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Demonstration of 9-*O*-Acetyl-*N*-acetylneuraminic Acid in Brain Gangliosides from Various Vertebrates Including Man

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Summary: Ganglioside fractions were isolated from brains of man, cow, horse, pig, sheep, cat, rabbit, rat, chicken and codfish. The acylneuraminic acid residues, liberated from these gangliosides by treatment with dilute aqueous acid or neuraminidase, were analysed by thin-layer chromatography and combined gas-liquid chromatography/mass spectrometry. Small amounts (up to 20%) of 9-*O*-acetyl-*N*-acetylneuraminic acid, and in bovine and porcine brain gangliosides also traces of *N*-glycoloylneuraminic acid, were found in addition to *N*-acetylneuraminic acid.

Vorkommen von 9-O-Acetyl-N-acetylneuraminsäure in Gangliosiden aus Gehirnen verschiedener Wirbeltiere einschließlich des Menschen

Zusammenfassung: Aus den Gehirnen von Mensch, Rind, Pferd, Schwein, Schaf, Katze, Kaninchen, Ratte, Huhn und Kabeljau wurde die Gangliosidfraktion isoliert. Die daraus durch Hydrolyse mit verdünnter wäßriger Säure oder Neuraminidase freigesetzten Acylneuraminsäuren wurden mit Hilfe von Dünnschichtchromatographie und der Kombination von Gaschromatographie mit Massenspektrometrie analysiert. In allen Proben wurden kleine Mengen (bis zu 20%) an 9-*O*-Acetyl-*N*-acetylneuraminsäure gefunden. Zusätzlich ließen sich in den Gehirn-Gangliosiden von Rind und Schwein neben *N*-Acetylneuraminsäure auch Spuren von *N*-Glycoloylneuraminsäure nachweisen.

Key words: Brain gangliosides, 9-*O*-acetyl-*N*-acetylneuraminic acid, *N*-glycoloylneuraminic acid, gas-liquid chromatography/mass spectrometry

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The occurrence of *O*-acyl groups as substituents of *N*-acylneuraminic acid residues in natural glycoconjugates has been extensively studied (for a review see ref.[1]). These investigations deal predominantly with such residues as components of glycoproteins. Little is known about their presence in glycolipids. *O*-Acetylated *N*-acetylneuraminic acid has only been found in brain

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Enzyme: Neuraminidase, acylneuraminyl hydrolase (EC 3.2.1.18).

gangliosides of some fish species[2-4] and the occurrence of *O*-acetylated *N*-glycoloylneuraminic acid in the hematosides of equine erythrocytes has been reported[5].

The present study has been made to establish the different types of acylneuraminic acids of vertebrate brain gangliosides and is connected with our interest in the action of mammalian neuraminidases towards substrates containing *O*-acetyl-*N*-acetylneuraminic acid*.

Materials and Methods

Brains from cow, horse, pig, sheep, cat, rabbit and rat were obtained immediately after death and human brains 12 to 24 h after death. Brains from codfish stored at 0 °C were obtained about one day after death. Heads of adult chicken were obtained in frozen state from a chicken farm. The materials were stored at − 20 °C until extraction.

Gangliosides were extracted from 50 gram portions of brain grey matter or total brain with chloroform/methanol, repartitioned with water according to Folch et al.[6] and Suzuki[7], dialysed against three changes of double distilled water and freeze dried. The delipidated tissue homogenates containing the brain glycoproteins were immediately dried in vacuo.

Acylneuraminic acids were liberated from 30 mg portions of the ganglioside fractions by hydrolysis in 5 m*l* of aqueous formic acid, pH 2.2, at 70 °C for 1 h or by incubation for 3 h at 37 °C with 2 U of highly purified neuraminidase from *Clostridium perfringens*[8] in 5 m*l* of 0.05M acetate buffer, pH 5.4 containing 0.2% Triton CF-54. Acylneuraminic acids were diffused for 5 h against 20 vol. of water at 3 °C. The diffusates were washed twice with 0.5 vol. of ether. The acylneuraminic acids were purified by passage through a column (2 x 10 cm) of Dowex 50 $H^{\oplus}$, 200 - 400 mesh, then adsorbed on a column (2 x 10 cm) of Dowex 2X8 $HCOO^{\ominus}$, 200 - 400 mesh, eluted with 1M formic acid and lyophilized. Acylneuraminic acids from the crude brain glycoprotein fractions (tissue residues after extraction of the gangliosides) were obtained in a similar way, using 5 g of delipidated tissue in 10 m*l* of formic acid for hydrolysis.

Thin-layer chromatography of gangliosides and acylneuraminic acids was carried out as described earlier [9,10]. The molar ester contents of the acylneuraminic acid mixtures were estimated with the hydroxamic acid method and the quantitative neuraminic acid assay[10,11].

Gas-liquid chromatography and combined gas-liquid chromatography/mass spectrometry of the methyl ester trimethylsilyl ethers of acylneuraminic acids were carried out as described previously[12,13]. Retention times (R_{NeuNAc}) are given relative to that of the methyl ester pertrimethylsilyl ether of *N*-acetylneuraminic acid.

Results and Discussion

Thin-layer chromatograms of the different ganglioside fractions are shown in Fig. 1. The predominance of slow moving tri-, tetra- and pentasialogangliosides in codfish brain is in accordance with the results of Ishizuka and Wiegandt[14]. Direct indications for the presence of *O*-acetylated gangliosides cannot be obtained from this chromatographic separation.

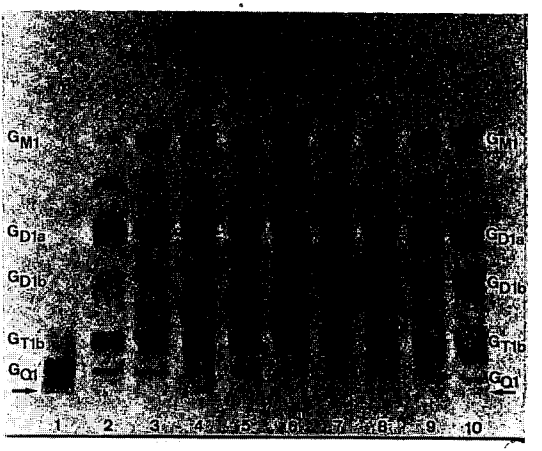

Fig. 1. Thin-layer chromatographic separation of brain ganglioside mixtures from various vertebrates on precoated silica gel plates (0.25 mm), using n-butanol/pyridine/water (9 : 6 : 4, v/v) containing 1 mg KCl/m*l* as solvent.

Arrows indicate origin. Lanes: 1, codfish; 2, chicken; 3, rabbit; 4, rat; 5, horse; 6, pig; 7, sheep; 8, cow; 9, cat; 10, man. Detection with orcinol/$Fe^{3\oplus}$/HCl spray reagent[10]. The nomenclature of gangliosides is that of Svennerholm[15] and according to the IUPAC-IUB recommendations;
G_{M1}, II^3NeuAc-GgOse$_4$Cer
G_{D1a}, IV^3NeuAc,II^3NeuAc-GgOse$_4$Cer
G_{D1b}, II^3(NeuAc)$_2$-GgOse$_4$Cer
G_{T1b}, IV^3NeuAc,II^3(NeuAc)$_2$-GgOse$_4$Cer
G_{Q1}, Tetrasialoganglioside

* Veh, R.W., Sander, M., Haverkamp, J. & Schauer, R., in preparation.

Thin-layer chromatography of acylneuraminic acids liberated from the different ganglioside fractions showed in each case the presence of an orcinol-positive component with an R_F-value corresponding to that of mono-*O*-acetyl-*N*-acetylneuraminic acid[10,11,13]. On two-dimensional thin-layer chromatography with intermediate ammonia treatment[10] this compound migrated as *N*-acetylneuraminic acid in the second direction. The amount of *O*-acetylated *N*-acetylneuraminic acid was about 15 - 20% of the total acylneuraminic acid in the case of codfish and between 4 and 15% in the other cases. No major differences could be detected in the amounts of *O*-acetylated *N*-acetylneuraminic acid relative to *N*-acetylneuraminic acid liberated by acid or enzymic hydrolysis.

The possibility that the *O*-acetylated *N*-acetylneuraminic acid present in the ganglioside fractions originates from contaminating glycoproteins can be excluded, as no orcinol-positive material was detected at the origin on the thin-layer chromatograms (Fig. 1, glycoproteins are immobile in the chromatographic system used). It has to be noted that *O*-acetylated *N*-acetylneuraminic acid was also found in the acylneuraminic acid fraction obtained from purified bovine brain G_{D1a} ganglioside[9].

Analysis by gas-liquid chromatography of the acylneuraminic acid mixtures confirmed the presence of an *O*-acetylated *N*-acetylneuraminic acid. On the chromatogram of each mixture a small peak was present with an R_{NeuNAc}-value of 1.13, corresponding to the R_{NeuNAc}-value of reference 9-*O*-acetyl-*N*-acetylneuraminic acid. (The other natural *O*-acetylated sialic acids have different R_{NeuNAc}-values, as is described in ref.[11].) The assignment of the *O*-acetyl residue at C-9 was proven by gas-liquid chromatography/mass spectrometry. The characteristic fragment ions of the 9-*O*-acetyl-*N*-acetylneuraminic acid are described in ref.[12]. On the basis of this analysis technique, no clear indications were found for the presence of other *O*-acyl-*N*-acylneuraminic acids. As a typical example, the chromatogram of the acylneuraminic acid mixture obtained from cat gangliosides is given in Fig. 2.

With the exception of the codfish, vertebrates were not known to contain *O*-acetylated sialic acids in their brain gangliosides. The *O*-acetylated *N*-acetylneuraminic acid from codfish brain gangliosides, however, was previously considered to be 8-*O*-acetyl-*N*-acetylneuraminic acid on the basis of periodate oxidation experiments[2]. Taking into account the results of model experiments on the periodate oxidation of *O*-acetylated *N*-acetylneuraminic acid derivatives[16], the mass spectrometric data of the present investigation are only consistent with the structure 9-*O*-acetyl-*N*-acetylneuraminic acid.

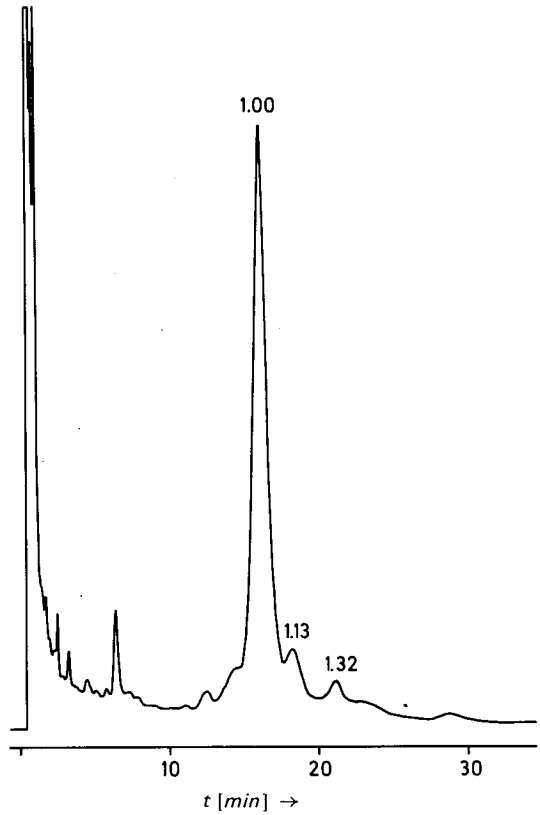

Fig. 2. Gas-liquid chromatography on 3.8% SE-30 of the mixture of acylneuraminic acids obtained from cat brain gangliosides.

Components identified as their methyl ester trimethylsilyl ethers by combined gas-liquid chromatography/mass spectrometry: R_{NeuNAc} 1.00, *N*-acetylneuraminic acid; R_{NeuNAc} 1.13, 9-*O*-acetyl-*N*-acetylneuraminic acid; R_{NeuNAc} 1.32, undersilylation product of *N*-acetylneuraminic acid.

The absence of 4-*O*-acetyl-*N*-acetylneuraminic acid in equine brain gangliosides is remarkable, since this compound has been found in considerable amounts in several equine tissues[10,12,17]. *N*-Glycoloylneuraminic acid (R_{NeuNAc} = 1.80) was detected as a minor component (about 1%) in the acylneuraminic acid mixtures isolated from porcine and bovine gangliosides. The presence of this compound in bovine brain gangliosides has already been described[18], but the knowledge of its occurrence in porcine brain gangliosides is quite new.

O-Acetylation of *N*-acetylneuraminic acid residues in gangliosides increases the possible number of these molecular species considerably, probably leading to a larger diversity in the pattern of cell-surface gangliosides. This phenomenon may be involved in the mechanism of cell recognition. Another aspect of *O*-acetylation of *N*-acetylneuraminic acid residues in gangliosides could be the alteration of the $Ca^{2\oplus}$-binding properties[19] of these molecules. Furthermore, *O*-acetyl groups could influence the turnover of the gangliosides by increasing their resistance towards the action of brain neuraminidase, as was observed for bacterial neuraminidases with glycoproteins as substrates[17]. With respect to the relatively low molar amount of *O*-acetyl groups found in the total ganglioside fractions, the possibility of preferential *O*-acetylation of only specific gangliosides or of gangliosides in special cell types in the brain should be considered.

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