy-nonulosonic acid (KDN) (Nadano *et al.*, 1986; Kanamori *et al.*, 1990). Most other sialic acids arise from substitution of one or more of the hydroxyl groups of Neu5Ac, Neu5Gc or KDN with acetyl, methyl, lactyl, phosphate or sulphate groups (Warren, 1964; Schauer, 1987; Iwasaki *et al.*, 1990; Manzi *et al.*, 1990a). Unsaturated and dehydro-forms, and different linkages to the underlying sugar chain, further increase the diversity of these molecules (see Table I for a listing of the currently known sialic acids in nature).

Problems of nomenclature and abbreviation

In any system with so much complexity, uniform nomenclature and abbreviations can greatly aid communication and progress. The complete descriptive names of the various sialic acids (see Table I) are too cumbersome for routine use. Initially, several systems of abbreviations were in use by investigators studying different types of modified sialic acids. Thus, 9-*O*-acetyl-*N*-acetyl-neuraminic acid was abbreviated as 9OAcNANA, 9OAcNeuNAc, NeuAc9OAc, etc. A uniform nomenclature was subsequently suggested (Scott *et al.*, 1982) based on using the root word sialose (Sia) for 2-keto-3-deoxy-nonulose, a common component of all known sialic acids. This system was comprehensive and accurate, but the required abbreviations were still quite cumbersome (e.g. the above-mentioned compound would be abbreviated SiaNAcA9OAc). A subsequently proposed nomenclature (Schauer, 1982, 1987) has proved to be the simplest and most widely used in recent years (see Table I). In this system, the above-mentioned compound would be abbreviated Neu5,9Ac$_2$, the root abbreviation Neu denoting the core neuraminic acid, and the acetate groups (Ac) assumed to substitute the amino group at the 5-position and the hydroxyl

Table I. Types of sialic acids reported in nature and suggestions for nomenclature and abbreviations

Full name[a]	Abbreviation	Group name[b]	Comments
Neuramininc acid	Neu	Sia	Unstable in free form. Indirect evidence for existence in stable glycosidic linkage
N-acetyl-neuraminic acid	Neu5Ac	Sia	Higher animals (echinoderms to man), certain bacteria and parasites
N-glycolyl-neuraminic acid	Neu5Gc	Sia	Most animals except adult humans and birds; immunogenic in the latter two
Keto-deoxy-nonulosonic acid	KDN	Sia	Sperm and eggs in teleost fish
9-*O*-acetyl-*N*-acetyl-neuraminic acid	Neu5,9Ac_2	Sia9Ac	Widespread throughout higher animals and in certain bacteria
9-*O*-acetyl-*N*-glycolyl-neuraminic acid	Neu5Gc9Ac	Sia9Ac	Widespread throughout higher animals, except in humans and birds
9-*O*-acetyl-keto-deoxy-nonulosonic acid	KDN9Ac	Sia9Ac	Fish egg glycoproteins
7-*O*-acetyl-*N*-acetyl-neuraminic acid	Neu5,7Ac_2	Sia7Ac	Widespreaad throughout higher animals and in certain bacteria
7-*O*-acetyl-*N*-glycolyl-neuraminic acid	Neu5Gc7Ac	Sia7Ac	Widespread throughout higher animals, except in humans and birds
4-*O*-acetyl-*N*-acetyl-neuraminic acid	Neu4,5Ac_2	Sia4Ac	Found in ungulates, monotremes, etc Difficult to detect when present in smaller amounts
4-*O*-acetyl-*N*-glycolyl-neuraminic acid	Neu4Ac5Gc	Sia4Ac	Found in ungulates. Difficult to detect when present in smaller amounts
7,9-di-*O*-acetyl-*N*-acetyl-neuraminic acid 8,9-di-*O*-acetyl-*N*-acetyl-neuraminic acid	Neu5,7(8)9Ac_3	SiaDiAc	Exist in 1:1 equilibrium Usually in smaller amounts with Neu5,9Ac_2
7,9-di-*O*-acetyl-*N*-glycolyl-neuraminic acid 8,9-di-*O*-acetyl-*N*-glycolyl-neuraminic acid	Neu5Gc7(8)9Ac_2	SiaDiAc	Exist in 1:1 equilibrium. Usually in smaller amounts with Neu5,9Ac_2
4,9-di-*O*-acetyl-*N*-acetyl-neuraminic acid	Neu4,5,9Ac_3	SiaDiAc	Found in ungulates Difficult to detect when present in smaller amounts
7,8,9-tri-*O*-acetyl-*N*-acetyl-neuraminic acid	Neu5,7,8,9Ac_4	SiaTriAc	Sometimes present in smaller amounts in places where Neu5,9Ac_2 is found
7,8,9-tri-*O*-acetyl-*N*-glycolyl-neuraminic acid	Neu5Gc7,8,9Ac_3	SiaTriAc	Sometimes present in smaller amounts in places where Neu5,9Ac_2 is found
9-*O*-lactyl-*N*-acetyl-neuraminic acid	Neu5Ac9Lt	SiaLt	Found in higher mammals, both in bound and free forms
9-*O*-lactyl-*N*-glycolyl-neuraminic acid	Neu5Gc9Lt	SiaLt	Porcine submandibular gland
4-*O*-acetyl-9-*O*-lactyl-*N*-acetyl-neuraminic acid	Neu4,5$Ac_2$9Lt	SiaAcLt	Found in horses, both in bound and free forms
4-*O*-acetyl-9-*O*-lactyl-*N*-glycolyl-neuraminic acid	Neu4Ac5Gc9Lt	SiaAcLt	Found in horses, both in bound and free forms
8-*O*-methyl-*N*-glycolyl-neuraminic acid	Neu5Gc8Me	SiaMe	Found in echinoderms. Major component of starfish gangliosides
8-*O*-methyl-*N*-acetyl-neuraminic acid	Neu5Ac8Me	SiaMe	Found in echinoderms. Minor component of starfish gangliosides
8-*O*-methyl-9-*O*-acetyl-*N*-glycolyl-neuraminic acid	Neu5Gc8Me9Ac	SiaAcMe	Found in echinoderms. Component of starfish gangliosides
8-*O*-methyl-7,9-di-*O*-acetyl-*N*-glycolyl-neuraminic acid	Neu5Gc8Me7,9Ac_2	SiaAc_2Me	Minor component of starfish gangliosides
8-*O*-sulpho-*N*-glycolyl-neuraminic acid	Neu5Gc8S	SiaS	Found so far in gangliosides from sea urchin and bovine stomach
9-*O*-phosphoro-*N*-acetyl-neuraminic acid	Neu5Ac9P	SiaP	Found so far only in free form as a cytosolic biosynthetic intermediate
2,3 didehydro 2,6 anhydro-*N*-acetyl-neuraminic acid	Neu2en5Ac	Sia-en	Found in biological fluids in free form. Product of spontaneous breakdown of CMP-Neu5Ac?
9-*O*-acetyl-2,3 didehydro 2,6 anhydro-*N*-acetyl-neuraminic acid	Neu2en5,9Ac_2	Sia-enAc	Rat urine and bovine submandibular gland
9-*O*-lactyl-2,3 didehydro 2,6 anhydro-*N*-acetyl-neuraminic acid	Neu2en5Ac9Lt	Sia-enLt	Porcine submandibular gland
2,3 didehydro 2,6 anhydro-*N*-glycolyl-neuraminic acid	Neu2en5Gc	Sia-en	Found in biological fluids in free form. Product of spontaneous breakdown of CMP-Neu5Gc?
9-*O*-acetyl-2,3 didehydro 2,6 anhydro-*N*-glycolyl-neuraminic acid	Neu2en5Gc9Ac	Sia-enAc	Porcine urine
9-*O*-lactyl-2,3 didehydro 2,6 anhydro-*N*-glycolyl-neuraminic acid	Neu2en5Gc9Lt	Sia-enLt	Porcine submandibular gland
8-*O*-methyl-2,3 didehydro 2,6 anhydro-*N*-glycolyl-neuraminic acid	Neu2en5Gc8Me	Sia-enMe	Starfish

Table I. Continued

Full name[a]	Abbreviation	Group name[b]	Comments
2,7 anhydro-*N*-acetyl-neuraminic acid	Neu2,7an5Ac	Sia-an	Found in biological fluids Formed during release of *N*-acetyl-neuraminic acid by certain sialidases?
2,7 anhydro-*N*-glycolyl-neuraminic acid	Neu2,7an5Gc	Sia-an	Found in biological fluids. Formed during release of *N*-glycolylneuraminic acid by sialidases?
8-*O*-methyl-2,7 anhydro-*N*-glycolyl-neuraminic acid	Neu2,7an5Gc8Me	Sia-an	Starfish
4,8 anhydro-*N*-acetyl-neuraminic acid	Neu4,8an5Ac	Sia-an	Formed during breakdown or release of 4-*O*-acetyl-*N*-acetyl-neuraminic acid

[a]The full names of the sialic acids can be written with the substitutions listed last (e.g. Schauer, 1982) or first (e.g. Higa and Paulson, 1985; Manzi *et al*, 1990a,b) The latter system (with maximum hyphenation) is followed in this table, to emphasize the substitutions.
[b]When the precise structure of the sialic acid is known, the complete nomenclature should be used. When incomplete data concerning a sialic acid are available, the group names suggested here can be used until full information is known (see the text for more details).

group at the 9-position. Until recently, this nomenclature system comprehensively covered all known sialic acids. The discovery of a family of sialic acids derived from KDN has shown that not all sialic acids share the amino group at the 5-position found in neuraminic acid (Nadano *et al.*, 1986; Iwasaki *et al.*, 1990). However, the terminology can be easily modified to include such compounds, e.g. 9-*O*-acetyl-2-keto-3-deoxy-nonulosonic acid can be written as KDN9Ac. However, a more general problem not addressed by this nomenclature is that, in many cases, the investigator is not certain of the exact type of sialic acid present at a given position in an oligosaccharide. In this circumstance, it is suggested that the generic abbreviation Sia be used (not to indicate sialose, but to indicate a sialic acid, type unknown). If other partial information is available, it could be incorporated into such an abbreviation, e.g. a sialic acid of otherwise unknown type with an acetyl substitution at the 9-position could be written as Sia9Ac, until further information became available. On the other hand, if the type of substitution is known, e.g. an *O*-acetyl group, but the location is not, it could be abbreviated as SiaOAc. If a substitution is present, but the type is unknown, it could be written with an X, e.g. SiaX. If past history is any judge, it is likely that further diversity in the sialic acids will be discovered, and that the terminology and abbreviations discussed here will require further modifications in the future.

Distribution of modified sialic acids in nature: the myth of species specificity

Sialic acids seem to have appeared late in evolution and are not generally found in plants, prokaryotes or most invertebrates (Warren, 1963; Schauer, 1982). However, certain strains of bacteria (usually pathogenic) contain sialic acids in their capsular polysaccharides (Robbins *et al.*, 1974; Edwards *et al.*, 1982; McCoy and Troy, 1987; Vimr *et al.*, 1989; Wessels *et al.*, 1989), sometimes modified by *O*-acetylation at the C-7 or C-9 positions (Ørskov *et al.*, 1979). Strains of *Escherichia coli* (K1OAc$^+$) which contain *O*-acetylated sialic acids can either be fixed, or undergo a reversible form variation between OAc$^+$ and OAc$^-$ at a characteristic frequency (Ørskov *et al.*, 1979), dependent upon the expression of a specific *O*-acetyltransferase (Higa and Varki, 1988). Since many of these bacteria are pathogenic, the possibility of gene transfer from eukaryotes has been suggested (Schauer, 1982; Higa and Varki, 1988), but no proof for this has yet been forthcoming. A more surprising report indicates that Neu5,9Ac$_2$ is present in a strain of *Rhizobium*, a root nodule-forming bacterium (Defives *et al.*, 1989).

With such notable exceptions, sialic acids are generally found only in higher invertebrates or in vertebrates. Early studies on mucins and erythrocyte membranes suggested species specificity in the modification of sialic acids, e.g. 4-*O*-acetylation in equine tissues, 9-*O*-acetylation in bovine and murine tissues, and *N*-glycolyl sialic acids in porcine tissues. Thus, it appeared that these substitutions were of interest to the comparative zoologist or animal virologist, but would have no significance for more general issues in biology. However, it is now evident that the relative insensitivity of earlier techniques (see below) had made it difficult to detect smaller quantities of substituted molecules in some species. With improvements in techniques and studies of more tissues, it has become clear that these modifications are not species specific. For example, 4-*O*-acetyl sialic acids were originally thought to be specific for the ungulates, but have now been found in monotremes (Kamerling *et al.*, 1982a), guinea pigs (Hanaoka *et al.*, 1989) and humans (Miyoshi *et al.*, 1986), and 9-*O*-acetyl sialic acids have been found in every vertebrate species studied to date (Haverkamp *et al.*, 1977; Schauer, 1982).

Tissue-specific and molecule-specific expression of sialic acid modifications

On the other hand, modifications of sialic acids show remarkable tissue-specific and developmentally regulated expression in a variety of systems. Some are molecule specific, i.e. found only on certain types of glycoconjugates in a given cell type (Manzi *et al.*, 1990c). Even within a particular group of molecules, the modification may be restricted to certain sialic acid residues. For example, with one possible exception (Gowda *et al.*, 1984), 9-*O*-acetylation in the ganglio-series gangliosides is found only on a specific terminal α2-8-linked sialic acid residue (Ghidoni *et al.*, 1980, 1984; Schwarting and Gajewski, 1983; Cheresh *et al.*, 1984a; Hirabayashi *et al.*, 1989; Chou *et al.*, 1990; Dubois *et al.*, 1990). Such findings predict highly specific roles for these modifications in tissue development and/or organization. They also predict the occurrence of specific enzymatic mechanisms for their generation and regulation (see below).

Reasons why sialic acid diversity might be missed during structural analysis

As mentioned above, many studies of sialoglycoconjugates have failed to take complexity in the sialic acids into account. To a large extent, the reasons for this omission are technical: the substitutions are often labile and can substantially alter the behaviour of sialic acids during release, purification and analysis. On the one hand, substitutions can markedly slow or even completely prevent the release of sialic acids by commonly used sialidases (Drzeniek, 1973; Rosenberg and Schengrund, 1976; Varki and Kornfeld, 1980b; Corfield *et al.*, 1981; Sander *et al.*, 1982; Varki and Diaz, 1983, 1984) or by acid hydrolysis (Neuberger and Ratcliffe, 1972, 1973; Varki and Kornfeld, 1980b; Varki and Diaz, 1984). On the other hand, when stronger acidic conditions are used, destruction of some types of substitutions can occur (Varki and Kornfeld, 1980b; Varki and Diaz, 1984; Schauer, 1987; Hanaoka *et al.*, 1989; Manzi *et al.*, 1990a). Furthermore, many methods commonly used in the structural analysis of glycoconjugates (base hydrolysis during purification of glycolipids, alkaline borohydride release of O-linked chains, hydrazinolysis, methylation analysis, etc.) cause the destruction of sialic acid modifications. Even if modified sialic acids are successfully released in intact form, their anomalous behaviour in many conventional colorimetric and chromatographic techniques can pose problems in subsequent analysis. Thus, conventional approaches to the study of sialic acids from biological sources could easily miss a significant amount of such modifications. However, these substitutions can clearly affect the size, shape, hydrophilicity, net charge and biological properties of the parent molecule. Thus, a careful analysis for their presence is worthwhile in all situations where sialic acids are believed to play biological roles.

Spontaneous migration of *O*-acetyl groups to the 9-position

The most common modification of the hydroxyl groups of sialic acids is the addition of *O*-acetyl esters. These ester groups are alkali labile, and can be present at the 4-, 7-, 8- or 9-positions in various combinations (up to three per molecule have been reported so far). One unusual aspect of the chemistry of *O*-acetylated sialic acids is of technical importance and could be of relevance to their biology: *O*-acetyl ester groups located at the 7- or 8-position can spontaneously migrate to the 9-position, if this hydroxyl group is not already substituted (Varki and Diaz, 1984; Kamerling *et al.*, 1987) (see Figure 2). The rate of migration of 8-*O*-acetyl groups is so rapid that such a compound cannot be obtained in a stable state (Kamerling *et al.*, 1987). Interestingly, the $T_{1/2}$ for *O*-acetyl migration in free 7-*O*-acetyl-Neu5Ac was 4–8 h at physiological pH (7.0) and temperature (37°C) (Kamerling *et al.*, 1987). However, at the pH values that might be encountered in the Golgi apparatus (<6.5) the rate of migration was substantially slower (>10 h). Although not yet accurately measured, the migration of *O*-acetyl groups in glycosidically bound molecules is probably similar. Thus, it is possible that *O*-acetyl esters on specific molecules can be first expressed at the cell surface in the 7-position, and subsequently migrate to the 9-position over a period of hours. The resulting change in structure of the molecule (see Figure 2) could alter the recognition or biological properties of the underlying molecule. Fortunately, recent technical improvements make it possible to release, purify and analyse these molecules without significant migration of the esters.

Improvements in methodologies for the study of sialic acid modifications

Prior to analysis, sialic acids from biological sources must be completely released and purified, with their modifications intact. With regard to the side-chain (7/8/9) *O*-acetylated sialic acids, chemical and enzymatic improvements have been introduced which allow near-quantitative release and purification, without loss or migration of the labile ester groups (Varki and Diaz, 1984; Diaz and Varki, 1985; Diaz *et al.*, 1989a). The correct choice of a sialidase, or the use of mildly acidic conditions, are critical in obtaining optimal non-destructive release of such molecules. Such release must be monitored and conditions may need to be individualized for the specific molecules under study. A key factor in preventing loss or migration of *O*-acetyl groups during purification is the avoidance of the strongly basic anion-exchange columns (e.g. Dowex 1 and Dowex 2) that have been traditionally used for the purification of sialic acids (Schauer, 1987). The substitution of weak anion exchangers, e.g. Dowex 3x4A, is successful, but requires careful control of pH and ionic strength conditions (Varki and Diaz, 1984; Diaz and Varki, 1985). However, even with all these improvements, certain types of sialic acids remain intractable to accurate analysis. For example, 4-*O*-acetylated sialic acids are practically resistant to all known sialidases (Rosenberg and Schengrund, 1976; Schauer, 1982; Hanaoka *et al.*, 1989). On the other hand, the use of even mildly acidic conditions for release results in substantial loss of the *O*-acetyl esters (Varki and Diaz, 1984; Schauer, 1987; Hanaoka *et al.*, 1989) and/or degradation to the 4,8 anhydro compound (Pozsgay *et al.*, 1987; Manzi *et al.*, 1990a; Sugiyama *et al.*, 1991). At present, indirect approaches, such as the use of enzymes that release the entire oligosaccharide intact (Damm *et al.*, 1989; Hanaoka *et al.*, 1989), or monoclonal antibodies that recognize the 4-*O*-acetyl groups (Miyoshi *et al.*, 1986), are the only practical alternative for the study of these molecules. With regard to rarer molecules such as *O*-methylated or sulphated sialic acids, much less is known about their susceptibility to sialidases or their optimal release with acid, and no other methods for their direct detection are available.

Once released and purified, the analysis of purified sialic acids has traditionally been done by colorimetry (Warren, 1959), TLC (Schauer, 1987), GLC and GLC/MS (Schauer, 1982; Reuter *et al.*, 1983). In recent years, substantial improvements in methodology have occurred, including NMR spectroscopy, several new and sensitive HPLC methods, and fast atom bombardment-mass spectrometry (FAB-MS) [see Table II, and Haverkamp *et al.* (1982), Shukla *et al.* (1982), Schauer *et al.* (1984), Diaz and Varki (1985), Higa and Paulson (1985), Powell and Hart (1986), Shukla and Schauer (1986), Kamerling *et al.* (1987), Schauer (1987), Damm *et al.* (1989), Diaz *et al.* (1989a), Hara *et al.* (1989), Manzi *et al.* (1990a, b) and Manuguerra *et al.* (1991) for examples]. The technique of derivatization with 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB) followed by HPLC analysis with fluorescent detection (Hara *et al.*, 1989) has proven to be particularly sensitive and specific, and applicable to most derivatives (Manzi *et al.*, 1990b). Several techniques have also been developed for the detailed analysis of substitutions on

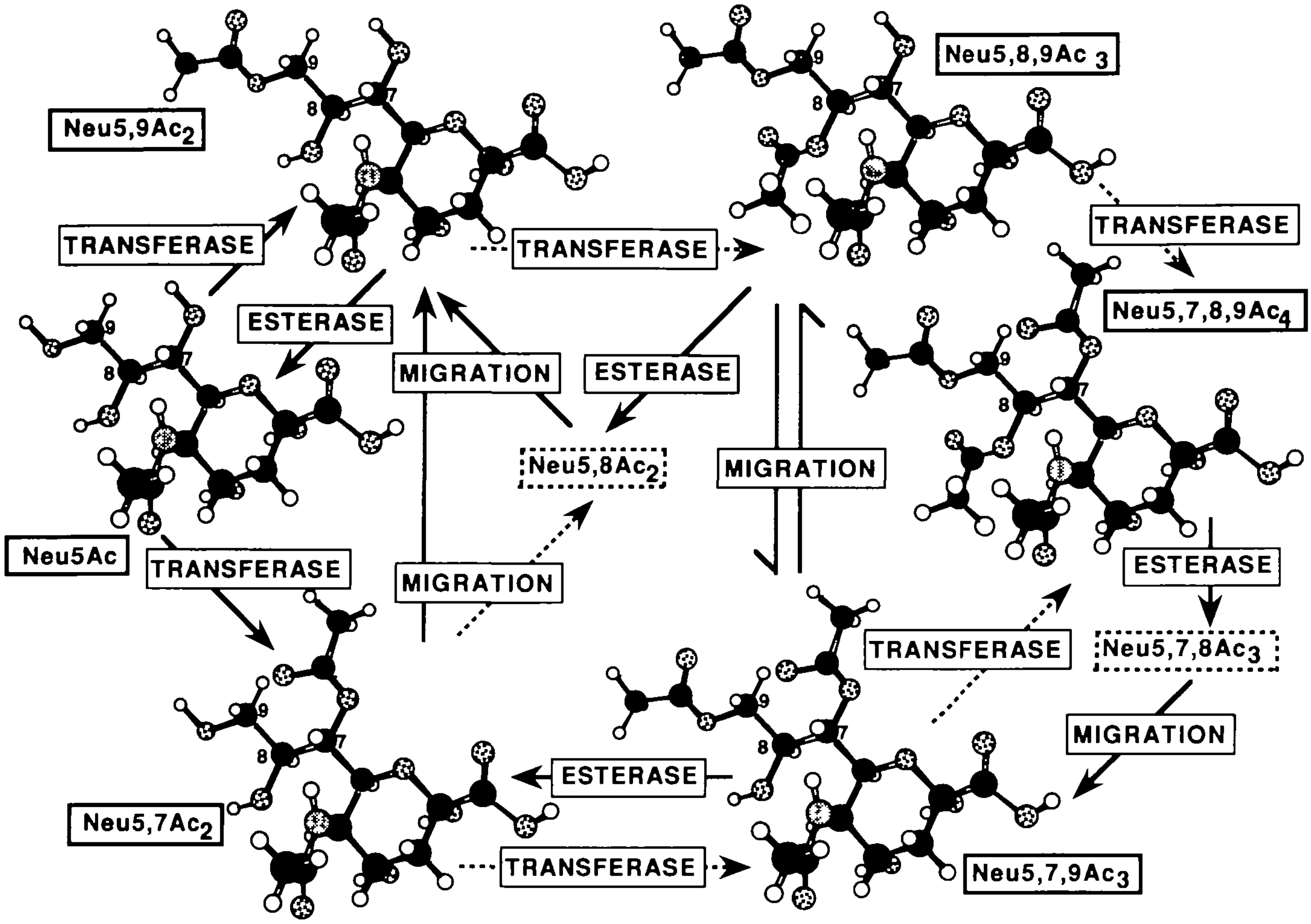

Fig. 2. Chemical and enzymatic relationships of side-chain *O*-acetylated sialic acids The migration of *O*-acetyl esters in the sialic acid side chain is shown, along with enzymatic relationships to the parent non-*O*-acetylated compound The di-*O*-acetyl sialic acids exist in a 1:1 equilibrium between the 7,9- and 8,9-di-*O*-acetyl forms, and migration cannot occur in 7,8,9-tri-*O*-acetyl molecules 'Transferase' refers to sialate: *O*-acetyltransferase(s) and 'esterase' refers to sialate: 9-*O*-acetylesterase(s). The solid arrows indicate reactions that have been conclusively demonstrated, while the dotted arrows indicate reactions that are likely or theoretically possible. The compounds indicated in the dotted boxes are transient intermediates which are presumed to exist, but are difficult to isolate and demonstrate

Table II. Improvements in the technology for the study of sialic acids

Method	Year introduced	Sensitivity range	Capability of studying mixtures
NMR spectroscopy	1982	μmol–nmol	Poor to fair
HPLC methods			
Amine resins	1984	nmol	Fair
Aminex resins	1986	nmol	Fair
High-pH anion exchange	1990	pmol	Fair to good
TSK-ODS with DMB derivatization	1989	pmol–fmol	Good
FAB-MS	1990	pmol	Excellent

metabolically labelled sialic acids (Diaz and Varki, 1985; Manzi *et al.*, 1990c). Monoclonal antibodies (MoAbs) and lectins have also been used to identify *O*-acetylated molecules (Cheresh *et al.*, 1984a; Thurin *et al.*, 1985; Sparrow and Barnstable, 1988; Ravindranath *et al.*, 1989; Chou *et al.*, 1990; Drazba *et al.*, 1991). The 9-*O*-acetyl-specific haemagglutinin of influenza C virus has been successfully used to probe for such molecules (Muchmore and Varki, 1987; Manuguerra *et al.*, 1991; Zimmer *et al.*, 1991). All other lectins described to date have rather poor affinity (Ravindranath and Paulson, 1987; Ravindranath *et al.*, 1988) or are only relatively specific for *O*-acetylated molecules (Mandal and Basu, 1987; Ahmed and Gabius, 1989). On the other hand, antibodies tend to be too specific, detecting the *O*-acetylated sialic acids only in the context of many other details of the underlying oligosaccharide structure. Thus, present technology makes it possible to confidently analyse only the alkali-resistant sialic acids (e.g. Neu5Ac, Neu5Gc and KDN) and *O*-acetylation of the 7/8/9 side chain of such sialic acids. Much remains to be done to improve the analysis of the other modified sialic acids.

Sialic acid modifications in development and malignancy

O-Acetylation at the 9-position appears to be developmentally regulated in a variety of systems and is re-expressed in certain

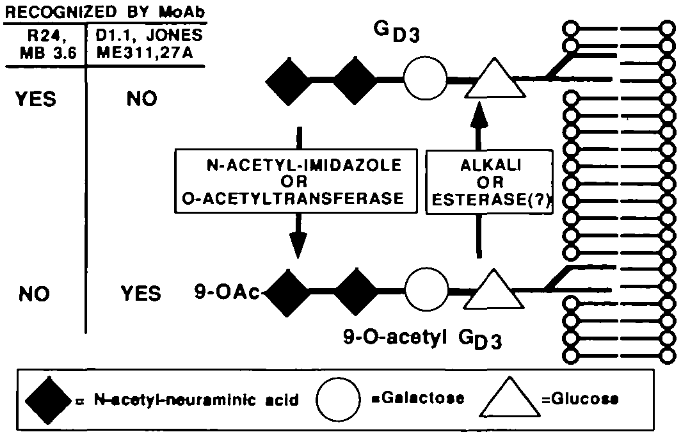

Fig. 3. Structural, biochemical and immunological relationships between G_{D3} and 9-O-Acetyl G_{D3}. The disialoganglioside G_{D3} is recognized by specific monoclonal antibodies as indicated Addition of a single *O*-acetyl ester to the 9-position of the terminal sialic acid molecule abolishes recognition by these antibodies and generates the epitopes recognized by the other antibodies indicated. The chemical and enzymatic interconversions between the two molecules are indicated.

malignancies in the form of 'onco-fetal' antigens, recognized by monoclonal antibodies (Cheresh *et al.*, 1984b; Constantine Paton *et al.*, 1986; Levine *et al.*, 1986; Blum and Barnstable, 1987; Muchmore *et al.*, 1987; Johnstone and Stallcup, 1988; Mendez Otero *et al.*, 1988; Schlosshauer *et al.*, 1988; Sparrow and Barnstable, 1988; Stallcup *et al.*, 1989; Chou *et al.*, 1990). The first example arose from the study of MoAb D1.1, which detected a developmentally regulated ganglioside (Levine *et al.*, 1984). This antibody was shown to recognize the addition of a single *O*-acetyl ester to the 9-position of the outer sialic acid residue of the disialoganglioside G_{D3} (Cheresh *et al.*, 1984a, b). Selective chemical acetylation of G_{D3} recreated the epitope (Cheresh *et al.*, 1984a), whereas alkali destroyed it (see Figure 3).

G_{D3} is the major sialoglycosphingolipid of human melanoma cells (Pukel *et al.*, 1982) and MoAbs against it are currently in clinical trials for the serotherapy of this cancer (Dippold *et al.*, 1985; Houghton *et al.*, 1985). However, G_{D3} expression is not unique to melanoma cells. In contrast, 9-*O*-acetyl-G_{D3} was not initially detected in any other human tumours nor in any human adult normal tissues studied (Cheresh *et al.*, 1984b), making it an oncofetal antigen of considerable interest. The proposed structure of 9-*O*-acetyl-G_{D3} was then confirmed by NMR and FAB-MS and another antibody (ME311) recognizing this epitope described (Thurin *et al.*, 1985), and its interaction with a crab lectin demonstrated (Ravindranath *et al.*, 1988). The importance of the *O*-acetylated molecule in the generation of the host immune response to melanoma gangliosides was then explored (Ravindranath *et al.*, 1989). Remarkably, *O*-acetylated G_{D3} has been reported as a tumour-associated antigen even in melanomas of the hamster (Ren *et al.*, 1989) and of the fish *Xiphophorus* (Felding-Habermann *et al.*, 1988). In parallel studies, others described the MoAb JONES, which recognized an epitope in the developing murine nervous system, and showed a striking dorsal–ventral gradient of expression across the developing retina (Constantine Paton *et al.*, 1986). It was then found that the primary epitope recognized by JONES was in fact 9-*O*-acetyl-G_{D3} (Blum and Barnstable, 1987; Sparrow and Barnstable, 1988). Further studies showed that the distributions of 9-*O*-acetyl-G_{D3} and G_{D3} were not congruent in several parts of the developing nervous system (Schlosshauer *et al.*, 1988; Sparrow and Barnstable, 1988). Altered expression of this molecule has been also reported in the weaver mouse, a genetic developmental abnormality whose primary defect is unknown (Johnstone and Stallcup, 1988). Very recently, other investigators described MoAb 27A (Dekan *et al.*, 1990) specific for the podocytes of the glomeruli in the rat kidney. This antibody also appears to recognize 9-OAcG$_{D3}$ (Reiviren *et al.*, 1990). Recent studies have also found this molecule in bovine milk products (Bonafede *et al.*, 1989; Hanagata *et al.*, 1991). Table III summarizes current knowledge concerning the restricted distribution and developmentally regulated expression of this interesting molecule in the rat, the species in which the most comprehensive studies have been carried out. Other alkali-labile, *O*-acetylated gangliosides in the nervous system bearing 9-*O*-acetyl residues have also been reported, including 9OAc-G_{T1b}, 9OAc-G_{Q1b}, 9OAc-G_{T3}, 9OAc-disialylparagloboside, 9OAc-G_{D2}, and 9OAc-G_{D1a} (Haverkamp *et al.*, 1977; Ghidoni *et al.*, 1980, 1984; Schwarting and Gajewski, 1983; Gowda *et al.*, 1984; Hirabayashi *et al.*, 1989; Chou *et al.*, 1990; Dubois *et al.*, 1990; Aubry *et al.*, 1991; Sjoberg and Varki, 1991). Of note, the 9-*O*-acetyl group is found on a terminal α2-8-linked sialic acid in all but one of these examples; however, the characterization of the latter was incomplete (Gowda *et al.*, 1984). Regulated expression of such molecules has also been described in other parts of the developing nervous system (Hirabayashi *et al.*, 1989; Chou *et al.*, 1990; Drazba *et al.*, 1991) and in human melanoma cells (Manzi *et al.*, 1990c). The various MoAbs against

Table III. Tissue-specific and developmentally regulated expression of 9-*O*-acetyl-G_{D3} in the rat

Central nervous system
ED8–9: neural plate and neural tube
ED10–11: neural tube and notochord: ?neural crest cells
ED13–18: germinal (neuroepithelial) cells in ventricular zones and/or process-forming cells
Post-natal proliferating germinal cells and/or process-forming cells
Adult trace amounts in cerebellum
Peripheral nervous system
ED13–18 dorsal root ganglia and their central and peripheral processes
Post-natal/adult: some dorsal root ganglia and their central and peripheral processes
Retina
ED12–13: central retinal cells and throughout vitreal surface
ED17–P3 dorsal–ventral gradient (except for ciliary rim and optic nerve-head)
Adult: outer plexiform layer only
Adrenal medulla
ED16 to adult. chromaffin cells
Kidney
ED12–16 metanephros
ED17–18 glomeruli only
Adult: podocyte cells of the glomerulus

ᵃBased on the data of Stallcup, Barnstable, Farquhar, and others using various monoclonal antibodies against 9-*O*-acetyl-G_{D3}

9-OAc-G_{D3} show cross-reactivity with certain other *O*-acetyl-gangliosides (Thurin *et al.*, 1985; Hirabayashi *et al.*, 1989; Stallcup *et al.*, 1989; Chou *et al.*, 1990). The existence of 7-*O*-acetyl-gangliosides has also been noted in fresh preparations from melanoma cells (Manzi *et al.*, 1990c; Sjoberg and Varki, 1991), further complicating the interpretation of many studies. However, a comprehensive study of the biosynthesis and immunological reactivity of this family of molecules has yet to be done. It should also be kept in mind that *O*-acetylated gangliosides can be confused with alkali-labile inner ganglioside lactones involving the carboxyl group of sialic acids and adjacent hydroxyl groups. These have been reported to occur both naturally and as artifacts of purification (Gross *et al.*, 1980; Riboni *et al.*, 1986; Ando *et al.*, 1989; Bosslet *et al.*, 1989; Maggio *et al.*, 1990; Terabayashi *et al.*, 1990; Kielczynski *et al.*, 1991).

There have been other reports of alkali-labile sialic acids that show developmental regulation and oncofetal changes in expression. In contrast to melanoma cells, colon cancer cells appear to lose the (7/8/9)*O*-acetylation that is normally found in the colonic mucosa, presumably on O-linked oligosaccharides (Reid *et al.*, 1984b; Muchmore *et al.*, 1987; Hutchins *et al.*, 1988). *O*-Acetylation in this case seems to appear post-natally in the normal animal. Thus, the loss of *O*-acetylation in tumours is once again essentially a reversal to the embryonic state. Expression of 4-*O*-acetylated sialic acids in human colon cancer has also been indirectly demonstrated with antibodies (Miyoshi *et al.*, 1986).

Neu5Gc can also be an onco-fetal antigen, specifically in humans and chickens. Although it is expressed in fetal human tissue and in certain human tumours (Higashi *et al.*, 1985; Hirabayashi *et al.*, 1987a, b) and human tumour cell lines (Carubelli and Griffin, 1968; Ohashi *et al.*, 1983), it is not found in normal adult human tissue (Schauer, 1982). In fact, glycoconjugates containing Neu5Gc are immunogenic in humans. Thus, upon exposure of humans to horse serum, a major epitope recognized in the resulting 'serum sickness' reaction is Neu5Gc (Merrick *et al.*, 1978; Fujii *et al.*, 1982). Spontaneously occurring 'Hanganutziu-Deicher' antibodies to Neu5Gc also occur in patients with cancer and with certain infectious diseases (Nishimaki *et al.*, 1979) and in chickens with Marek's disease, a malignant herpes-virus infection (Naiki *et al.*, 1982; Higashi *et al.*, 1984). It appears that post-natal suppression of Neu5Gc expression is complete prior to immune tolerization in humans and birds, but that re-expression of this sialic acid can occur in certain disease states. In contrast, Neu5Gc is found in adult primates (Schauer, 1982) and is a major component of many adult murine tissues. However, Neu5Gc expression in the rat does show developmental regulation in tissues such as the colon and small intestine (Bouhours and Bouhours, 1983, 1988; Muchmore *et al.*, 1987).

Biosynthesis and turnover of *O*-acetylated sialic acids

Neu5Ac is believed to be the precursor for all the other sialic acids. The tissue-specific and developmentally regulated expression of *O*-acetyl esters suggests that their synthesis and/or turnover are very carefully regulated. Early studies with bovine submaxillary gland slices and extracts showed that the donor for the *O*-acetylation of the 7- and 9-positions was acetyl-coenzyme A (AcCoA) (Schauer and Wember, 1971; Corfield *et al.*, 1976; Schauer, 1978). An analogous 4-*O*-acetyltransferase activity was found in equine submaxillary gland (Schauer, 1978). In each case, the acceptor substrate was reported to be either a free sialic acid molecule in the cytosol, or a bound sialic acid in the membrane fraction. However, at the time these studies were carried out, the topological relationships between the secretory pathway, the Golgi apparatus and the cytosol were not well understood. Later, others showed that glycosylation reactions occurred primarily in the Golgi apparatus (Palade, 1975; Hirschberg and Snider, 1987) and required the transport of intact sugar nucleotides into the lumen of this organelle, to serve as donors for luminally oriented transferases (Hirschberg and Snider, 1987). The utilization of [^{3}H-acetyl]AcCoA by isolated intact rat liver Golgi vesicles was therefore studied (Varki and Diaz, 1985; Sambasivam and Murray, 1988; Diaz *et al.*, 1989a, b; Higa *et al.*, 1989a). The results indicate that *O*-acetylation in this system is primarily a post-polymerization reaction in which acetyl groups from cytosolic AcCoA are transferred to luminally oriented sialic acids by a novel mechanism, probably involving a trans-membrane transfer of acetate groups (Higa *et al.*, 1989a). *O*-Acetyl esters can be transferred to 7- or the 9-position of endogenous sialic acids in this system, and the esters at the 7-position migrate to the 9-position if the pH conditions are appropriate (see Figure 2). Similar results were obtained for the *O*-acetylation of gangliosides in Golgi-enriched vesicles from human melanoma cells (Manzi *et al.*, 1990c; Sjoberg and Varki, 1991).

With regard to the question of cytosolic acetylation, other facts must also be considered. *O*-Acetylated sialic acids are poor substrates for CMP-sialic acid synthetase (Kean and Roseman, 1966) and in some cases cannot be utilized at all (Higa and Paulson, 1985). Even if CMP-*O*-acetylated sialic acids can be formed, they are poor substrates for sialyltransferases. In fact, certain sialyltransferases cannot utilize substituted CMP-sialic acids at all, including the major α2-6 sialyltransferase of bovine submaxillary gland, a tissue rich in *O*-acetylated sialic acids (Higa and Paulson, 1985). On the other hand, there is no evidence for separate sialyltransferases that utilize CMP-*O*-acetyl-sialic acids. In spite of these findings, *O*-acetyl groups are found selectively expressed between glycoproteins and glycolipids in a given cell type (Manzi *et al.*, 1990c) and even between sialic acid residues in the same molecule (Ghidoni *et al.*, 1980, 1984; Sonnino *et al.*, 1982; Manzi *et al.*, 1990c), indicating that the *O*-acetylation reaction can be highly specific. Finally, there are no other known examples of enzymes with identical substrate specificities that exist both in the cytosol and within the lumen of the Golgi apparatus. Taken together, current data suggest that most if not all *O*-acetylation of sialic acids may take place within the lumen of the Golgi apparatus or in Golgi-like organelles, after the transfer of sialic acids to glycoconjugates (see Figure 4). However, the existence of a true cytosolic *O*-acetyltransferase still cannot be ruled out. It also remains to be seen if distinct *O*-acetyltransferases are involved in the acetylation of specific positions on sialic acids. The selective distribution of *O*-acetyl esters also suggests a family of *O*-acetyltransferases, presumably specific for sialic acids on different classes of glycoconjugates (e.g. gangliosides versus N-linked oligosaccharides). Alternatively, a single enzyme could exist whose specificity is altered by different modifier proteins, in a manner similar to α-lactalbumin for galactosyltransferase (Hill and Brew, 1975). Unfortunately, purification of these extremely labile *O*-acetyltransferases has proven to be an intractable problem.

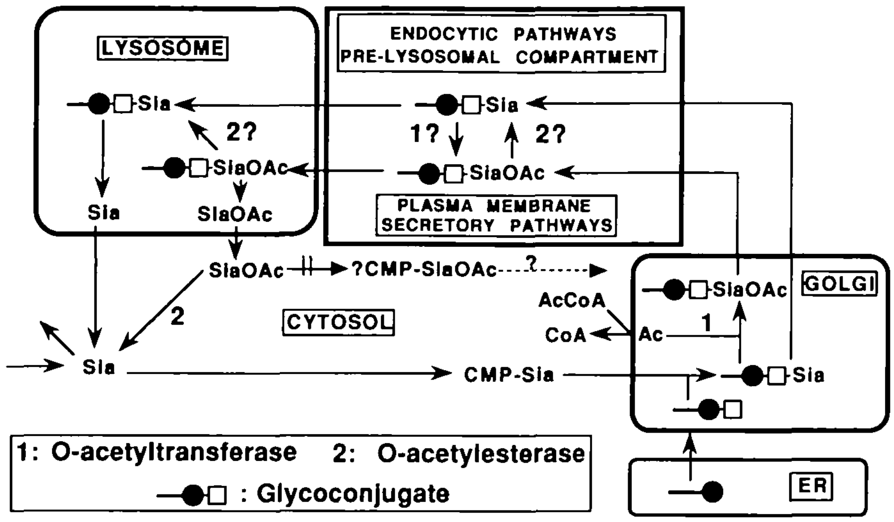

Fig. 4. Probable pathways for the *O*-acetylation and de-*O*-acetylation of sialic acids in mammalian cells The normal pathways for the biosynthesis, activation, transfer and turnover of Neu5Ac are indicated Steps at which *O*-acetylation and de-*O*-acetylation of sialic acids are known to occur are noted, with question marks indicating uncertainties

Once attached to sialic acids, *O*-acetyl esters need to be removed at some point in the life cycle of the parent molecule, either for terminal degradation or as part of an acetylation–deacetylation cycle (see Figures 2 and 4). Mammalian sialic acid-specific acetylesterases have recently been discovered and characterized, and may be involved in the turnover of 9-*O*-acetyl groups in a variety of different systems (Varki *et al.*, 1986; Schauer *et al.*, 1988, 1989; Higa *et al.*, 1989b, c). Evidence has also been presented suggesting that these are a unique family of DFP-sensitive esterases with critical arginine residues required for their action (Hayes and Varki, 1989). Notably, most of these enzymes are specific for esters at the 9-position, and are incapable of working on *O*-acetyl esters at the 7-position (see Figure 2). However, these 7-*O*-acetyl groups can eventually migrate to the 9-position and thus become substrates for these enzymes (Schauer *et al.*, 1988; Higa *et al.*, 1989b, c). In the case of the rat liver glycoprotein esterase (Higa *et al.*, 1989c), it was shown that the enzyme could eventually de-*O*-acetylate even 7,8,9 tri-*O*-acetylated sialic acids by sequential rounds of enzymatic cleavage and non-enzymatic migration of ester groups (see Figure 2). An esterase activity against 4-*O*-acetyl groups has also been found in equine tissues (Schauer *et al.*, 1988), which are rich in these substituents.

The best interpretation of current data is that there are at least two 9-*O*-acetylesterases in mammalian systems. One appears to be a cytosolic enzyme with specificity for 9-*O*-acetyl sialic acids in the free form (Varki *et al.*, 1986; Schauer *et al.*, 1988). As discussed above, *O*-acetylated sialic acids are poor substrates for re-utilization by enzymes such as CMP-sialic acid synthase and several sialyltransferases (Higa and Paulson, 1985). Thus, this cytosolic esterase activity could serve to 'recycle' *O*-acetylated sialic acids that are exported from lysosomes into the cytosol (see Figure 4). The other 9-*O*-acetylesterase is a water-soluble glycoprotein with complex- and high-mannose-type N-linked chains. It traverses the endoplasmic reticulum (ER)-Golgi pathway like other soluble glycoproteins, but is sequestered in intracellular membrane-bound compartments. This enzyme has been purified to homogeneity from rat liver and its properties characterized (Higa *et al.*, 1989b, c). It appears to be highly specific for cleavage of 9-*O*-acetyl groups from sialic acids. However, esters from the 7- or 8-position can be indirectly removed, after they have migrated to the 9-position. Thus, this single enzyme can de-*O*-acetylate even 7,8,9 tri-*O*-acetylated sialic acids by sequential cleavage from the 9-position, under mildly alkaline conditions (Higa *et al.*, 1989b). Although the activity was first detected in the Golgi apparatus (Diaz *et al.*, 1989a), it now appears that most of the enzyme is in later compartments, including true lysosomes (Butor,C., Higa,H.H., Griffiths,G. and Varki,A., unpublished observation). On the other hand, it has a relatively high K_m for its substrate and, unlike classical lysosomal enzymes, has a neutral pH optimum. At the present time, it is difficult to reconcile these properties with a specific role for this enzyme in the lysosomal turnover of *O*-acetylated sialic acids.

More precise information regarding the subcellular localization and contributions of each of these enzymes to the regulation of *O*-acetylation is being explored in a variety of different systems. Ultimately, the purification and molecular cloning of each will be required to achieve this goal, and to fully understand the regulation of *O*-acetylation.

Origin and fate of the *N*-acetyl group of sialic acids

The *N*-acetyl group at the 5-position of Neu5Ac normally originates from AcCoA (Warren and Felsenfeld, 1962; Kornfeld *et al.*, 1964; Roseman, 1970; Neufeld and Pastan, 1978) during conversion of GlcNH$_2$-6-P to GlcNAc-6-P, the precursor of UDP-GlcNAc. The latter undergoes irreversible epimerization to ManNAc, which is eventually converted to CMP-Neu5Ac via several intermediates (Warren, 1962; Kornfeld *et al.*, 1964; Roseman, 1970; Schauer, 1982) (see Figure 5). After Neu5Ac is transferred to macromolecules from the nucleotide sugar, it can later be released in the lysosomes, exported into the cytosol (Hildreth *et al.*, 1986; Renlund *et al.*, 1986), and either re-utilized or degraded to ManNAc and pyruvate (Warren, 1986). The *N*-acetyl group can also be hydroxylated to an *N*-glycolyl group by a specific hydroxylase (Schoop *et al.*, 1969). In earlier studies, a model was proposed in which such hydroxylases were active both in the cytosol and in the membrane fraction

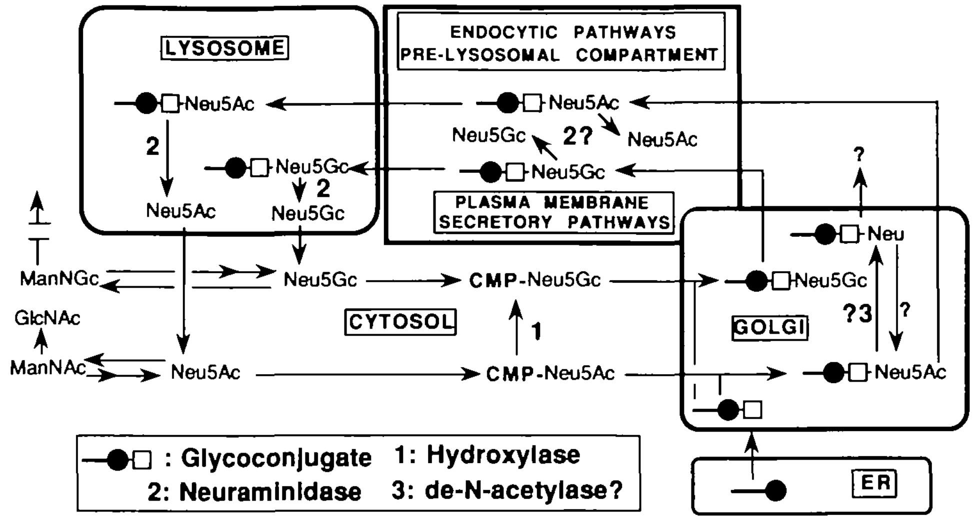

Fig. 5. Origin and fate of the *N*-acetyl group of sialic acids. The general pathways for the biosynthesis, activation and transfer of Neu5Ac are indicated. Steps at which the *N*-acetyl group can be removed, added back or hydroxylated are indicated, with question marks indicating uncertainties.

(Buscher *et al.*, 1977; Schauer, 1978). However, several groups have now found that only the nucleotide donor CMP-Neu5Ac can be hydroxylated and that very little of this enzyme activity is present in membrane fractions (Shaw and Schauer, 1988; Bouhours and Bouhours, 1989; Muchmore *et al.*, 1989; Lepers *et al.*, 1990). A model has been suggested to reconcile the earlier finding that Neu5Gc is a significant component of the free sialic acid pool (Muchmore *et al.*, 1989). It is proposed that the free cytosolic Neu5Gc arises from lysosomal release and export, and can then be re-utilized for synthesis of CMP-Neu5Gc (see Figure 5). Support for this model comes from the finding that Neu5Gc from extracellular glycoproteins can be taken up and incorporated into cellular glycoconjugates (Carubelli and Griffin, 1968; Furukawa *et al.*, 1988). If this cycle were to be exclusive, Neu5Gc would progressively accumulate in cells, a phenomenon that is indeed observed to some extent. Since Neu5Gc is also a substrate for acylneuraminate pyruvate-lyase, this cytosolic enzyme would result in the production of ManNGc (Jourdian and Roseman, 1962). While this compound is a substrate for re-condensation in sialic acid synthesis, no alternate fate for it is currently known. This should further tend to favour accumulation of Neu5Gc within the cell. More recent studies indicate that the hydroxylase reaction may occur in a multi-step process and involve the cytochrome b_5 complex (Kozutsumi *et al.*, 1990). Wheat germ agglutinin (WGA)-resistant mutants with high-level expression of Neu5Gc have recently been found (Shaw *et al.*, 1991; E.A.Muchmore, C.Hubbard, L.D.Powell and A.Varki, unpublished observations) that may help shed further light on this pathway.

Throughout all biological reactions of sialic acids, the *N*-acyl group was presumed to remain associated with the rest of the molecule. Since the de-*N*-acetylated form, neuraminic acid (Neu) is very unstable in the free state (Gielen, 1967), it had always been assumed that it did not exist in nature. However, the glycosidically bound form of neuraminic acid is at least as stable as the *N*-acetylated compound (Karkas and Chargaff, 1964). In 1988, it was reported that the MoAb DH5, raised against chemically synthesized de-*N*-acetyl G_{M3}, reacted with lipid extracts from A431 cells (Hanai *et al.*, 1988). Chemical re-*N*-acetylation with labelled acetic anhydride suggested that very small amounts of de-*N*-acetyl G_{M3} were present in the cells. A survey with this MoAb was positive in certain tumour cells, but not in some normal tissues and cell lines. It was therefore postulated that a de-*N*-acetylation/re-*N*-acetylation cycle existed involving the sialic acid moiety of G_{M3}. More recently, direct evidence for such turnover of *N*-acetyl groups on the sialic acids of both G_{M3} and G_{D3} in human melanoma cells was reported (Manzi *et al.*, 1990c). Taken together, these studies have provided evidence for the presence of de-*N*-acetyl gangliosides in certain cell types. However, direct proof of their existence will require isolation and positive identification by physical techniques such as FAB-MS (Nores *et al.*, 1989). There appear to be no other reports of de-*N*-acetyl-gangliosides in the literature, nor have there been any reports of non-acetylated neuraminic acid residues on other glycoconjugates in any other system. However, such molecules are likely to be missed during the conventional purification steps: for example, anion ion-exchange chromatography could result in separation and loss of these zwitterionic compounds, and their migration on TLC can be quite anomalous (Hanai *et al.*, 1988). Also, glycosidically bound neuraminic acids are resistant to all known sialidases (Drzeniek, 1973; Rosenberg and Schengrund, 1976). If acid hydrolysis were used instead to release these residues, the resulting free neuraminic acid would be very unstable, decomposing spontaneously (Gielen, 1967). Thus, it might not be surprising if small amounts of de-*N*-acetyl gangliosides are present in many cell types. Assuming that they exist, how might such molecules be made? The most likely scenario is the action of a specific de-*N*-acetylase, working directly on intact gangliosides. However, the search for such an enzyme has not yet been fruitful.

Biosynthesis of other types of sialic acid substitutions

Various types of dehydrated or unsaturated sialic acids are found in nature as free molecules, and appear to arise during enzymatic or chemical degradation processes. Thus, 2,7-anhydro sialic acids can arise following the release of bound sialic acids by certain unusual sialidases (Li *et al.*, 1990), 2,3-didehydro 2,6-anhydro compounds can result from mild alkali-catalysed breakdown of CMP-sialic acids (Beau *et al.*, 1984), and 4,8-anhydro compounds can form during the release or deacetylation of 4-*O*-acetylated compounds (Pozsgay *et al.*, 1987; Manzi *et al.*, 1990a). Many of these compounds are found in free form in biological fluids (Saito and Rosenberg, 1984; Nohle *et al.*, 1985; Suzuki *et al.*, 1985; Shukla *et al.*, 1987). It is not clear if these arise from enzymatically catalysed reactions, or from spontaneous chemical processes occurring at a slow rate in physiological conditions.

The phosphate group of Neu5Ac9P arises from ManNAc-6-P; however, this compound has only been reported so far as a free biosynthetic intermediate and not in glycosidically linked form. Very little is known about the biosynthesis of the other types of modified sialic acids. The deaminated form of sialic acid (KDN) could arise from sequential de-*N*-acetylation and deamination of Neu5Ac at some step. Other types of substitutions of the hydroxyl groups presumably arise from utilization of the appropriate donors (e.g. S-adenosylmethionine for methylated sialic acids, 3′-phosphoadenosine 5′-phosphosulphate for sulphated molecules). In some cases, e.g. the *O*-lactyl group, it is hard to predict what the natural donor might be. Appropriate enzymes should also exist to permit the turnover of the substitutions. The subcellular sites and levels of regulation of such enzymes also cannot be predicted at the present time.

Clues to the biological roles of sialic acid substitutions

On a quantitative level, some would argue that these modifications of sialic acids are usually minor in amount, and therefore raise questions about their biological relevance. However, there are several examples in which apparently minor modifications of carbohydrates play major biological roles. In the case of mannose 6-phosphate residues on lysosomal enzymes, only a small proportion of the N-linked oligosaccharides carried this modification at steady state (Varki and Kornfeld, 1980a), but they have a crucial function in the subcellular trafficking of lysosomal enzymes (Kornfeld, 1987). Another example is heparin, in which the rare 3-*O*-sulphate group is critical in mediating its anticoagulant action (Lindahl *et al.*, 1980).

While less clear cut than the classic examples cited above, there are now many clues to the biological roles of *O*-acetylated sialic acids, in widely disparate tissues and times in development. For example, B-cells have been reported to have *O*-acetylated sialic acids, whereas T-cells do not (Kamerling *et al.*, 1982b). On the other hand, thymocytes appear to have *O*-acetylated sialic acids (Schwarting and Gajewski, 1983) and T-cells of patients with various malignancies have been reported to acquire *O*-acetylation (Holzhauser *et al.*, 1988; Stickl *et al.*, 1991). The biological significance of these observations to the function of lymphocytes is currently unknown.

O-Acetylated sialic acids are frequently found in neural gangliosides, where their expression varies with developmental stage. The restricted distribution of *O*-acetylation of G_{D3} discussed above implies a critical role for the *O*-acetyl group in the morphogenesis and development of organs such as the retina, cerebellum and adrenal gland. Differences in the *O*-acetylation of brain gangliosides have been reported between cold-blooded and warm-blooded species, and between awake and hibernating animals (Ghidoni *et al.*, 1984; Rahmann *et al.*, 1984). As discussed earlier, other alkali-labile gangliosides have also been reported to show developmental changes and regional distribution in a variety of systems. The implication of these findings is that *O*-acetylation of gangliosides may play a role in the organization of neural tissues. However, no direct proof of this is currently available. Developmental regulation of *O*-acetylation is also found in tissues such as the gut mucosa, with a chronology quite distinct from that seen in the brain. In this case, it has been suggested that the *O*-acetylation may appear as a response to microbial colonization, and play a role in protecting against certain microorganisms (Muchmore *et al.*, 1987). A similar argument has been made for the *O*-acetylation of sialic acids on murine erythrocytes that appears to confer resistance to the binding of the malarial parasite (Reuter *et al.*, 1991).

Expression of *O*-acetyl and *N*-glycolyl groups on cell surfaces can also alter the action of bacterial sialidases (Drzeniek, 1973; Corfield *et al.*, 1981, 1986; Varki and Diaz, 1983; Reid *et al.*, 1984a) and the binding of pathogenic viruses (Herrler *et al.*, 1985; Higa *et al.*, 1985; Rogers *et al.*, 1986; Muchmore and Varki, 1987; Vlasak *et al.*, 1987, 1988). In most cases, the consequence would be protection of the host from the corresponding microbial pathogen. Taken together with the frequent expression of these modifications on mucosal surface, it is reasonable to postulate that these modifications play a role in host defenses against primary attack or recognition by pathogens. However, clear exceptions are seen in the case of influenza C and certain coronaviruses which specifically bind to 9-*O*-acetylated sialic acids (Herrler *et al.*, 1985; Rogers *et al.*, 1986; Muchmore and Varki, 1987; Vlasak *et al.*, 1987, 1988; Schultze *et al.*, 1991). However, these are relatively benign pathogens compared to the influenza A and B viruses, whose binding to sialic acids is abrogated by *O*-acetylation (Higa *et al.*, 1985; Rogers *et al.*, 1986). Thus, the price paid for using *O*-acetylation to protect from the more dangerous viruses might be susceptibility to the less pathogenic ones. With regard to the 2,3 didehydro 2,6-anhydro sialic acids found in biological fluids, it has been hypothesized that they provide protection by virtue of their powerful inhibition of microbial sialidases (Schauer, 1982). In the final analysis the data are supportive, but no conclusive proof exists that sialic acid modifications provide crucial protection from pathogens.

The 9-*O*-acetylesterase found as a 'receptor-destroying enzyme' in the coat protein of influenza C viruses (Herrler *et al.*, 1985; Vlasak *et al.*, 1987) has now been found in several coronaviruses (Vlasak *et al.*, 1988; Holmes and Williams, 1990; Parker *et al.*, 1990; Schultze *et al.*, 1991; Yokomori *et al.*, 1991). All those studied to date are similar to the mammalian 9-*O*-acetyl esterases in having DFP-sensitive serine active sites. Primary sequencing of an influenza C HE protein (Vlasak *et al.*, 1989) and database sequence comparisons with other influenza CHE clones indicate that they all share the common sequence Phe-Gly-Asp-Ser-Arg-Thr(Ser)-Asp (FGDSRSD) (Hayes and Varki, 1989). The arginine residue immediately adjacent to the active site serine is unique to this family of serine esterases and is presumed to be involved in recognition of the anionic sialic acid substrate (Hayes and Varki, 1989). Notably, the identical sequence is conserved in several strains of coronavirus glycoproteins which have the same substrate specificity (Kienzle *et al.*, 1990; Parker *et al.*, 1990; Yokomori *et al.*, 1991), but substantial sequence divergence in flanking areas of the polypeptide. A notable variation occurs in most strains of the MHV coronaviruses, in which the gene is almost always rendered silent by stop codons (Luytjes *et al.*, 1988; Yokomori *et al.*, 1991). It could be postulated that the high expression of

O-acetylated sialic acids on murine red cells (Sarris and Palade, 1979; Reuter *et al.*, 1980; Varki and Kornfeld, 1980b) provides a natural barrier against coronaviruses with active HE proteins reaching the liver via the circulation. Thus, the MHV coronaviruses may have been selected for loss of expression of the HE function. In keeping with such a hypothesis, the incomplete open reading frames of several of these viruses still contain the FGDSRSD sequence that appears to be characteristic of this esterase function (Hayes and Varki, 1989).

The fact that both the 9-*O*-acetyl-specific haemagglutination and 9-*O*-acetyl esterase activities are encoded by the same polypeptide raises the question of whether they are mediated by the same binding pocket. However, in the case of influenza C, it has been shown that inactivation of the esterase with DFP does not decrease the haemagglutination 'receptor' activity contained in the same protein. Rather, it stabilizes the haemagglutinin activity by inactivating the esterase and preventing destruction of the receptor (Muchmore and Varki, 1987). In spite of this, the DFP-treated virus showed a markedly diminished infectivity (Muchmore and Varki, 1987). Similar results were obtained using reversible inhibitors of the esterase (Vlasak *et al.*, 1989). These data indicate that the 9-*O*-acetyl esterase activity is important for these viruses in the initial phase of infection. It is possible that the virus needs the esterase activity to avoid peripheral proteins bearing 9-*O*-acetylated sialic acids so that it can reach the membrane-bound receptors that will ensure its uptake. Alternatively, the esterase activity might be required immediately after endocytosis to permit detachment of the virus from the endosomal wall.

Modifications can also alter recognition by arthropod lectins that bind sialic acids (Ravindranath *et al.*, 1985, 1988; Mandal and Basu, 1987; Ravindranath and Paulson, 1987; Mandal, 1990). However, since these organisms do not themselves contain sialic acids, the meaning of these observations is not clear; perhaps they have other natural ligands that are structurally related. 9-*O*-Acetylation can also abrogate the normal function of sialic acid in preventing activation of the alternate complement pathway (Fearon, 1979; Michalek *et al.*, 1988; Meri and Pangburn, 1990). This was demonstrated by strain-specific differences in the *O*-acetylation of erythrocyte sialic acids in mice, which seemed to explain differences in their sensitivity to lysis by complement (Varki and Kornfeld, 1980b). The exocyclic side chain of sialic acid is important in its binding of factor H, the key regulatory molecule of the alternative complement pathway (Fearon, 1979; Meri and Pangburn, 1990). Thus, it is reasonable to hypothesize that the addition of a bulky acetyl group to this side chain could cause a loss of binding of factor H. However, this has not been proven directly. The significance of this phenomenon for the normal physiology of complement is also not clear.

As indicated in earlier sections, sialic acid modifications can provide epitopes for recognition by antibodies in a variety of situations. It is not as well appreciated that the modifications can also prevent recognition by other antibodies. This is of practical importance because of the large number of antibodies that recognize sialic acid-dependent epitopes on specific proteins [see Bazil *et al.* (1989), Shelley *et al.* (1989), Cyster *et al.* (1991), Poppema *et al.* (1991) and Taylor-Papadimitriou (1991) for examples]. In most such instances, the effects of sialic acid modifications on antibody recognition have not been explored. *O*-Acetylation can also affect the immunogenicity and pathogenicity of bacteria with sialic acids in their capsular polysaccharides (Ørskov *et al.*, 1979). Thus, isolates of K1 *E.coli* from disease states are frequently non-*O*-acetylated (non-antigenic and non-stimulatory for complement), whereas free-living isolates are frequently *O*-acetyl-positive (more antigenic, but presumably more resistant to attack by endo- and exosialidases from other organisms).

The role of the common *N*-glycolyl group is difficult to discuss, given its apparent absence in the adult human, and its strain-specific expression in certain tissues of adult rats and dogs (Yasue *et al.*, 1978; Bouhours and Bouhours, 1988). It is possible that it has critical roles in embryogenesis, but is trivial or vestigial in the post-natal animal, apart from its moderate retardation of bacterial sialidase action. Possible roles for de-*N*-acetyl sialic acids have been suggested (Hanai *et al.*, 1988). While G_{M3} inhibited tyrosine phosphorylation of the epidermal growth factor (EGF) receptor, synthetic de-*N*-acetyl G_{M3} had a stimulatory effect and proved to be a stimulator of growth of intact cells. Neither compound had any effect on the number of EGF receptors, nor their affinity for EGF. However, another recent study obtained somewhat different results regarding the effects of de-*N*-acetyl G_{M3} on growth (Song *et al.*, 1991). Regardless, all of these results suggest that de-*N*-acetyl gangliosides could be involved in growth regulation.

The discovery of mammalian lectins recognizing ganglioside oligosaccharides (Ahmed and Gabius, 1989; Tiemeyer *et al.*, 1989, 1990) predict many biologically important recognition processes involving sialic acids. In each case, the effects of various modifications of sialic acids will bear close scrutiny. In at least one case (Ahmed and Gabius, 1989), *O*-acetylation appears to significantly improve the recognition of sialic acids. More recently discovered sialic acid-binding lectins include the selectins (Bevilacqua *et al.*, 1989; Camerini *et al.*, 1989; Stoolman, 1989; Lowe *et al.*, 1990; True *et al.*, 1990; McEver, 1991; Moore *et al.*, 1991; Polley *et al.*, 1991), the macrophage sialoadhesin (Crocker and Gordon, 1989; Crocker *et al.*, 1991) and CD22β (Stamenkovic *et al.*, 1991) of B-lymphocytes. In these cases, there has been no systematic investigation of the effects of sialic modification on ligand recognition.

Experimental approaches to understanding the biological roles of sialic acids

In spite of all these tantalizing clues, the precise biological roles of modified sialic acids remain obscure in most cases. Marked variations in *O*-acetylation can be found between otherwise similar cell lines in culture, with no obvious consequences to the growth and housekeeping functions of the single cell. Furthermore, sialic acids have not been detected in simpler developmental systems such as *Dictyostelium discoideum* (Amatayakul-Chantler *et al.*, 1991) and the nematode *Caenorhabditis elegans* (Bacic *et al.*, 1990). Thus, we must conclude that the more important biological roles of sialic acid substitutions have to be studied in intact, complex mammalian systems. To date, no naturally occurring genetic defects in sialic acid modification have been discovered in animals. Perhaps such mutations are lethal *in utero* and would never be observed in live animals. We are therefore left with the need to create conditional mutants in sialic acid modification in intact higher animals. In diploid mammalian species, recessive mutations are still somewhat difficult to obtain (Capecchi, 1989) and, furthermore, could be lethal. The expression of genes that bestow a dominant phenotype can circumvent many of these problems (Hanahan, 1989). Transgenic mice were therefore developed, expressing the HE protein from influenza C virus under the control of specific promoters (Varki *et al.*, 1991). Under physiological pH and temperature conditions, the only known activity of this cell-surface protein is an esterase specific for 9-*O*-acetylated sialic acids. The goal was to disrupt critical functions of these molecules during development, and to study the resulting consequences. The initial results in this regard are encouraging (Varki *et al.*, 1991). Expression of the enzyme in the fertilized egg consistently arrested development at the two-cell stage, suggesting that *O*-acetylated sialic acids might be involved in segmentation of the embryo. Late expression in specific organs caused developmental abnormalities. Further work is needed to prove that these abnormalities are indeed due to the destruction of *O*-acetylated sialic acids, and not due to some other unexpected consequence of expression of the viral protein. The same approach could presumably be taken towards uncovering the roles of 9-*O*-acetylated sialic acids in other tissues.

Future directions

A great deal remains to be done in the study of sialic acid modifications. Further improvements in analytical methods are needed to permit the non-expert to routinely identify and characterize these substitutions. The discovery of new sialic acids is likely to continue. For each substitution, the relevant enzymes involved in biosynthesis and turnover need to be purified , characterized and molecularly cloned. The molecular basis of the tissue-specific and developmentally regulated expression of the substitutions must then be explored. The many clues to the biological roles of these substitutions need to be explored and new ones must be actively sought. The future promises to be exciting.

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Abbreviations

Unless stated otherwise, all sugars are in the D-configuration and the pyranose form. The various sialic acids are abbreviated according to the nomenclature of Schauer, described in the text and in Table I. A parallel nomenclature based upon the root word 'Sia' is proposed here for sialic acid residues whose structure is incompletely characterized (see the text and Table I). The various ganglio-series gangliosides are designated according to Svennerholm *et al.* (1989). Other abbreviations used include: AcCoA, acetyl-coenzyme A; 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride, DMB; EGF, epidermal growth factor, ER, endoplasmic reticulum; FAB-MS, fast atom bombardment-mass spectrometry, KDN, 2-keto-3-deoxy-nonulosonic acid, MoAbs, monoclonal antibodies; WGA, wheat germ agglutinin.

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