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Note

Identification of the *N*-glycolylneuraminyl-(2→8)-*N*-glycolylneuraminyl group in a trout-egg glycoprotein by methylation analysis and gas–liquid chromatography–mass spectrometry

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Recently, methylation analysis has been applied to the elucidation of the type of linkage of neuraminic acids[1–4]. By this method, Finne *et al.*[5,6] could show the occurrence of disialosyl [*N*-acetylneuraminyl-(2 → 8)-*N*-acetylneuraminyl] groups in glycoproteins of mammalian tissues. We have isolated, from the soluble fraction from the eggs of rainbow trout[7], a novel glycoprotein in which *N*-glycolylneuraminic acid accounts for ~50% of the weight, and occurs mostly as disialosyl groups. This paper describes the results of methylation analysis used for the identification and quantitative determination of *N*-glycolylneuraminyl-(2 → 8)-*N*-glycolylneuraminyl groups in the trout-egg glycoprotein.

The g.l.c. pattern of the neuraminic acid derivatives obtained from an oligosaccharide fraction (BH_4-III) of the trout-egg glycoprotein, after methylation by the method of Hakomori[8], followed by methanolysis (0.5M hydrogen chloride, 16 h, 80°), and acetylation is given in Fig. 1a. The retention time, peak area ratio, and identification of each peak are given in Table I. In Fig. 2, the mass spectra of Peaks 1, 2, and 3 are given. Only Peak 2 was observed for a sample of standard *N*-glycolylneuraminic acid similarly treated, and for a trisaccharide obtained from pig submaxillary mucin, in which an *N*-glycolylneuraminosyl group occupies the nonreducing end.

Fragment ions used for the identification are listed in Table II; the fragmentation scheme (Scheme 1) was drawn according to Halbeek *et al.*[4]. The molecular ion (M) and fragment ions (A and B) indicate the mol. wt. of each compound. Fragment F (*m/e* 89) is observed only in Peak 2, indicating[9] *O*-methyl groups at O-8 and O-9, whereas this ion is absent in Peaks 1 and 3, suggesting that O-8 is acetylated. Thus, Peak 2 is fully methylated and arises from the terminal sialosyl groups, whereas Peaks 1 and 3 arise from internal sialic acid residues that are substituted at C-8.

As neuraminidase from *Clostridium perfringens* released all the sialic acid residues from the glycoprotein as *N*-glycolylneuraminic acid, the substituent at O-8 of the penultimate *N*-glycolylneuraminic acid residue was another *N*-glycolylneuraminic acid residue.

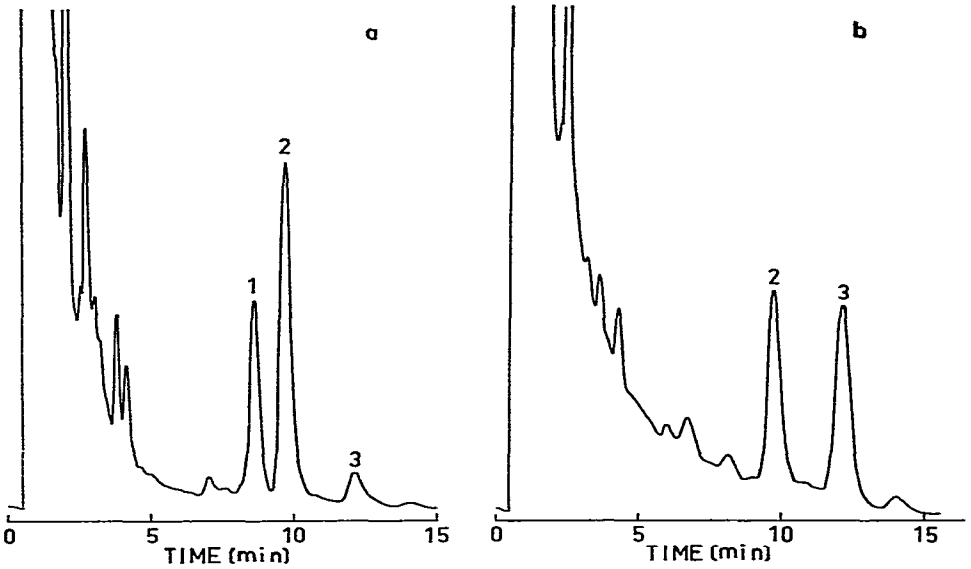


Fig. 1. Gas–liquid chromatography of the neuraminic acid derivatives obtained from an oligosaccharide fraction (BH_4-III) of the trout-egg glycoprotein after methylation, methanolysis, and acetylation: (a) Methanolysis in 0.5M methanolic HCl for 16 h at 80°; (b) methanolysis in 0.05M methanolic HCl for 1 h at 80°. G.l.c. was performed on an OV-101 column at 230°; carrier gas: N_2, 38 ml/min; detection: flame ionization.

TABLE I

RELATIVE RETENTION TIMES, PEAK AREA RATIOS, AND IDENTIFICATION OF G.L.C. PEAKS

	Peak		
	1	*2*	*3*
Relative retention-time[a]	0.89	1.00	1.25
Peak-area ratio[a]	0.55	1.00	0.12
Compound[b] identified by m.s.	8-OAc-4,7,9-OMe-NeuN(Ac,Me)	4,7,8,9-OMe-NeuN(Gl,Me)	8-OAc-4,7,9-OMe-NeuN(Gl,Me)

[a] Values are given relative to Peak 2. [b] *O*-Acetyl-*O*-methyl derivatives of methyl (methyl 5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulopyranosid)onate: NeuN(Ac,Me), *N*-acetyl-*N*-methylneuraminic acid; NeuN(Gl,Me), *N*-glycolyl-*N*-methylneuraminic acid.

Fragment G, which appears as an intense signal in each peak, is useful for differentiating *N*-acetyl (Peak 1) from *N*-glycolyl (Peaks 2 and 3) derivatives. Deuterium-labeling of this fragment shows that the *N*-acetyl group (Peak 1) was introduced by acetylation after methanolysis (*m/e* 129 → 132, Table II). Under standard conditions of methanolysis (0.5M hydrogen chloride, 16–18 h, 80°) similar to those used by others[2–4], the *N*-glycolyl group of the terminal sialosyl group is stable, whereas a large proportion of *N*-glycolyl groups located on internal sialic acid residues are split off. Thus, milder conditions of methanolysis (0.05M hydrogen chloride, 1 h, 80°), under which *N*-deacylation of nonmethylated, lipid-bound sialic acid residues is insignificant[10],

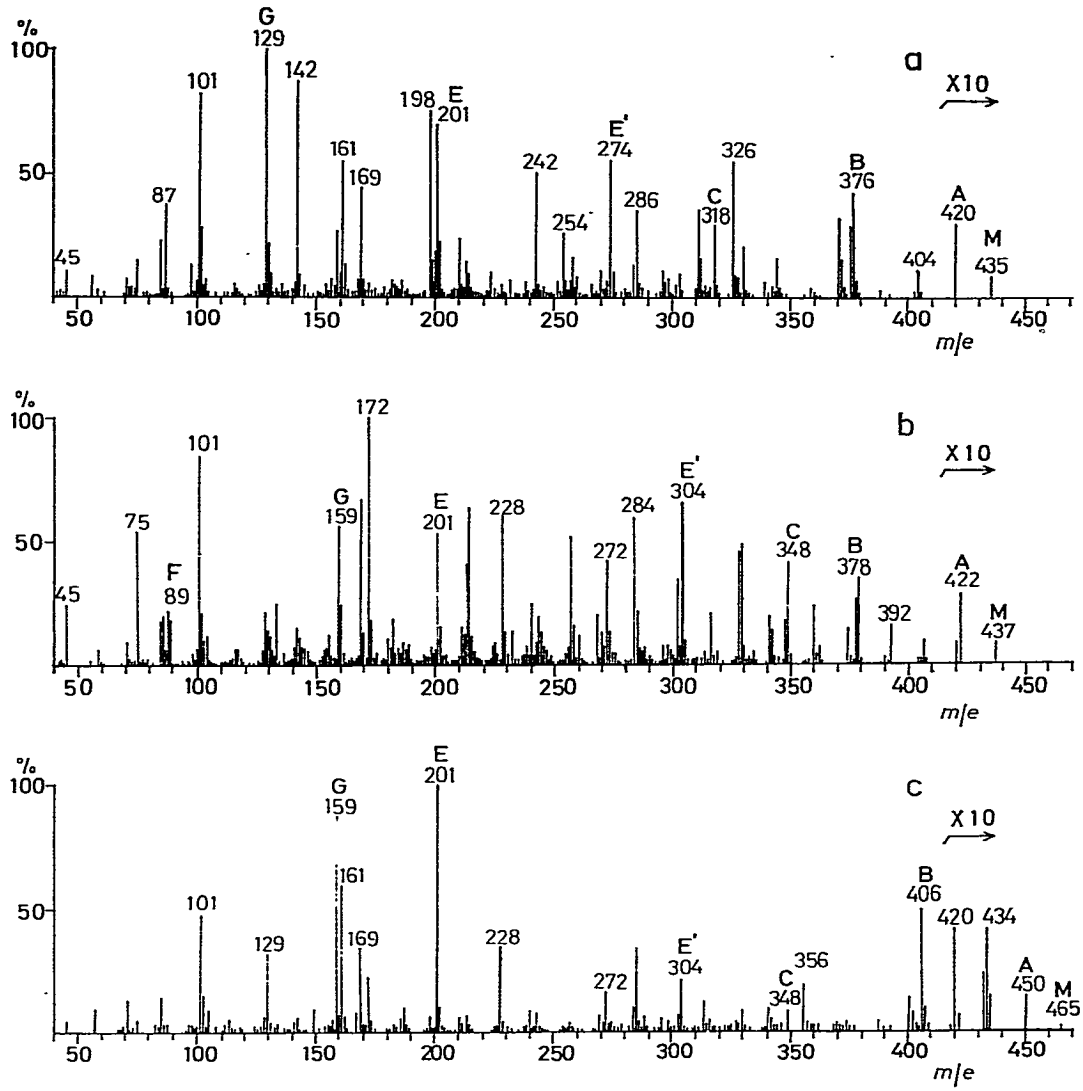


Fig. 2. Mass spectra of the neuraminic acid derivatives separated by g.l.c.: (a) Peak 1; (b) Peak 2; and (c) Peak 3.

were investigated. Fig. 1b shows the gas–liquid chromatogram obtained from the same sample as used for Fig. 1a, but methanolyzed under milder conditions. In contrast to Fig. 1a, Peak 1 has disappeared and Peak 3 increased, indicating that the *N*-glycolyl group was stable under the milder conditions of methanolysis.

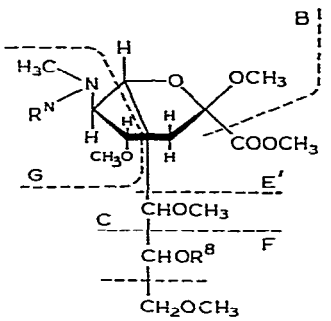
The content of 8-*O*-acetyl derivative(s) was compared to the content of fully methylated derivative under the two conditions of methanolysis, for the intact glycoprotein and for each of the oligosaccharide fractions (see Table III). All oligosaccharide fractions, except BH_4-IV (the smallest chain arising as a minor component), contain sequences in which two *N*-glycolylneuraminic acid residues are linked to each

TABLE II

CHARACTERISTIC FRAGMENT-IONS USED FOR THE IDENTIFICATION OF G.L.C. PEAKS

Fragment	*Peak 1*		*Peak 1(d)*[a]		*Peak 2*		*Peak 3*	
	m/e	%[b]	m/e	%	m/e	%	m/e	%
M	435	0.9	441	<0.5	437	0.9	465	<0.5
M − Me (A)	420	3	426	1	422	3	450	0.9
M − CO_2Me (B)	376	42	382	17	378	35	406	51
M − (CHOR-8)CH_2OMe (C)	318	29	321	8	348	42	348	9
M − CHOMeCHO(R-8)CH_2OMe (E′)	274	55	277	12	304	65	304	20
E′ − $NMeHR^N$ (E)	201	71	201	97	201	52	201	100
$R^NNMe = CHCH = COMeCH = CHOMe$	198	91	201		228	60	228	34
$CH_2OMeCHO(R\text{-}8)CHOMe$	161	55	164	37	133	25	161	60
$R^NNMe = CHCH = CHOMe$ or $R^NNMe = CHCOMe = CH_2$	142	86	145	55	172	100	172	22
$R^NNMeCH = CHOMe$ (G)	129	100	132	100	159	55	159	88
$CH_2OMeCHO(R\text{-}8)$ (F)	[c]		[c]		89	18	[c]	

[a]Peak 1 from the sample acetylated with acetic anhydride-d_6. For this sample the mass spectrum was obtained with a DuPont spectrometer, whereas other samples were analyzed with a Hitachi instrument. [b]Intensities are expressed relative to the base peaks in the region $m/e > 40$. [c]Not observed.



	R^N	R^8
Peak 1	CH_3CO	CH_3CO
Peak 2	CH_3OCH_2CO	CH_3
Peak 3	CH_3OCH_2CO	CH_3CO

Scheme 1

other by a (2 → 8)-linkage. Under the milder conditions of methanolysis, the proportion of 8-*O*-acetyl to fully methylated derivative was close to unity for the intact glycoprotein and for the oligosaccharide fractions, except for BH_4-IV. Under the standard conditions of methanolysis, however, the proportion of 8-*O*-acetyl to fully methylated derivative(s) was ~2:1 for the intact glycoprotein and for the oligosaccharide fraction BH_4-I. These results suggest the presence of large oligosaccharide chains containing oligosialosyl groups.

Methylation analysis has been useful for the determination of the type of linkage of sialic acid residues in meningococcal capsular polysaccharides[1], glycolipids[2,3], and animal glycoproteins[5,6]. A standard technique has been permethylation

TABLE III

YIELDS[a] OF 8-*O*-ACETYL DERIVATIVES RELATIVE TO THE FULLY METHYLATED DERIVATIVE UNDER DIFFERENT CONDITIONS OF METHANOLYSIS

Conditions of methanolysis	*Glyco-protein*	*Oligosaccharides*			
		BH_4-I	*BH_4-II*	*BH_4-III*	*BH_4-IV*
0.5M HCl, 16 h, 80°, *N*-acetyl	1.9	1.8	1.0	0.55	[b]
N-glycolyl	0.30	0.46	0.08	0.12	
0.05M HCl, 1 h, 80°, *N*-glycolyl	1.2	0.75	0.92	1.1	0.0
Yield of oligosaccharide (% of total)		20	40	33	7

[a]Values are given relative to the amount of the fully methylated derivative. [b]Not determined.

of the polymers by the method of Hakomori[8], followed by methanolysis, and acetylation or trimethylsilylation of the free hydroxyl groups. The products may be separated by g.l.c. and identified by characteristic fragment-ions observed in mass spectrometry.

Previous studies have been confined to the linkage between *N*-acetylneuraminic acid residues. The presence of a linkage between two *N*-glycolylneuraminic acid residues has been shown in glycolipids from cat erythrocyte stroma[11], but not by the technique just discussed. We have recently reported the occurrence of disialosyl linkages between *N*-glycolylneuraminic acid residues in a glycoprotein isolated from eggs of the rainbow trout[7]. The present paper describes the application of methylation analysis to the elucidation of the linkage of *N*-glycolylneuraminic acid residues in the trout-egg glycoprotein. The presence of a (2 → 8) linkage between the residues, as suggested by the previous study by periodate oxidation[7], has been confirmed.

An interesting stability of the *N*-glycolyl group of methylated neuraminic acid was observed in the present study. It was found that methanolysis at a concentration of 0.5M hydrogen chloride results in complete *N*-deacylation of nonmethylated *N*-glycolylneuraminic acid, as it was observed for *N*-acetylneuraminic acid[12]. In previous methylation analyses of sialic acid-containing polysaccharides, the methanolyzed samples were sometimes *N*-reacetylated with acetic anhydride, prior to *O*-acetylation or *O*-trimethylsilylation[1,4]. In the present study, the fully methylated *N*-glycolylneuraminic acid residues that are free or located at the nonreducing end of the carbohydrate chains were not *N*-deacylated during methanolysis with 0.5M methanolic hydrogen chloride for 16–18 h at 80°. In contrast, the internal residues of *N*-glycolylneuraminic acid were extensively *N*-deacylated; subsequently they were reacylated during *O*-acetylation to give an 8-*O*-acetyl-4,7,9-tri-*O*-methyl derivative (Peak 1, Fig. 1a), which was identified by acetylation with acetic anhydride-d_6. These results suggest that the free hydroxyl group at C-8 produced by the methanolysis is responsible for the release of the *N*-glycolyl group. A space-filling Corey–Pauling–Koltum (CPK) model shows that the oxygen atom of OH-8 is located near the amide carbon atom and may act as a nucleophile agent in attacking the amide carbonyl group. The C–N

bond fission occurs through a tetrahedral intermediate, an ester linkage formed at C-8 being subsequently methanolyzed.

Under the milder conditions of methanolysis (0.05M hydrogen chloride, 1 h, 80°), which do not cause significant *N*-deacylation of nonmethylated sialic acid[10], the methylated, internal residues of *N*-glycolylneuraminic acid were not *N*-deacylated either. Under the milder conditions, however, quantitative liberation of sialic acid from the polymer was not achieved. For the intact glycoprotein and the high mol.-wt. oligosaccharide BH_4-I, the yields of methylated sialic acid derivatives obtained under the milder conditions of methanolysis were about one fifth of those obtained under the standard conditions of methanolysis. For oligosaccharide BH_4-III, which is a tetrasaccharide consisting of one D-galactose, one 2-acetamido-2-deoxy-D-galactitol, and two *N*-glycolylneuraminic acid residues, the yields of methylated derivatives of sialic acid were not different under either condition of methanolysis. Moreover, the ratio of the 8-*O*-acetyl to the fully methylated derivative(s) was the same (1:1) under both conditions, suggesting that the liberation of methylated sialic acid residues from this oligosaccharide was quantitative. Thus, it may be concluded that one disialosyl [*N*-glycolylneuraminyl-(2 → 8)-*N*-glycolylneuraminyl] group is linked to either a D-galactose or a 2-acetamido-2-deoxy-D-galactose residue in this carbohydrate chain.

The yields of methylated products were also estimated by use of hexa-*O*-acetylinositol as a standard for the determination of the peak areas in g.l.c. Under both conditions of methanolysis, the recovery of sialic acid derivatives from oligosaccharide BH_4-III was 100% on the basis of the molar proportion of *N*-glycolylneuraminic acid. The recovery from the oligosaccharide fraction BH_4-I and from the intact glycoprotein under the standard conditions of methanolysis was 80% and 30%, respectively, and the ratio of the 8-*O*-acetyl to the fully methylated derivative(s) was 2:1 for both samples, thus suggesting the presence of oligomeric chains of *N*-glycolylneuraminic acid residues in the original glycoprotein. The occurrence of polymeric sequence of *N*-glycolylneuraminic acid in Nature has not been reported.

EXPERIMENTAL

Materials. — The ovulated eggs of rainbow trout were obtained in January 1977 at the Okutama Fish Farm. *N*-Acetylneuraminic acid was a product of Seikagaku Kogyo Co. (Tokyo, Japan), and *N*-glycolylneuraminic acid was isolated, by gel filtration after mild acid hydrolysis, from pig submaxillary mucin. Sephadex and DEAE-Sephadex were products of Pharmacia Fine Chemicals (Uppsala, Sweden). All reagents used in the methylation analysis were distilled and stored in all-glass containers.

Preparation of the trout-egg glycoprotein and reduced oligosaccharide fraction. — The fraction representing "phosvitin–lipovitellin complex" was obtained from the eggs of rainbow trout, essentially according to the procedure of Wallace *et al.*[13]. Any "membrane" material was removed by a high-speed (100,000 *g* for 30 min) centrifugation of the extract. The phosvitin–lipovitellin complex was dissolved in 0.15M NaCl

containing 1% of dodecyl sodium sulfate, and mixed with an equal volume of 90% phenol. The mixture was stirred for 3 h at room temperature. The aqueous phase separated by centrifugation contained, in addition to phosvitin, a glycoprotein that has not previously been described. The glycoprotein and phosvitin were deposited on a column of DEAE-Sephadex A-25 and eluted with 0.3–0.4M NaCl at pH 8.0, and further separated from each other by gel filtration on Sephadex G-150. The yield of the glycoprotein was ~500 mg from 500 g of wet eggs.

The oligosaccharides obtained after alkaline borohydride treatment (M $NaBH_4$–0.1M NaOH, 72 h, 37°) of the glycoprotein were fractionated on Sephadex G-25 as described by Spiro and Bhoyroo[14]. Fractions BH_4-I to BH_4-IV were eluted from the Sephadex column in this order. Partial characterization of the trout-egg glycoprotein and the oligosaccharide fractions has been published[7].

Porcine submaxillary mucin was prepared by the method of De Salegui and Plonska[15], and its reduced oligosaccharides were prepared as described for the trout oligosaccharides.

Methylation. — A dried sample of each oligosaccharide fraction (BH_4-II–BH_4-IV, 100–200 μg) was dissolved in dry dimethyl sulfoxide (0.1 ml) and treated with methylsulfinylmethylsodium (~2M, 0.1 ml) for 2 h, and then with methyl iodide (0.1 ml), for 2 h at room temperature with occasional agitation. The reaction mixture was diluted with water (1 ml), and extracted with chloroform (1 ml). The chloroform phase was washed 4 times with water (1 ml each) and evaporated to dryness. Glycoprotein (500 μg) and a high mol.-wt. oligosaccharide (BH_4-I, 300–500 μg) were methylated as just described. The rection mixture was applied to a 1.0 × 60 cm column of Sephadex G-10, which was eluted with 10% methanol. The material eluted in the void volume was pooled and evaporated to dryness.

Methanolysis and acetylation. — The methylated sample was methanolyzed in a sealed glass tube under either of the following conditions: (i) 0.5M methanolic HCl for 16–18 h at 80°; (ii) 0.05M methanolic HCl for 1 h at 80°. After methanolysis, the acid and methanol were evaporated, and the residue was acetylated[16] by heating with 1:1 (v/v) pyridine–acetic anhydride (0.2–0.4 ml) for 30 min at 80°. Deuterioacetylation was performed with acetic anhydride-d_6 (Merck, min. D 99%). The acetylating reagents were removed by evaporation with toluene (0.5 ml). When the sample contained impurities, as judged by g.l.c., it was dissolved in a small amount of chloroform, and the solution was applied to a small column (0.7 × 0.7 cm) of silica gel (Mallinckrodt, 100 mesh). The column was washed with chloroform, and the methylated sugars were eluted with methanol.

G.l.c. and m.s. — The sample was dissolved in chloroform (50 μl) and a portion (2–5 μl) was injected on the top of a glass column (2 m × 4 mm, i.d.) of 2% OV-101 on Gas Chrom Q (Applied Science Co.) operated at 230° or 4% OV-1 on Chromosorb WAWDMCS (Johns Manville Co.) operated at 220°. A better resolution was obtained under the former conditions, whereas a higher detector response was obtained with the latter column. G.l.c.–m.s. was performed (by courtesy of Hitachi Co.) with a Hitachi M-60 mass spectrometer at 20 eV. A part of the g.l.c.–m.s. analyses were performed with a DuPont Dimaspec model 321 GC/MS instrument at 70 eV.

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