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Assays of sialate-*O*-acetyltransferases and sialate-*O*-acetylesterases

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Abstract The *O*-acetylation of sialic acids is one of the most frequent modifications of these monosaccharides and modulates many cell biological and pathological events. Sialic acid-specific *O*-acetyltransferases and *O*-acetylesterases are responsible for the metabolism of esterified sialic acids. Assays were developed for the analysis of the activities and specificities of these enzymes. The methods had to be varied in dependence on the substrate assayed, the kind of biological source, and the state of enzyme purity. With the new techniques the primary site of *O*-acetyl incorporation at C-7, catalyzed by the animal sialate-*O*-acetyltransferases studied, was ascertained. Correspondingly, this enzyme, for example from bovine submandibular gland, can be denominated as AcCoA:sialate-7-*O*-acetyltransferase (EC 2.3.1.45). Methods for assaying the activity of esterases de-*O*-acetylating sialic acids and their metabolic cooperation with the *O*-acetyltransferases are presented.

Keywords Sialic acids · *O*-acetylated · *O*-Acetyltransferases · *O*-Acetylesterases · Thin-layer chromatography · High-performance liquid chromatography · Radiometry · Colorimetry

Introduction

One of the most abundant modifications of sialic acids (Sia) in man, animals and microorganisms is *O*-acetylation, either at the pyranose ring at C-4, or at the Sia side-chain, at C-7, C-8 or C-9 [1–3]. One Sia molecule may carry up to three acetyl residues and these may be combined with an *N*-acetyl or *N*-glycolyl group and with additional *O*-substituents like methyl and lactyl groups [1–4]. In individual animal species and tissues the relative degree of Sia *O*-acetylation can vary from very low to practically full esterification of these monosaccharides. The richest known sources of *O*-acetylated Sia are bovine submandibular gland mucin and human colon mucosa.

In animals, two enzyme systems are known, which were found to be responsible for ring and side-chain *O*-acetylations. These are AcCoA:sialate-4-*O*-acetyltransferase (EC 2.3.1.44), best characterized in guinea pig liver [5, 6], and AcCoA:sialate-7(9)-*O*-acetyltransferase (EC 2.3.1.45) studied in rat liver [7], human colon [8], and bovine submandibular gland [9]. An *O*-acetyltransferase exclusively transferring acetyl groups to C-9 was investigated in *Campylobacter jejuni* [10]. In mammalian tissues the acetyltransferases are localized in the Golgi membrane [1–3, 6, 7, 9]. Identification of the sialate-*O*-acetyltransferase (SOAT) gene was possible in some microorganisms like *Neisseria meningitides* [11], *Escherichia coli* [12], group B *Streptococcus* [13], and *C. jejuni* [10], but not yet in eukaryotic cells.

Interest in *O*-acetylated Sia is increasing due to their wide occurrence and involvement in many cell biological and pathophysiological phenomena. They retard the degradation of glycoconjugates by negatively influencing the activity of sialidases and sialate lyases, hinder or promote the recognition of molecules, cells or pathogens, thus playing roles in microbial infections, immunity, apoptosis as well as differentiation and tumor growth [1–3]. They can be considered as potential markers for skin tumors such as melanoma and basalioma [14, 15] and acute lymphatic leukemia (ALL) [16].

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These functions demand more knowledge about the regulation of the expression of *O*-acetylated Sia on the molecular genetic level and characterization of the acetyltransferases involved. This article describes methods suitable for identification of the activities of these enzymes.

The occurrence of the amount of *O*-acetylated Sia in a special tissue or cell is believed to depend not only on SOAT activity alone, but also on the activity of sialate-*O*-acetylesterases (SOAEs), which are involved in saponification of ester groups of Sia. In this way they "switch off" the function of *O*-acetylated Sia and also facilitate the catabolism of these monosaccharides. It was shown in human colon mucosa that the quantity of *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac_2) depends on the relationship of both the SOAT and SOAE activities [17]. Both activities are also high in rat liver, which also seem to cooperate in the expression of *O*-acetylated Sia in this tissue [7, 18]. Furthermore, a regulatory influence of these two enzymes on the expression of Neu5,9Ac_2 in *E. coli* [12] and group B *Streptococcus* [19] has been discussed. Sialate-*O*-acetylesterases have frequently been detected in animals of the deuterostome lineage, in bacteria and in viruses, some of which were partly or totally purified and the respective genes cloned [20–24]. The techniques of the analysis of SOAE activities will therefore be included.

Methods

Assays of sialate-*O*-acetyltransferases

Preparation of enzyme samples

The activity of animal SOAT can be determined in tissue homogenates, whole cell lysates, isolated membrane fractions, microsomal solubilisates or protein fractions from enzyme purification steps by testing with endogenous or exogenous substrates.

SOAT assay using endogenous substrates

The best source known for assaying SOAT activity with endogenous substrates are microsomal membranes enriched by Golgi vesicles. SOAT activity was found predominantly in this fraction from the eukaryotic cells and tissues studied. Microsomes are prepared by differential or sucrose gradient centrifugation of crude subcellular membrane fractions obtained after homogenization of fresh or frozen tissues or cells. Sialyltransferase activity [25] may be used as marker enzyme of these membranes. Most experience in subcellular membrane preparation containing highest SOAT activity was obtained with rat liver [7, 26], guinea pig liver [6] and bovine submandibular gland ([9]; Srinivasan *et al.*, unpublished).

Protein in the microsomal fractions is determined by the bicinchononic acid assay as per manufacturer's (Interchim, Montluçon, France) instruction. Endogenous Sia are quantified by a microadaptation of the orcinol/ferric chloride method [27].

The quality of bound Sia as endogenous substrate is analyzed after hydrolysis of the microsomes in 2 M propionic acid for 4 h at 80°C [28, 29]. This method enables quantitative release of Sia from their glycosidic linkages and preservation of most *O*-acetyl groups. Since a propionic acid/water mixture evaporates anisotropically, Sia can be gently concentrated in an acid hydrolyzate. Their analysis is described below.

SOAT activity is estimated in a total volume of 200 µl potassium phosphate (50 mM)–KCl (50 mM) buffer, pH 6.5, containing routinely 25 µg microsomal protein and 0.4 µCi [^{14}C]AcCoA (Amersham, Braunschweig, Germany). The assay is carried out in the presence or absence of protease inhibitors (2 mM Pefabloc SC and Mini Complete™, Roche Molecular Biochemicals, Mannheim, Germany). The incubation routinely is at 37°C for 30 min, and the reaction is terminated by cooling in ice.

The quantity of acetyl groups transferred by membrane-bound SOAT can be determined in two ways, either by counting of radioactivity incorporated into microsomes or by analysis of the Sia released by mild acid hydrolysis after incubation. In the first approach microsomes are washed two times in ice-cold incubation buffer, with intermediate centrifugation at 100,000×*g*. The final microsomal sediment is solubilized in 400 µl 1 N NaOH overnight at room temperature, neutralized with 200 µl 2 N HCl, mixed with 1 ml of scintillation cocktail and counted for radioactivity in a β-counter. In order to determine the increment of radioactive *O*-acetyl groups formed, which were found to be largely due to neo-*O*-acetylated Sia (*i.e.* Sia esterified during enzyme incubation), microsomes are incubated with 0.1 N NaOH for 1 h on ice, washed three times with buffer, before solubilization and counting. The radioactivity difference between these two batches, of which one was mildly saponified, gives an approximate value of glycoconjugate-bound neo-*O*-acetylated Sia. In the second approach, Sia are liberated from the washed microsomes by 2 M propionic acid at 80°C in the course of 4 h. After centrifugation Sia are purified and analyzed as described below.

This SOAT assay with microsomes is relatively quick and easy to carry out. It is well suited for screening of SOAT activity in animal tissues and cells, since in all cases the transferase is bound to microsomes, probably the Golgi. It is accompanied by nascent glycoconjugates, the Sia of which serve as *O*-acetyl acceptors. Residual CMP-Neu5Ac in the Golgi may also be neo-*O*-acetylated before being transferred to glycoconjugates during the incubation time of

SOAT reaction. The excellent SOAT acceptor property of CMP-Neu5Ac and GD3 will be described below. Use of microsomes allows kinetic measurements of acetyl transfer from AcCoA, but not with bound sialyl acceptors, since with increasing microsome quantities SOAT activity also raises. Activators and inhibitors can well be tested with the bound enzyme. Since the microsomal fractions may contain both glycoproteins and glycolipids, neo-*O*-acetylated gangliosides may be extracted by chloroform/methanol/25 mM $CaCl_2$ in water (50:40:10), and analyzed by TLC as described below, before acid or alkaline treatment of microsomes.

This assay with endogenous substrates can also be carried out with tissue homogenates or cell lysates (before incubation, it is recommended to precipitate the larger particles by centrifugation at 18,000×*g*). The specific activities of SOATs are, however, much lower than in isolated microsomes.

SOAT assay using exogenous substrates

As enzyme probe tissue homogenates, cell lysates, microsomes or solubilized enzyme in different stages of purification may be used.

Assay with CMP-Neu5Ac This sugar nucleotide revealed to be an excellent substrate of eukaryotic SOATs. In a typical assay, total 200 µl of 50 mM potassium phosphate–50 mM KCl buffer, pH 6.5, contain 25 µg protein, 36 µg CMP-Neu5Ac (Calbiochem-Novabiochem, Bad Soden, Germany) and 0.4 µCi [^{14}C]AcCoA. After 30 min at 37°C, the reaction is terminated with 200 µl 4 M propionic acid (final concentration must be 2 M acid). The CMP-glycoside bond is hydrolyzed at 80°C for 15 min followed by cooling in ice for 10 min (under these conditions, Sia linked to glycoconjugates, which may be present in the assay as endogenous substrates, are not yet released to a significant degree). The incubation mixture is centrifuged at 14,000×*g* for the solubilized enzyme and 100,000×*g* for the microsomes for 30 min at 4°C. The supernatant is concentrated to dryness in a centrifugal vacuum dryer for about 3 h. The residue with neo-*O*-acetylated Sia is dissolved in 50 µl water and analyzed as described below. This assay can also be performed with Neu5Ac or other free Sia and does not require the short hydrolysis step after incubation. It allows kinetic measurement of both donor and acceptor substrates.

Assay with GD3 Ganglioside GD3 is also a very suitable substrate for the analysis of SOAT from different mammalian sources. Microsomes, solubilized crude or purified SOAT (25 µg protein) are mixed with 25 µg GD3 (Snow Brand Milk Products, Tokyo, Japan) and 0.04 µCi [^{14}C] AcCoA in the above phosphate–KCl buffer, pH 6.5. The mixture is sonicated for 10 s and incubated for 30–60 min at 37°C, and the reaction is terminated by freezing in liquid nitrogen. After thawing some precipitated material is removed by centrifugation at 14,000×*g* and the supernatant is concentrated to dryness by centrifugal vacuum drying for 3 h. For TLC analysis of the neo-*O*-acetylated ganglioside (see below) the residue is taken up in 50 µl water and sonicated for 10 s in case of testing membrane-bound enzyme. In case of assaying solubilized enzyme, chloroform/methanol (2:1) can be used for dissolution of the sample. If direct identification of the nature of neo-*O*-acetylated Sia of gangliosides is desirable, these monosaccharides can be released by propionic acid hydrolysis before chromatographic analysis.

The assay described can also be applied to other gangliosides or to sialylated glycoproteins and oligosaccharides. In the latter two cases, Sia have to be hydrolyzed from glycosidic linkage before analysis. Otherwise, separation of the reaction products by techniques, such as capillary electrophoresis, is possible [10]. It was shown that radioactivity was incorporated into these substrates only as ester groups of Sia, as was proven by saponification and quantitative analysis of Sia after release from glycosidic linkage.

Analysis of SOAT reaction products

Ion-exchange purification of Sias Free Sia obtained from the assay described in "Assay with CMP-Neu5Ac" and dissolved in 50 µl ultrapure water are purified by anion-exchange chromatography on a 1 ml column of Dowex 2× 8, 200–400 mesh, chloride form at 4°C [27]. After the adsorption of Sia, the column is washed with 5 ml water. Sia are eluted with 5 ml of 1 M formic acid or 2 M propionic acid and the column is washed with 5 ml water. Both eluates are combined, lyophilized, dissolved in 800 µl water and dried in a speed vac concentrator. The residue is dissolved in 20 µl water. One half is used for TLC (see below) and the other for radioactivity counting.

Thin-layer chromatography of neo-O-acetylated Sia Purified Sia from ion-exchange chromatography are resolved on (10×10 cm) cellulose plates in *n*-butanol/*n*-propanol/0.1 N HCl (1:2:1) at room temperature. At least 5 µg Sia should be added to a lane of 1–4 cm width. A standard Sia mixture (10 µg) isolated from bovine submandibular gland mucin ("BSM standard") containing various *N*- and *O*-acetylated Sia [4, 27] is run in parallel for comparison. It is recommended to cochromatograph this standard also with the radioactive Sia from the SOAT assay, since small differences in the R_f-values between assay and standard Sia may arise, due to residual contaminants in the test sample. After drying the plate with air and scanning for the quantity

and localization of radioactivity, the location of Sia is visualized by spraying the plate with the orcinol/ferric chloride reagent diluted with water by one third and heating at 120°C for 20 min [27].

A much simpler method for the identification of free neo-*O*-acetylated Sia from SOAT assays was developed ("Assay with CMP-Neu5Ac"). The samples are directly chromatographed, without prior ion-exchange chromatography, on cellulose in ethanol:1 M ammonium acetate (7:3) at room temperature. This solvent allows Sia separation even in the presence of salts from the incubation medium. Figure 1 shows a typical example of this kind of TLC. While residual radioactive AcCoA (Fig. A) did not migrate into the Sia area, a second peak (R_f 0.54) is located at the position of *N*-acetyl-7-*O*-acetylneuraminic acid (Neu5,7Ac_2). This shows that the SOAT of bovine submandibular gland, primarily acetylates *O*-7 of the Sia molecule yielding Neu5,7Ac_2. This was observed repeatedly with crude and purified bovine SOAT but also with the corresponding enzyme from human and animal sources (unpublished data). In former studies with bovine submandibular gland SOAT [9, 27] mainly Neu5,9Ac_2 was identified on TLC. We also observed this Sia type after prior purification on ion-exchange resin and believe that migration of the acetyl groups from *O*-7 to *O*-9 has occurred during adsorption of Sia to the strong basic anion-exchange resin, in accordance with Varki's and Diaz' observation [30].

Thus, chromatography of neo-*O*-acetylated Sia directly on TLC in ethanol/ammonium acetate, which is much less sensitive to impurities of the sample has two advantages: First, it shortens the SOAT assay and secondly, it yields the native reaction product showing the position-specificity of the acetyltransferase.

Analysis of gangliosides Another relatively short and easy method of detection of SOAT reaction products is the direct chromatography of neo-*O*-acetylated GD3 from the supernatant of enzymatic assays ("Assay with GD3"). About 12 μg of GD3 from the assay are applied alone to a lane of maximum 4 cm width or, if available, together with standards of 7-*O*-acetyl- and 9-*O*-acetyl-GD3 (GD3 having Neu5,7Ac_2 or Neu5,9Ac_2 as terminal Sia, respectively) and developed on 10×10 cm silica gel in chloroform/methanol/25 mM $CaCl_2$ in water (50:40:10). After drying with cold air and radio-scanning, the gangliosides are visualized by staining the plates with the orcinol/ferric chloride reagent as described for free Sia in "Thin-layer chromatography of neo-*O*-acetylated Sia". Figure 2 shows a typical experiment resulting in the biosynthesis of 7-*O*-acetylated GD3.

Fluorimetric and radiometric high-performance liquid chromatography of neo-O-acetylated Sia Free Sia, radioactively labelled or unlabelled from SOAT assays are fluorimetrically labelled by incubation with DMB reagent (1,2-diamino-4,5-methylene-dioxybenzene◦2 HCl; Dojindo Laboratories, Tokyo, Japan) at 56°C for 90 min in about 70 μl [27, 31]. The incubation conditions are slightly modified when compared with the original procedure of Hara *et al.* [31]. After the addition of 1 μg derivatized BSM Sia standards, maximum 100 μl, the samples are injected into a reversed phase RP 18 column (Lichrospher 100, particle size 5 μm; Merck, Darmstadt, Germany) and eluted with acetonitrile/methanol/water (9:7:84) at a flow rate of

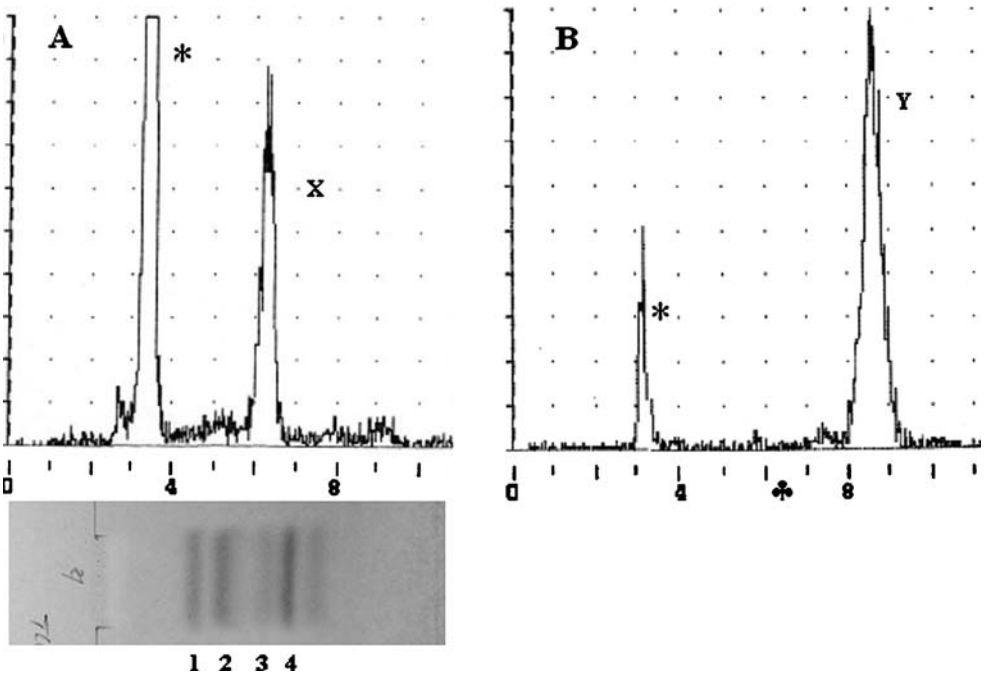

Fig. 1 Radio-thin-layer analysis of neo-*O*-acetylated sialic acids from sialate-*O*-acetyltransferase reaction. CMP-Neu5Ac was used as substrate, shortly hydrolyzed after incubation with enzyme from bovine submandibular gland and co-chromatographed with BSM Sia standard on cellulose (10 cm) in ethanol/ammonium acetate. The TLC plate was radio-scanned and stained by orcinol/ferric chloride reagent. **A**: Neu5,7Ac_2 (*X*) was formed in this reaction; *asterisk* represents residual [^{14}C]AcCoA. *1*, Neu5Gc; *2*, Neu5Ac; *3*, Neu5,7Ac_2; *4*, Neu5,9Ac_2. **B**: Alkali saponification of the neo-synthesized 7-*O*-acetylated Sia (original position is marked with ♣); *Y*, free acetate

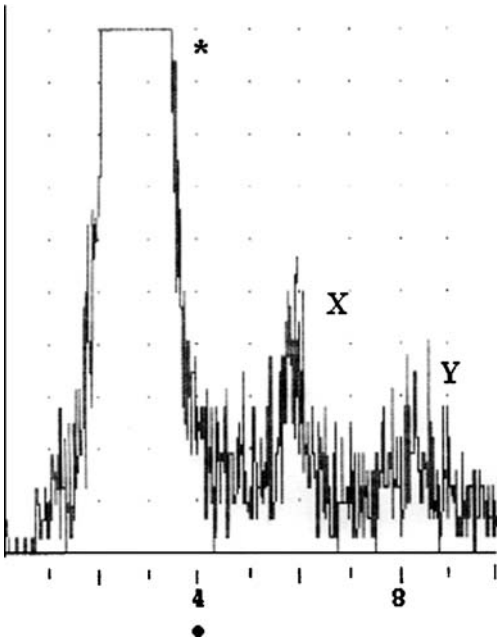

Fig. 2 Identification of *O*-acetylated GD3 neosynthesized by human lymphocyte SOAT by chromatography on silica gel in chloroform/methanol/$CaCl_2$ in water, followed by radio-scanning and Sia staining. *Asterisk*, Residual AcCoA; *X*, 7-*O*-acetylated GD3; *Y*, free acetate; *filled circle*, GD3 migratory position

0.5 ml/min. As internal control, 1,000 dpm of fluorimetrically labeled [^{14}C]Neu5Ac standard can be added to the sample from enzyme assay or chromatographed separately. The fluorescence of the eluate is measured at 362 nm for excitation and 460 nm for emission. The eluate is then monitored for radioactivity by mixing with scintillator fluid (Optiflow plus) which is pumped at a flow rate of 6 ml/min with the aid of a T-piece. Radiometry is carried out in a Radioflow Detector LB (Bertholds Technologies, Bad Wildbad, Germany). Radioactivity eluted at a delay of 1–2 min when compared with the fluorimetric scan of a distinct Sia peak, as tested with standard radioactive Neu5Ac. Figure 3 presents a typical example of these SOAT experiments. It was obtained from an enzyme assay with CMP-Neu5Ac and again shows neo-*O*-acetylated Neu5,7Ac_2. In some experiments, also smaller amounts of Neu5,8Ac_2 were found, but never significant amounts of Neu5,9Ac_2, if contact with ion-exchange resin or other slightly alkaline conditions had been avoided. Mainly Neu5,7Ac_2 was also identified by radio-HPLC after mild acid hydrolysis of neo-*O*-acetylated GD3 (not shown).

To increase the sensitivity of this HPLC analysis it is recommended to use the tenfold (4 μCi) amount of radioactive AcCoA than described for TLC assays and to prefer [^{14}C]-labelled acetyl donor instead of tritium-labelled coenzyme. Minimum 1,000 dpm (counted by a β-counter) per eluting Sia peak can well be identified (Fig. 3).

Further analyses to confirm the identity of neo-O-acetylated Sia The methods comprising treatment of radioactive neo-*O*-acetylated Sia with mild alkali, influenza C virus esterase, and sialate pyruvate lyase are described in detail by Reuter and Schauer [27]. Here a short version with some modifications is given.

Alkaline treatment Mild saponification of free or GD3-bound Sia, radio-labelled in the *O*-acetyl group, is carried out by placing the sample at the start area (2.5 cm from

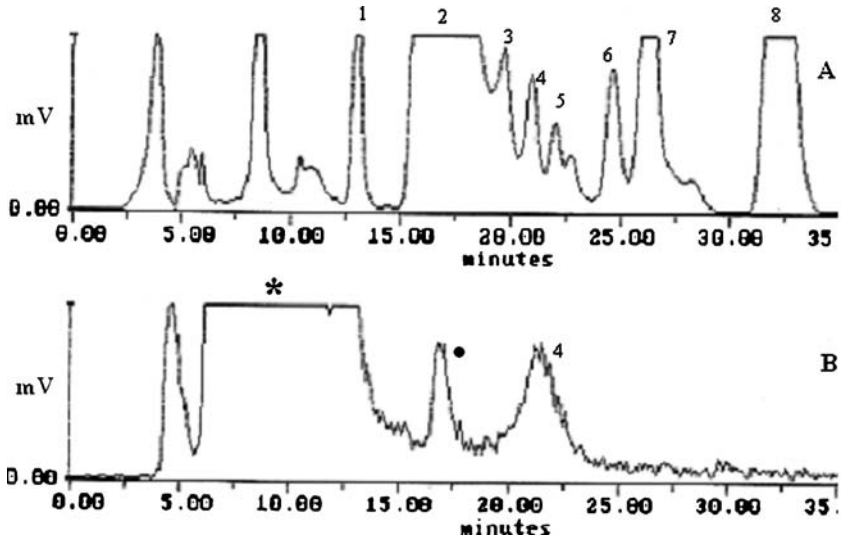

Fig. 3 Fluorimetry-coupled radio-HPLC analysis of neo-*O*-acetylated Sia from sialate-*O*-acetyltransferase reaction. CMP-Neu5Ac was used as substrate of the enzyme from bovine submandibular gland. After short hydrolysis, the reaction product and BSM Sia standard are derivatized with DMB reagent and co-injected into the HPLC instrument. For details see "Methods." **A**: Fluorescence detection of BSM Sia standard. *1*, Neu5Gc; *2*, Neu5Ac; *3*, Neu5Gc7Ac; *4*, Neu5,7Ac_2; *5*, Neu5Gc9Ac; *6*, Neu5,8Ac_2; *7*, Neu5,9Ac_2; *8*, reagent peak. **B**: SOAT reaction product Neu5,7Ac_2 detected by radio-HPLC; *asterisk*, residual [^{14}C]AcCoA; *filled circle*, co-injected [^{14}C]-labelled Neu5Ac (1,000 dpm) standard; *4*, [^{14}C]Neu5,7Ac_2

bottom) of a TLC plate, which is put overnight at room temperature into a chamber with saturated ammonia vapour. After 1 h "drying," BSM Sia standard is added and the plate developed in the respective cellulose or silica gel systems (see above). Using these treatments, radioactive Sia with alkali-labile, radioactive *O*-acetyl groups disappear from the chromatograms. An example is shown in Fig. 1B.

Influenza C virus esterase treatment This technique allows a putative discrimination between an *O*-acetyl group bound either to C-7 or C-9, because only 9-*O*-acetyl groups can be hydrolyzed with esterase from influenza C virus [22], in accordance with most viral, bacterial and animal sialate-*O*-acetylesterases investigated [23]. Samples of neo-*O*-acetylated free Sia or GD3 are dissolved in 20 μl 100 mM Tris/HCl buffer, pH 7.4, and incubated with 1–5 mU virus esterase for 30 min at 37°C. They are then chromatographed in the TLC or HPLC systems as described above and compared with a control sample without esterase incubation.

Sialate lyase treatment In order to further prove the Sia nature of TLC bands or HPLC peaks, either radioactively labelled or unlabelled, degradation of Sia by the lyase and comparison of the result with the analysis of untreated Sia samples revealed to be useful. This test is only possible with free Sia. Decrease or disappearance of a stainable or radioactive TLC band or HPLC peak strongly indicates the presence of a Sia, because this lyase is specific for Sia degradation. However, it should be considered that Sia *O*-acetylated at the side-chain are cleaved at slower rate than non-*O*-acetylated Sia. 4-*O*-Acetylated Sia are resistant towards that enzyme. The lyase reaction is carried out in 50 μl 50 M phosphate buffer, pH 7.2, using 0.6 enzyme units, by incubation for 1 h at 37°C before analysis. An assay without enzyme is run in parallel.

Bacterial sialate-O-acetyltransferases

Evidence for the *O*-acetylation of poly-Sia in bacteria is increasing and the involvement of these modifications in pathogenicity is discussed, as described in "Introduction." Two non-radioactive enzyme assays *O*-acetylating α2,8-bound Sia will be described.

Campylobacter jejuni sialate-O-acetyltransferase assay [10, 32] The activity of affinity-purified recombinant enzyme is determined in 50 mM MES [2-(*N*-morpholino) ethane sulfonic acid] buffer, pH 6.5, containing 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM AcCoA and 0.5 mM Neu5Acα2-8Neu5Acα2-3Galβ1-4Glc-FCHASE [6-(5-fluorescein-carboxamido)-hexanoic acid succimidyl ester] incubated at 37°C for 5 min. After termination of the reaction by the addition of acetonitril, the assay product is analyzed using capillary electrophoresis and TLC on silica gel in ethylacetate/methanol/water/acetic acid (4:2:1:0.1). This *O*-acetylation reaction is also followed by real time NMR spectroscopy showing preferred incorporation of the acetyl group at C-9 of α2-8-linked Sia.

Escherichia coli sialate-O-acetyltransferase assay [33] A different and simple strategy is followed for assaying the activity of a recombinant, affinity-purified SOAT from *E. coli*. The thiol groups of CoA resulting from the enzyme reaction are quantified by Ellman's reagent. The assay is carried out in 10 μl 20 mM Tris–HCl buffer, pH 7.5, containing 25 mM EDTA, 4 mM DTNB [dithio-bis(2-nitrobenzoic acid)], 6.85 mM colominic acid, 1 mM AcCoA, and 24 pmol of enzyme protein at 25°C by continuous monitoring the enzyme reaction at 405 nm using a microplate spectrophotometer reader.

Sialate-*O*-acetylesterase assays

Esterases are abundant in animal cells and in micro-organisms. Some of these animal enzymes seem to be involved in Sia metabolism, since they show high activity with *O*-acetylated Sia (see "Introduction"). There it was mentioned that de-*O*-acetylation facilitates the degradation and thus turnover of Sia and sialylated macromolecules. In bacteria and viruses evidence is accumulating that SOAEs are involved in infection of the host. Especially viral sialate esterases are attracting more attention. SOAEs can also hydrolyze *O*-acetyl esters from synthetic substrates, crude or purified protein fractions can easily be screened for esterase activity by methylumbelliferyl acetate in microtiter scale. However, these tests do not prove the Sia specificity of the esterase under study. In order to achieve this the use of radioactive Neu5,9Ac_2 or HPLC analysis showing the Sia nature and its possible de-*O*-acetylation directly, has to be applied occasionally during the purification process as a control. It can also be applied for the specificity and quantitative activity determination of SOAE occurring in crude samples like tissue homogenates or cell lysates. The specificity regarding the position of *O*-acetyl groups is best identified by HPLC using purified isomers of *O*-acetylated Sia. The procedures are shortly described in the following. For more details see [21–23, 27].

Analysis of SOAE activity on microtiter plates

Methylumbelliferyl acetate at 60 μM concentration is incubated in 100 μl 100 mM Tris/HCl buffer, pH 7.4, with 25–50 μg protein for 30 min at 37°C. Heat-denatured

protein (3 min, 95°C) serves as blank. Fluorescence is read at 365 nm immission and 462 nm emission wavelengths in a microtiter plate reader.

Analysis of SOAE activity by HPLC

Neu5,9Ac$_2$ (1 mM) is incubated in 50 mM Tris–HCl buffer, pH 7.5, with esterase from influenza C virus (about 2,000 hemagglutinin units) or SOAE samples from other origin for 15 min at 37°C, followed by cooling on ice. An assay without viral suspension is used as blank. For HPLC analysis Sia are fluorescence-labelled with DMB either directly or after the removal of particles and disturbing proteins from the test solution (see "Fluorimetric and radiometric high-performance liquid chromatography of neo-*O*-acetylated Sia"). An example for the decrease of Neu5,9Ac$_2$ concentration with concomitant increase of the Neu5Ac peak resulting from esterase activity is shown in Fig. 4. When using crude materials from microorganisms or animal tissues or cells, which often contain only little esterase activity, the incubation time of the enzyme reaction may be extended. Esterase activity can be quantified by following the amount of the *O*-acetylated Sia under study with time, protein amount etc. Influenza C virus esterase may serve as standard for calibration.

Analysis of SOAE using radioactive Neu5,9Ac$_2$

A very sensitive and specific method for the determination of sialate-*O*-acetylesterase activity using Neu5,9Ac$_2$ as substrate, radioactively labeled in the *O*-acetyl moiety was developed by Higa *et al.* [21]. This enzyme is incubated at 37°C for 30–60 min in Tris/HCl buffer of pH 8. The reaction is terminated by the addition of a mixture containing 1 M chloroacetic acid, 0.5 M NaOH and 2 M NaCl, followed by centrifugation at 10,000×*g* for 5 min at room temperature. The supernatant is then mixed with a toluene-based scintillation cocktail and kept at room temperature for 15 min before β-counting. Only the protonated acetate released by the esterase can enter the toluene phase and is counted, in contrast to original radioactive Sia remaining in the aqueous phase.

Discussion

The assays of sialate-*O*-acetyltransferases and sialate-*O*-acetylesterases described demonstrate the present knowledge for determination of the activities of these enzymes. The availability of suitable tests is a prerequisite for investigation especially of the acetyltransferases. Eukaryotic SOATs are Golgi-bound enzymes which resisted purification for a long time. They are part of a protein complex

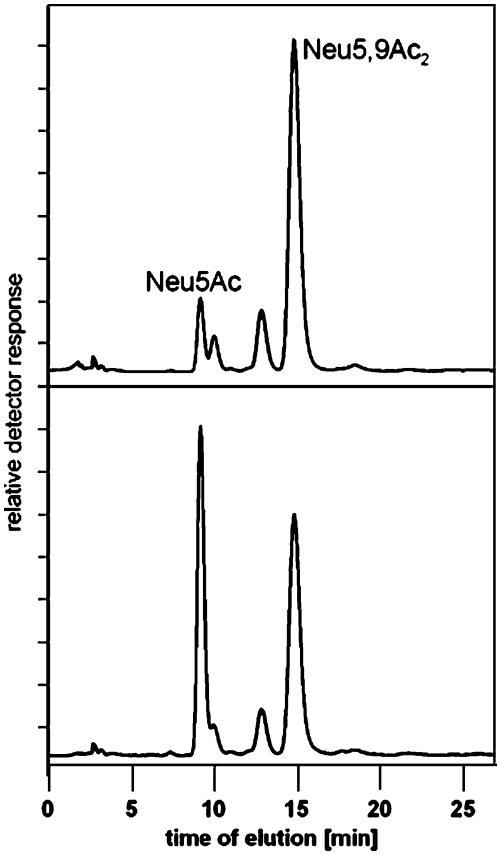

Fig. 4 Assay of influenza C virus esterase by fluorimetric HPLC. The esterase is incubated with Neu5,9Ac$_2$ as substrate at conditions described in "Methods." The *upper panel* represents untreated Neu5,9Ac$_2$ which contains a small amount of Neu5Ac. The *lower panel* shows a decrease of the Neu5,9Ac$_2$ peak which corresponds to an increase of the Neu5Ac peak, due to hydrolysis of the *O*-acetyl groups by the viral esterase

([9, 26], Srinivasan *et al.*, unpublished) which hampered access and yielded only low enzyme quantities. Only now could the SOAT from bovine submandibular gland be solubilized in higher amounts by detergents and purified ([9], Srinivasan *et al.*, unpublished) by using newly developed, faster and more sensitive test systems, which are the basis of this report. The improvements are the direct analysis by TLC and HPLC of the particle-free supernatants from enzyme incubation. This was possible by the finding that CMP-Neu5Ac and GD3 are most suitable substrates and that analytical systems are available allowing the separation and identification of neo-*O*-acetylated Sia, in spite of the presence of salts etc. from the incubation medium. This made ion-exchange chromatography of Sia from the assay prior to chromatographic analysis in most cases superfluous and shortens the analytical procedure and avoids migration of the *O*-acetyl group. Identification of neo-*O*-acetylated Sia was also assisted by treatments with mild alkali, virus esterase and sialate lyase. A further improvement, when compared to the previous attempts on

isolating SOATs [6, 9] is the observation that the yield of enzyme products is higher when protease and esterase inhibitors were omitted.

The methods described for the identification and characterization of SOATs proved their value in various animal systems. It is a disadvantage to use radioactive substances, however, in the eukaryotic systems studied so far endogenous *O*-acetylated Sia were always present and the yields of neo-*O*-acetylated Sia from the assays rather low, which allowed exact and unequivocal activity determinations only with radioactive (co-) substrates. Even during partial purification of SOAT from bovine submandibular gland *O*-acetylated Sia could not completely be removed [9]. Non-radioactive assays will be possible as soon as pure SOAT protein with high activity, to be obtained best by recombinant expression, will be available. Such assays were developed for some bacterial (*C. jejuni* and *E. coli*) SOATs ("Bacterial sialate-O-acetyltransferases"). Lack of endogenous Sia facilitates identification of the exact structure of *O*-acetylated Sia formed in the enzyme reaction under study by mass spectrometry or NMR spectroscopy. The latter method was applied to the *C. jejuni* SOAT reaction showing primary incorporation of the *O*-acetyl group at C-9 of Sia [10, 32]. With the radioactive methods described it was clearly shown that in bovine submandibular gland *O*-acetyl incorporation primarily occurs at C-7 of Sia and not at C-9. The same reaction product was obtained with corresponding enzyme activities from human lymphocytes, cow liver and starfish *Asterias rubens* (unpublished results). Significant 9-*O*-acetylation was not observed if the precautions described above were applied. In rat liver both 7- and 9-OH were found to be neo-*O*-acetylated, although the authors also took possible migration from C-7 to C-9 into consideration [26]. Based on the studies described here the eukaryotic SOAT can therefore more precisely be denominated AcCoA:sialate-7-*O*-acetyltransferase in contrast to the earlier [1–4, 9] name AcCoA:sialate-7(9)-*O*-acetyltransferase. The ester group may migrate from position 7 to the primary alcohol group of Sia side chain, as was studied by various techniques [30, 34]. This is mirrored by the composition of *e.g.* bovine submandibular gland mucin mainly by 7- and 9-*O*-acetyl isomers of Sia [35]. Lower amounts of the 8-*O*-acetyl derivative, probably resulting from the migration process, regularly occur, too. Based on this migration it can be explained that another *O*-acetyl group is incorporated at C-7 by the same SOAT, leading to oligo-*O*-acetylated Sia frequently found in bovine mucin. It is summarized in Fig. 5 that three SOATs were identified on the basis of the position-specificity of Sia *O*-acetylation: 4-*O*-acetyl-, 7-*O*-acetyl- and 9-*O*-acetyltransferases (4-SOAT, 7-SOAT and 9-SOAT).

Regarding side-chain *O*-acetylation evidence was obtained that there may exist various SOATs showing specificity towards the type of complex carbohydrate and linkages of Sia [36]. However, the microsome-bound 4-SOAT from guinea-pig liver, which strictly esterifies only the hydroxyl at C-4, is active with a variety of free and bound Sia at rather variable rates [6]. Especially most gangliosides tested were excellent substrates. This is similar to GD3 which revealed to be a good substrate in the experiments presented ("Assay with GD3" and "Analysis of gangliosides"). The addition of GD3 to Chinese hamster ovary cells was found to result in rapid 9-*O*-acetylation of the terminal Sia residue [37] which seems to be caused by an induction mechanism of Sia *O*-acetylation. A crucial question is furthermore, whether CMP-Neu5Ac, being an excellent substrate for the 7-SOAT from the cells and tissues we have studied, is the physiological SOAT substrate *in vivo*. In that case the rate of *O*-acetylation in the Golgi could be determined by the nature of sialyltransferases. The possible influence of sialyltransferases on *O*-acetylation was discussed by Shi *et al.* [36]. All these questions may be better answered after complete purification or recombinant expression of the enigmatic sialate-*O*-acetyltransferases.

In Fig. 5 the position-specificity of the action of the two sialate-*O*-acetylesterases (4-SOAE and 9-SOAE) best characterized is included. The occurrence and substrate specificity of the large family of eukaryotic, prokaryotic and viral esterases is described with (updated) references [1, 22–24]. Interestingly, with the exception of bovine torovirus esterase, which prefers the di-*O*-acetylated substrate *N*-acetyl-7(8)9-di-*O*-acetylneuraminic acid [24], all Sia esterases known cannot hydrolyze 7-*O*-acetyl groups. This can be used for identification of the acetyl position at the Sia side-chain (see "Influenza C virus esterase treatment"). From the metabolic aspect, a 7-SOAE is not absolutely

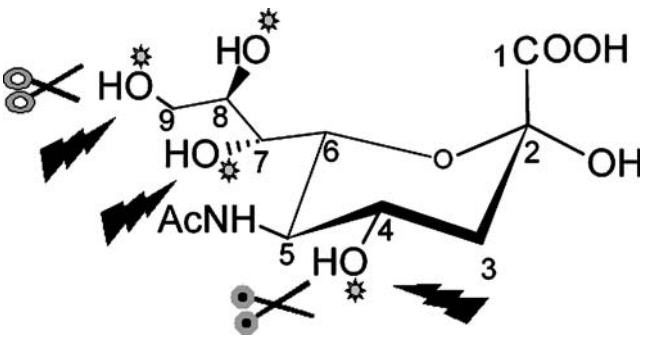

Fig. 5 Metabolism of sialic acid *O*-acetylation. The cartoon represents our knowledge of enzymes involved in the transfer and removal of Sia *O*-acetyl groups. The stars indicate the hydroxyls found to be *O*-acetylated. The flashed arrows symbolize the position-specificity of the SOAT activities discovered: sialate-4-*O*-acetyltransferase; sialate-7-*O*-acetyltransferase and sialate-9-*O*-acetyltransferase. The scissors represent the sialate-9-*O*-acetylesterase and sialate-4-*O*-acetylesterase involved in hydrolysis of the ester groups: The *O*-acetyl group at the Sia glycerol side-chain can migrate between C-7 and C-9, whereas the 4-*O*-acetyl group seems to be immobile. For further details see the text

necessary, due to *O*-acetyl migration from C-7 to C-9, where enzymatic saponification is possible.

In order to judge the extent of *O*-acetylation of glycoconjugates in a cell or tissue the activities of both, SOAT and SOAE, which seem to cooperatively regulate Sia *O*-acetylation, should be known. Since this question has been experimentally addressed only in a few animal tissues or bacteria addressed [38], we hope that the methods described in this manuscript will facilitate further studies. These will also contribute to the development of better methods for the *O*-acetylation of glycoconjugates required for binding studies, *e.g.* of siglecs and pathogens, or for the preparation of substances for clinical use.

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