STABILITY OF *N*-ACYL GROUPS OF NEURAMINIC ACID RESIDUES IN 2→8 LINKED POLYMERS TOWARD METHANOLYSIS USED IN METHYLATION ANALYSIS

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1. Introduction

Acid methanolysis has been found preferable to hydrolysis in methylation analysis of oligosaccharide chains of hexosamine-containing glycolipids [1], and of di- and polysialosyl structures which have recently been found to occur in glycolipids [2] and glycoproteins [3–5] as well as in colominic acid [2,6].

Although the stability of *N*-acyl groups of non-methylated sialic acid toward methanolysis has been described [7], no description on the corresponding stability of methylated sialic acid occurs in the literature. While non-methylated *N*-acetylhexosamines undergo de-*N*-acetylation under the conditions of methanolysis used to cleave glycosidic linkages and re-*N*-acetylation is necessary before gas–liquid chromatography (GLC) [8], the applied methanolysis conditions for fully methylated carbohydrate did not accompany de-*N*-acetylation of *N*-acetylglucosamine residues [9].

In the course of methylation analysis of the structure of novel carbohydrate chains recently found in the glycoproteins from rainbow trout [4,5], the internal 8-*O*-substituted *N*-glycolylneuraminic acid residues of methylated saccharides were found to undergo de-*N*-acylation whereas the terminal fully methylated *N*-glycolylneuraminic acid residues do not under the conditions of methanolysis widely employed in similar studies, and suggested participation of the free hydroxyl group at C-8 in de-*N*-acylation [10].

This work shows that this difference in the stability of *N*-acyl groups is generally observed in polysialosyl chains comprising either *N*-glycolylneuraminic acid or *N*-acetylneuraminic acid. The finding is especially important in unequivocal identification of polymers made up of *N*-glycolylneuraminic acid since de-*N*-acylation results in the formation of the derivative of *N*-acetylneuraminic acid.

Abbreviations: Neu*N*Ac, *N*-acetylneuraminic acid; Neu*N*Gl, *N*-glycolylneuraminic acid; Me, methyl; Ac, acetyl; Gl, glycolyl; 1,2,4,7,8,9-*O*Me-Neu*N*(Ac,Me), 4,7,8,9-tetra-*O*-methyl-*N*,*N*-acetyl,methylneuraminic acid methyl ester β-D-methyl glycoside; other partially methylated and acetylated derivatives of neuraminic acids are abbreviated in a similar way

2. Materials and methods

Colominic acid was purchased from Nakarai Chemicals (Kyoto). A 2→8-linked trimer of *N*-acetylneuraminic acid was isolated from a partial acid hydrolysate of colominic acid and kindly given by Mr S. Tsukada (University of Tokyo). Carbohydrate chains containing various length of *N*-glycolylneuraminosyl groups have been obtained from the trout egg glycoprotein as in [5]. Methylation was carried out according to Hakomori and the products were purified as in [10]. The methylated samples were methanolyzed in sealed glass tubes under either of the following conditions: (i) 0.5 M methanolic HCl for 16–18 h at 80°C; (ii) 0.05 M methanolic HCl for 1 h at 80°C. After methanolysis, the acid and methanol were evaporated, and the residue was acetylated by heating with 1:1 (v/v) pyridine–acetic anhydride-d_6 (Merck, min D 99%) for 30 min at 80°C [1].

Gas–liquid chromatography–mass spectrometry (GLC–MS) measurements were performed with a JMS-D300 mass spectrometer/JGC-20KP gas chromatograph (a glass column 2 m × 2 mm packed with 1% OV 101 on Chromosorb WHP, 80–100 mesh,

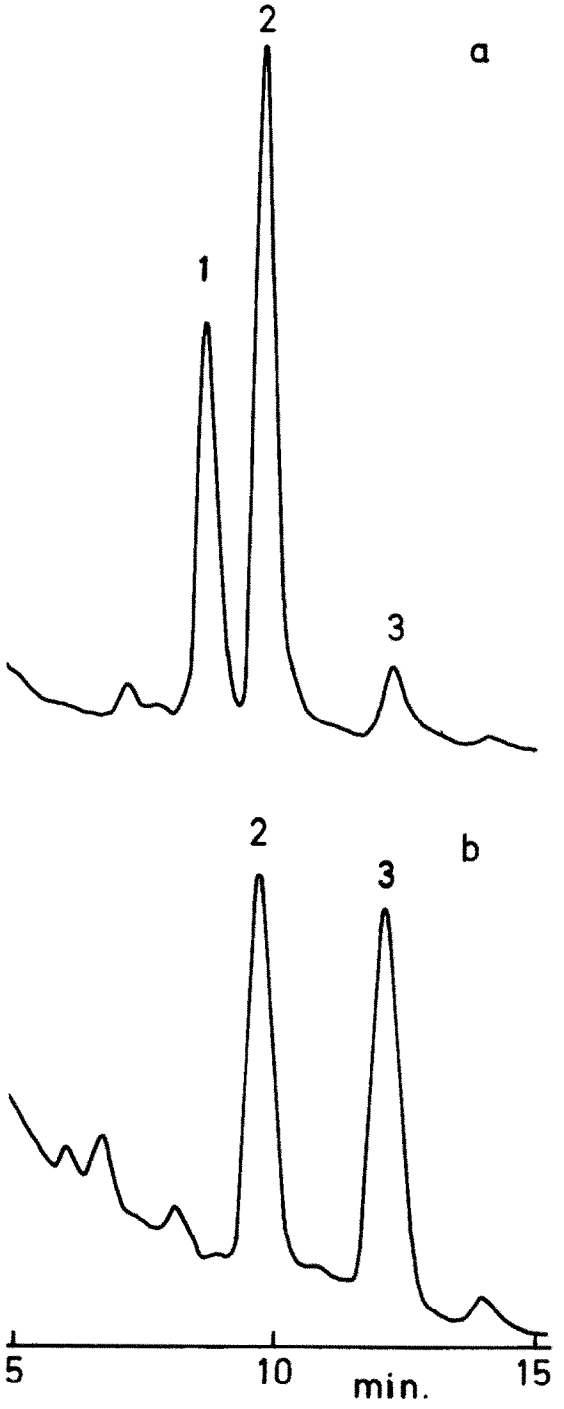

Fig.1. GLC of neuraminic acid derivatives obtained from methylated oligosaccharide containing *N*-glycolylneuraminyl-(2→8)-*N*-glycolyl-neuraminyl group under different conditions of methanolysis: (a) 0.5 M methanolic HCl, 16 h, 80°C; (b) 0.05 M methanolic HCl, 1 h, 80°C. GLC was performed on 2% OV-101 column (2 m × 4 mm) at 230°C; detection: flame ionization.

column-oven at 210°C, electron energy 70 eV, accelerating voltage 3 kV, trap current 300 μA). Ammonia was used in chemical ionization (CI) mass spectrometry: this technique was used to facilitate the assignment of gas–liquid chromatographic peaks.

3. Results and discussion

Difference in the GLC profiles obtained for the methylated oligosaccharide containing *N*-glycolylneuraminyl-(2→8)-*N*-glycolylneuraminyl group methanolyzed under two different conditions is shown in fig.1a,b. Three peaks obtained under condition (i) have been identified as follows: peak 1, 1,2,4,7,9-*O*Me-8-*O*Ac-Neu*N*(Ac,Me); peak 2, 1,2,4,7,8,9-*O*Me-Neu*N*(Gl,Me); and peak 3, 1,2,4,7,9,-*O*Me-8-*O*Ac-Neu*N*(Gl,Me) [10]. Under condition (ii), peak 1 does not appear and peak 3 increases. This observation leads to the following conclusions:

(1) The *N*-glycolyl group on the terminal *N*-glycolylneuraminic acid of the methylated saccharide is stable under either condition of methanolysis;
(2) The *N*-glycolyl group on the internal 8-*O*-substituted *N*-glycolylneuraminic acid is stable toward methanolysis under condition (ii) whereas it is cleaved during methanolysis under condition (i) and re-*N*-acetylated with acetic anhydride.

The methylation product of a trimer of *N*-acetylneuraminic acid was methanolyzed under either of the two conditions and acetylated with acetic anhydride-d_6. The substitution profile at the nitrogen atom in the products was analyzed by mass fragmentometry using the fragment ion G [2] which arises by cleavage of the C-3–C-4 and C-5–C-6 bonds. This ion has either *m/e* 129 (when not de-*N*-acetylated) or *m/e* 132 (when de-*N*-acetylated and re-*N*-deuterioacetylated). Mass fragmentograms of the products obtained by methanolysis under condition (i) are given in fig.2. Fig.2a (*m/e* 129) shows peak 1 corresponding to 1,2,4,7,8,9-*O*Me-Neu*N*(Ac,Me) and peak 2 corresponding to 1,2,4,7,9-*O*Me-8-*O*Ac-Neu*N*(Ac,Me), whereas in fig.2b (*m/e* 132) only peak 2 is predominant. The results indicate that de-*N*-acetylation occurs only in the 8-*O*-substituted neuraminic acid. A small peak behind peak 1 has been identified as 1,2,4,7,9-*O*Me-Neu*N*(Ac,Me) and must stem from incomplete *O*-acetylation. The degree of de-*N*-acetylation under given conditions of methanolysis was estimated from the degree of deuterio-

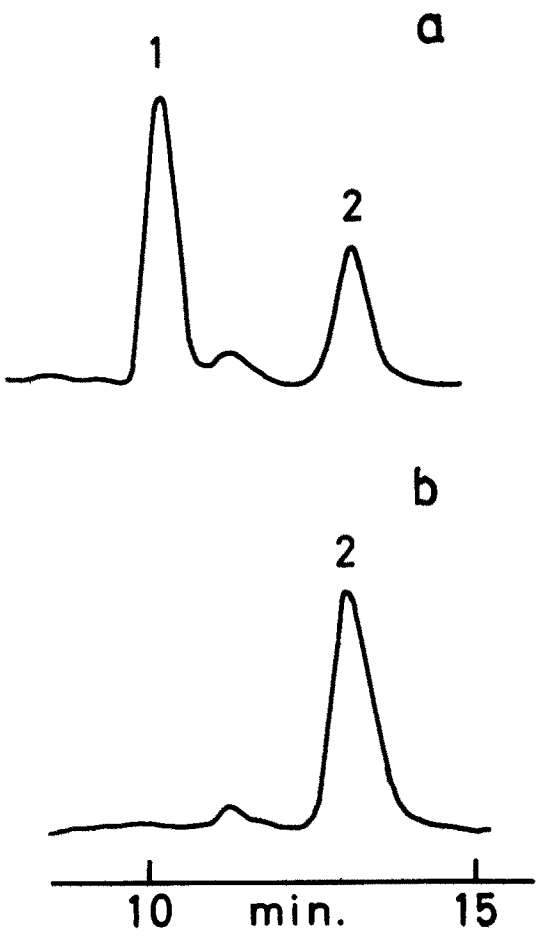

Fig.2. Mass fragmentograms of neuraminic acid derivatives obtained from methylated trimer of *N*-acetylneuraminic acid after methanolysis (0.5 M methanolic HCl, 16 h, 80°C) and acetylation with acetic anhydride-d_6. Detection at: (a) *m/e* 129; (b) *m/e* 132.

Table 1
Degree of de-*N*-acylation in 8-*O*-acetyl derivatives of methylated neuraminic acid

	Conditions of methanolysis	Method of analysis	Degree of de-*N*-acylation in 8-*O*-acetyl derivatives (%)
$(NeuNAc)_3$	(i)	a	50
		b	50
	(ii)	a	0
		b	0
Colominic acid	(i)	a	59
	(ii)	a	0
$(NeuNGl)_2$-R	(i)	c	82
	(ii)	c	0
$(NeuNGl)_4$-R	(i)	c	74
	(ii)	c	0
$(NeuNGl)_6$-R	(i)	c	88
$(NeuNGl)_8$-R	(i)	c	94
$(NeuNGl)_{10}$-R	(i)	c	86
$(NeuNGl)_{12}$-R	(i)	c	84
	(ii)	c	0
$(NeuNGl)_{16}$-R	(i)	c	90

R denotes asialooligosaccharides comprising galactose and *N*-acetylgalactosamine. Method of analysis: (a) proportion of deuterium labelling in the fragment ion B ($M-COOCH_3$); (b) proportion of deuterium labelling in the $M+1$ ion obtained by CI mass spectrometry; (c) the area of peak 1 relative to the sum of peak 1 and peak 3 in gas–liquid chromatograms similar to fig.1

acetylation monitored by the fragment ion B ($M-COOCH_3$, *m/e* 379 or 382). This can be estimated also from the $M+1$ ion (*m/e* 439 or 442) in the CI-mass spectrum. As shown in table 1, methanolysis under condition (i) resulted in 50% de-*N*-acetylation from the 8-*O*-substituted residues of the trimer of *N*-acetylneuraminic acid whereas no de-*N*-acetylation occurred under condition (ii). The degree of de-*N*-acetylation from the 8-*O*-substituted residues of colominic acid (degree of polymerization ~30) was 59% under condition (i).

In the case of polymers of *N*-glycolylneuraminic acid, the degree of de-*N*-acylation was estimated from the areas of peak 1 and peak 3 in fig.1. The values obtained for poly-*N*-glycolylneuraminosyl groups in the saccharide chains isolated from the trout egg glycoprotein are also given in table 1. As shown in table 1, >80% of the 8-*O*-substituted internal residues of *N*-glycolylneuraminic acid from each polymer were found to undergo de-*N*-acylation when methanolyzed under condition (i): i.e., peak 1 in fig.1a is predominant among the products. Only a small portion of the internal residues of *N*-glycolylneuraminic acid resists de-*N*-acylation and appears as peak 3. In this connection we do not agree with the gas–liquid chromatogram presented in [11] as evidence of the occurrence of disialosyl groups in pig submaxillary mucin which contains only *N*-glycolylneuraminic acid by chemical analysis. Analytical methods similar to ours (condition (i)) were used but only two peaks appear in the chromatogram [11]. Moreover they did not assign the nature of the *N*-substituent. In our hands a peak originating from an impurity often appears near the position

of peak 3 in fig.1a and we must be very careful in drawing conclusions from the GLC-profile alone. It is essential to obtain gas–liquid chromatograms under several different conditions to eliminate the interferance from impurities or ideally to obtain mass fragmentograms for the unequivocal assignment of the peaks.

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