Novel Oligosaccharide Chains on Polysialoglycoproteins Isolated from Rainbow Trout Eggs. A Unique Carbohydrate Sequence with a Sialidase-Resistant Sialyl Group, DGalNAcβ1→4(NeuGc2→3)DGalNAc†

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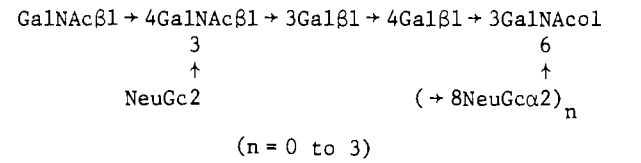
ABSTRACT: A series of novel carbohydrate chains all possessing a hitherto unknown trisaccharide unit, GalNAcβ1→4-(NeuGc2→3)GalNAcβ1→, have been isolated from trout egg polysialoglycoproteins, a new class of glycoproteins, on alkali–borohydride treatment. On the basis of chemical (methylation, Smith degradation, and hydrazinolysis–nitrous deamination) and direct-probe mass spectrometric methods, the structures of a series of the first major type of oligosaccharide alditols having a sialidase-resistant *N*-glycolylneuraminic acid residue in each molecule were determined. The structures thus determined are novel and all possess a unique carbohydrate sequence (sialidase-resistant unsubstituted sialyl group italicized): GalNAcβ1→4(*NeuGc2→3*)GalNAcβ1→3Galβ1→4Galβ1→3[(→8NeuGcα2)$_n$→6]GalNAcol (n = 0 through 3).

The first indication for the existence of a novel class of glycoproteins in trout eggs became apparent in 1978 (Inoue & Iwasaki). Sialic acid is the predominant sugar component of the rainbow trout polysialoglycoproteins accounting for 50–60% of the total weight, and the sialic acid is exclusively *N*-glycolylneuraminic acid (Inoue & Iwasaki, 1978). The occurrence of oligosialyl groups in these glycoproteins is unusual, and the only protein-bound oligo- or polysialyl group containing sugar chains known to occur in other animal tissue are those recently found in glycoproteins of developing rat brain (Finne, 1982).

Polysialoglycoproteins have been found to be ubiquitous components of certain fish eggs, and the subject of structural elucidation of such glycoproteins is an intriguing one even though no definite proof of their biological significance exists at present. Recently, using alkali–borohydride reductive cleavage, we have isolated from the trout egg polysialoglycoproteins a series of oligosaccharide alditols each of which contains one oligosialyl group (Inoue & Iwasaki, 1980; Nomoto et al., 1982). Our preliminary investigation using the conventional biochemical analysis of these fractions suggested that the structures of numerous oligosaccharides from the polysialoglycoproteins may be classified into three classes within each of which these structures are homologous and there exist common asialooligosaccharide core structures. These three classes of sialocarbohydrate chains are named (a) short-core units, (b) long-core units, and (c) fucose-containing units. Consequently, varying size of the oligosialyl groups attached to the core structures results in a large variety of carbohydrate chains present in polysialoglycoproteins.

We have now separated a series of oligosaccharide alditols of the long-core units with mono- to tetrasialyl groups and determined their structures from the data on the basis of (a) methylation analysis, (b) Smith degradation and hydrazinolysis–nitrous deamination, (c) direct-probe mass spectrometry of permethylated oligosaccharide alditols, and (d) CrO_3 oxidation.

The structures thus determined are novel:



The NeuGc[1] residue attached in 2→3 linkage to the penultimate GalNAc residue was found resistant to bacterial sialidases. This is the first demonstration of the occurrence of sialidase-resistant unsubstituted sialyl group in glycoprotein carbohydrate units.

Materials and Methods

Enzymes. Three bacterial sialidases were purchased for use: *Arthrobacter ureafaciens* sialidase (Nakarai Chemicals), *Clostridium perfringens* sialidase (Boehringer-Mannheim-Yamanouchi), and *Streptococcus sp IID6646* sialidase (Seikagaku Kogyo Co.).

Preparation of Polysialoglycoproteins. Isolation procedure for polysialoglycoproteins from trout eggs was described previously by Inoue & Matsumura (1979).

Isolation and Fractionation of Oligosaccharide Alditols from Polysialoglycoproteins. The details for preparation of oligosaccharide alditols by alkali–borohydride treatment of polysialoglycoproteins followed by fractionation on a DEAE-Sephadex A-25 column have been published (Nomoto et al., 1982). Material under each peak denoted as T1b, T3, T6, or T9 in Figure 1 of Nomoto et al. (1982) was loaded on a column (0.8 × ~40 cm) of Iatrobeads (Iatron Chemical Products Co.) and eluted with solvent I (1-propanol–concentrated NH_4OH–H_2O, 6:1:1.5) for T1b and solvent II (1-propanol–concentrated NH_4OH–H_2O, 6:1:2) for T3, T6, and T9. The fractionation was monitored by the phenol–sulfuric acid method (Dubois et al., 1956) or TLC (solvent II, resorcinol stain). Of materials thus obtained, the long-core units were designated as T1bL, T3L, T6L, and T9L, and they were

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[1] Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; SRO, sialidase-resistant oligosaccharide; GalNAcol, *N*-acetylgalactosaminitol; Fuc, fucose; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; Cer, *N*-(fatty acyl)sphingosine; G_{M1}, Galβ1→3GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glcβ1→1Cer; G_{M2}, GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glcβ1→1Cer; G_{D1a}, NeuAcα2→3Galβ1→3GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glcβ1→1Cer; NMR, nuclear magnetic resonance.

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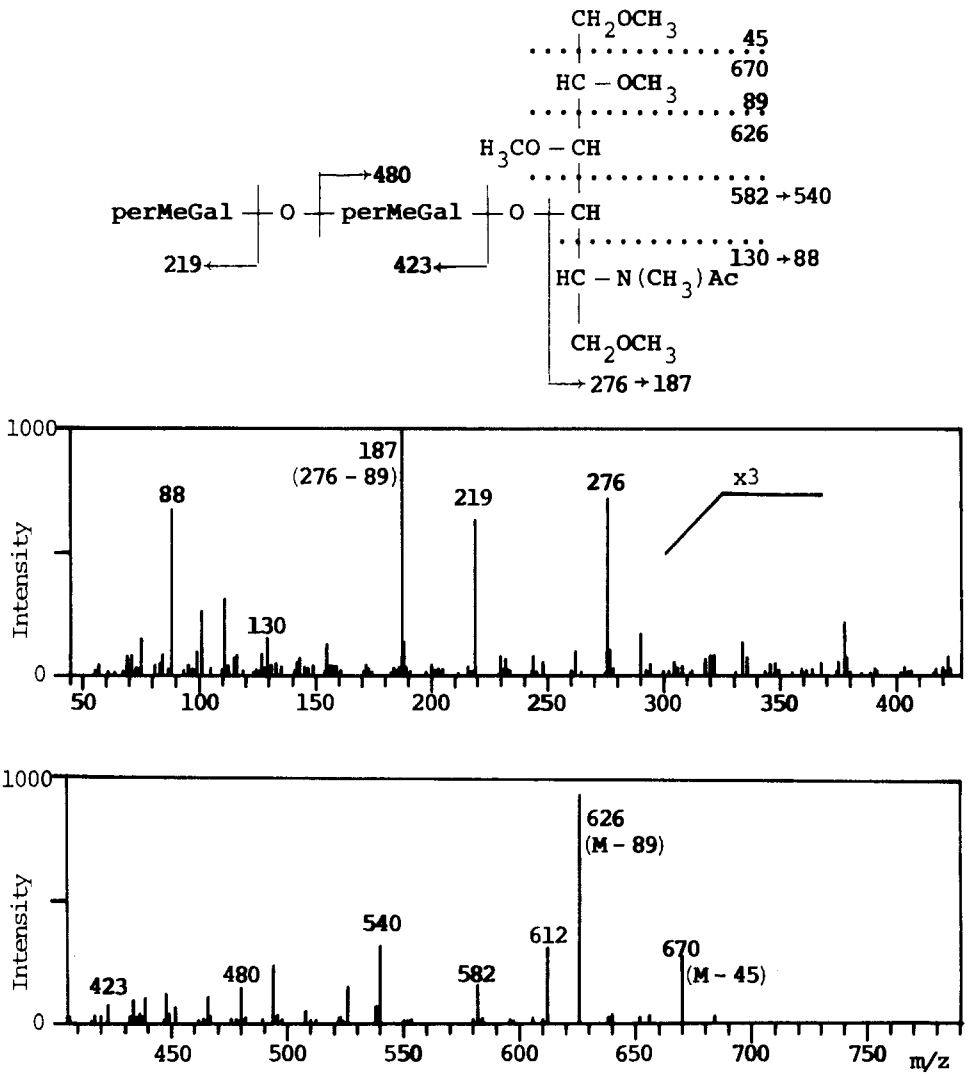


FIGURE 1: Direct-probe mass spectrum of permethylated derivative of a major product of hydrazinolysis–nitrous deamination of asialo-SRO. The structures and masses of the proposed fragments are shown at the top.

found to be pure by sugar composition analysis, by TLC in solvent II, and by ^{1}H NMR spectral measurements [see the following paper in this issue (Kitajima et al., 1984)].

Preparation of SRO and Asialo-SRO. The sialidase-resistant oligosaccharide (SRO = T1bL) was obtained from each oligosaccharide alditol, T3L, T6L, or T9L, by exhaustive digestion with sialidase. The core pentasaccharide moiety of SRO, asialo-SRO, was prepared by mild acid hydrolysis of SRO: SRO (2.76 mg) was partially hydrolyzed in 0.01 N HCl at 80 °C for 1 h. The partial hydrolysate was then applied to a column (1.1 × 20 cm) of DEAE-Sephadex A-25 after neutralization with 0.1 N NaOH and dilution with H_2O (2 mL). The neutral component obtained was subjected to Sephadex G-25 column chromatography (1.8 × 142 cm). The only products observable were asialo-SRO and free NeuGc besides the unreacted SRO. The yield of asialo-SRO was 0.55 mg.

Carbohydrate Analysis. The molar ratios of component sugars were determined by GLC as described previously (Nomoto et al., 1982). Free sialic acid released by hydrolysis was determined by the thiobarbituric acid method (Aminoff, 1961) with a modification (Uchida et al., 1977) using NeuGc as the standard.

Methylation Analysis. Permethylated oligosaccharides and partially methylated alditol acetates were prepared by the method of Stellner et al. (1973). A method of methylation analysis of sialyl groups was described previously (Inoue & Matsumura, 1979; Inoue et al., 1982). The partially methylated alditol acetates were identified by GLC–mass spectrometry (Lindberg, 1972) with a JEOL JMS-300 mass spectrometer–JGC-20KP gas chromatograph in a glass column (2 mm × 1 m) of 1.5% OV-17 on Chromosorb 750 (80–100 mesh).

Smith Degradation, Hydrazinolysis–Nitrous Deamination, and CrO_3 Oxidation. Smith degradation of SRO was conducted according to the procedures described by Spiro (1966). Hydrazinolysis–nitrous deamination was also carried out for asialo-SRO according to Strecker et al. (1981). The products were subjected to TLC on a sheet of Merck Kieselgel 60 (8.5 cm × 20 cm) with ethyl acetate–pyridine–acetic acid–water (5:5:1:3) as developing solvent (2.5 h). The major product (H) was eluted with water and subjected to methylation analysis and direct-probe mass spectrometric analysis. CrO_3 oxidation of asialo-SRO was conducted as described by Laine & Renkonen (1975).

Direct-Probe Mass Spectrometry. The permethylated oligosaccharide alditols were analyzed by direct-probe mass spectrometry with a JEOL JMS-300 spectrometer: electron energy 20 eV; ionization current 0.3 mA; chamber temperature 200 °C; probe temperature 200–400 °C.

Sialidase Treatments. All incubations were at 37 °C. In a typical experiment, an oligosaccharide sample containing 3 μmol of NeuGc was incubated with 0.2 unit of sialidase from *C. perfringens* in 0.1 M sodium acetate buffer (pH 4.7, 0.2

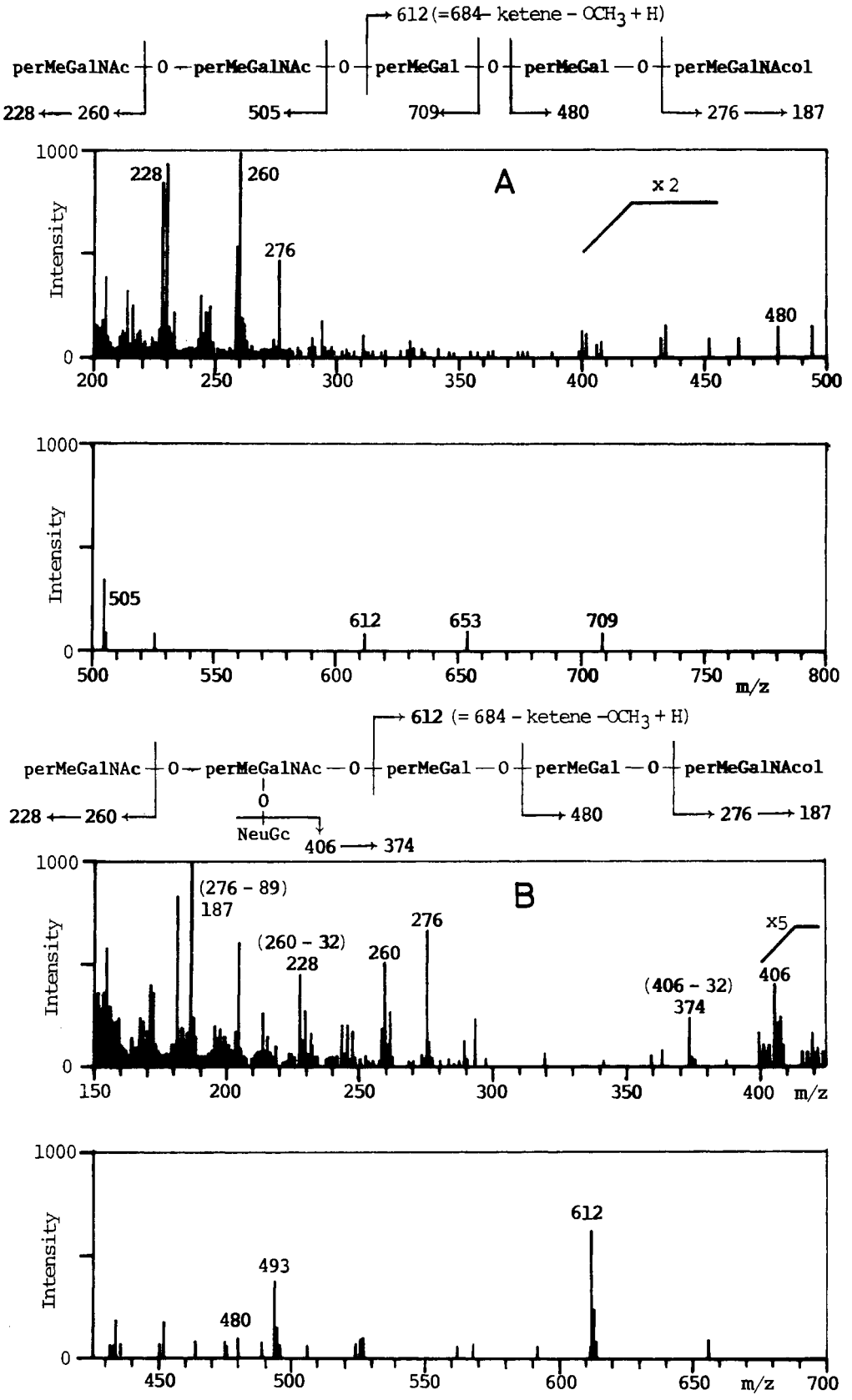


FIGURE 2: Direct-probe mass spectra of permethylated derivatives of (A) asialo-SRO and (B) SRO. The structures and masses of the proposed fragments are shown at the top of each part.

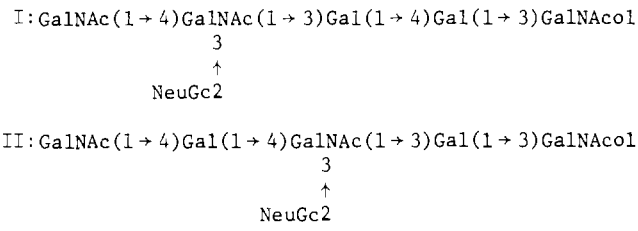
mL). Digestion with sialidase from *A. ureafaciens* was performed as described previously (Uchida et al., 1977).

Results and Discussion

Carbohydrate Composition. The molar ratios of the sugar in the purified long-core oligosaccharide alditols are presented in Table I.

Carbohydrate Sequence and Interglycosidic Linkages of Long-Core Unit with a Single NeuGc Residue (SRO). The analysis of partially methylated alditol acetates derived from SRO revealed the presence of 1 mol each of nonreducing terminal GalNAc, 3,4-di-O-substituted GalNAc, 3- and 4-O-substituted Gal, and 3-O-substituted GalNAcol. On removal of the NeuGc from SRO, 4-O-substituted GalNAc was newly

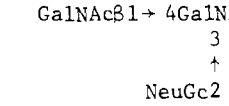
formed at the expense of 3,4-di-O-substituted GalNAc on methylation analysis, indicating that the NeuGc residue is attached to C-3 of the internal GalNAc. Methylation analysis of the product of Smith degradation of SRO revealed the presence of terminal GalNAc and 3-O-substituted Gal (about 1:1) but no terminal Gal nor 4-O-substituted GalNAc. These results suggest the presence of →4(NeuGc2→3)GalNAc1→-3Gal in SRO. Two structures that could accommodate these data for SRO are



Our decision in favor of I was made through the identification of the major oligosaccharide product H of hydrazinolysis–nitrous deamination of asialo-SRO. The measurement of direct-probe mass spectrum of the permethylated oligosaccharide H revealed the structure of H to be GalGalGalNAcol, showing that under the hydrazinolysis condition used in our experiment the GalNAcol residue in asialo-SRO apparently did not undergo deacetylation though the GalNAc residue was deacetylated. The fragment ions containing the structural information are seen at m/z 670, 626, 480, 423, and 219 (Figure 1). Sites of bond cleavages leading to the formation of these ions are also depicted in Figure 1. The linkages were established by methylation analysis of H: since in H the presence of 4-O-substituted Gal and the nonreducing terminal Gal was shown, the precursor cannot be asialo-II.

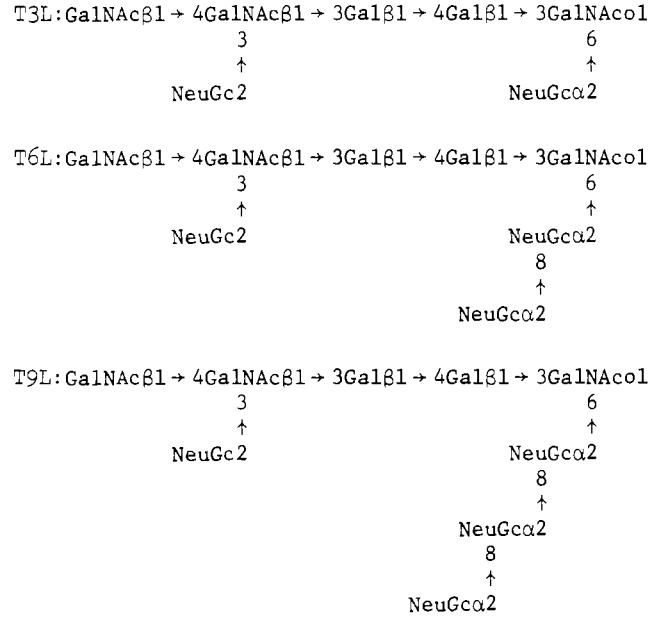
The direct-probe mass spectra of the permethylated SRO and asialo-SRO are shown in Figure 2, which offer compelling support for the assigned structure I for SRO. The spectrum of the permethylated asialo-SRO (Figure 2A) differs from that of the corresponding SRO (Figure 2B) by the presence of a reasonably strong peak at m/z 505 only in the former. We believe that the peak at m/z 505 is formed by the rupture of the glycosidic bond of the penultimate GalNAc residue, producing a diagnostic sequence ion, $(GalNAc\text{-}O\text{-}GalNAc)^+$. The sequence ion, $(GalNAc\text{-}O\text{-}GalNAc\text{-}O\text{-}Gal)^+$, is also observed, giving rise to the peak at m/z 709. When we reported the preliminary results (Inoue et al., 1981) on the structure of SRO, we relied too much on the appearance of the fragment ion at m/z 464 in the direct-probe mass spectra for permethylated SRO, T3L, and T6L (in the original report these were denoted as NGNA-oligosaccharide, fraction II-2a, and fraction II-3, respectively), and thereby, we assumed the presence of the terminal GalNAc→Gal common sequence (structure II) in these oligosaccharides. It is evident from the structures of I and II that methylation analysis of the intact SRO and Smith degradation product of SRO would not distinguish between them. The present results of hydrazinolysis–nitrous deamination studies of asialo-SRO require that the earlier reported sequence of the core pentasaccharide (structure II) be revised such that the correct structure is I. The revised structures of the long-core units now conform to the biogenetic sequence as well as the proton nuclear magnetic resonance spectral data (the latter point will be discussed in the following paper).

All the GalNAc and Gal residues of asialo-SRO were degraded by CrO_3 oxidation, indicating that these residues are β-linked. These data are consistent with the proposed structure for SRO:



Nature of Sialidase-Resistant NeuGc Residue in SRO. The sialyl group of SRO was resistant to three kinds of bacterial sialidases examined. Evidence for the location of this residue as nonreducing terminal was obtained from the formation of a 4,7,8,9-tetra-*O*-methyl derivative of NeuGc on methylation analysis. Hydrolysis of SRO under mild conditions (0.01 M trifluoroacetic acid, 70 °C, 1 h) gave only the products, asialo-SRO and sialic acid having an identical mobility with NeuGc by TLC, suggesting the absence of *O*-acyl groups, which have been shown to preserve under these conditions of hydrolysis (Buscher et al., 1974). The sialyl group of SRO did not become susceptible to sialidases after alkaline treatment (Ghidoni et al., 1980): this result also supports the absence of *O*-acyl groups and/or an intramolecular ester (McGuire & Binkley, 1964). Methanolysis of SRO under milder conditions (Yu & Ledeen, 1970) resulted in the appearance of only the derivatives of unsubstituted NeuGc on GLC. All of these results indicate that the sialidase-resistant sialic acid in SRO is the unsubstituted NeuGc.

Structures of Long-Core Units with Oligosialyl Groups. Removal of the sialyl groups from T3L, T6L, and T9L by treatment with sialidase produced monosialooligosaccharide, shown to be identical with SRO by the migratory behavior on TLC. Attachment of sialidase-sensitive sialyl groups to O-6 of the GalNAcol residue has been shown for compounds of the family of long-core units by methylation analysis: T3L, T6L, and T9L revealed the presence of 3,6-di-O-substituted GalNAcol instead of 3-O-substituted GalNAcol (Inoue et al., 1981). The presence of di- and trisialyl groups in T6L and T9L in the form of NeuGcα2→8NeuGcα2→ and NeuGcα2→8NeuGcα2→8NeuGcα2→ was also shown by methylation analysis, which revealed the molar ratio of 8-O-substituted NeuGc relative to the terminal NeuGc to be about 0.5 and 1.0 for T6L and T9L [cf. Inoue & Matsumura (1979) and Inoue et al. (1982)]. These data offer convincing proof of the structural formulations of T3L, T6L, and T9L:



Conclusions

The present study has revealed hitherto unknown structures in carbohydrate units of trout egg polysialoglycoproteins. In

Table I: Molar Ratios of Sugars in Long-Core Oligosaccharide Units

compound	NeuGc	Gal	GalNAc	GalNAcol
SOR (or T1bL)	1.0 (1)[a]	2.0 (2)	2.0 (2)	1.0[b]
T3L	1.9 (2)	1.7 (2)	1.7 (2)	1.0[b]
T6L	2.8 (3)	1.7 (2)	2.0 (2)	1.0[b]
T9L	4.2 (4)	1.7 (2)	2.0 (2)	1.0[b]
asialo-SRO		2.1 (2)	2.1 (2)	1.0[b]

[a] The values in parentheses are the nearest integral. [b] The data are normalized to one alditol residue.

addition to the demonstration of α2→8-linked oligo-NeuGc (Inoue & Iwasaki, 1980), the present work showed a novel asialooligosaccharide core structure and the occurrence of a sialidase-resistant sialyl group attached in 2→3 linkage to the penultimate GalNAc residue for the first time in glycoconjugate carbohydrate units. The structures of mono- through tetrasialyl long-core units have been determined. In view of the isolation of oligosaccharide fractions containing larger amounts of NeuGc and the demonstration of higher oligosialyl groups in the fractions (Inoue & Iwasaki, 1980; Nomoto et al., 1982; Kitajima et al., 1984), the occurrence of long-core units with higher oligosialyl groups is likely. Thus the long-core units present as the first major carbohydrate chains in polysialoglycoproteins of trout eggs are suggested to have the structure: GalNAcβ1→4(NeuGc2→3)-GalNAcβ1→3Galβ1→4Galβ1→3[(→8NeuGcα2)$_n$→6]GalNAcol.

Two points of special interest about the structure of long-core units need comments. The first is concerned with the presence of the disaccharide unit GalNAcβ1→4GalNAcβ1→. The occurrence of this structural element is novel in both glycolipids and glycoproteins though the sequence GlcNAcβ1→4GlcNAcβ1→ is ubiquitous in glycoproteins (Dawson, 1978). The second point relates to the first demonstration of the sialidase-resistant GalNAc-linked NeuGc residue in glycoproteins although sialidase-resistant sialic acid residues are well-known to occur in glycosphingolipids such as G_{M1}, G_{M2}, G_{D1a}, etc. Just as this paper was being prepared for publication, we became aware of three interesting papers on (a) the capsular polysaccharide antigen of type II group B *Streptococcus* by Jennings et al. (1983), (b) blood group Sd^a-active Tamm–Horsfall urinary glycoprotein by Morgan et al. (1983), and (c) glycophorin A from Cad erythrocyte membrane by Blanchard et al. (1983). They have reported that the terminal sialic acid residues of these antigenic oligo- or polysaccharides are attached to the internal Gal residue in 2→3 linkage and are resistant to both viral and bacterial sialidases. However, in contrast to the above three cases and gangliosides in which Gal is the site of attachment of sialic acid [e.g., Ledeen (1978)] with an exception (Watanabe & Hakomori, 1979), the sialidase-resistant NeuGc is linked to GalNAc in the carbohydrate units of trout egg polysialoglycoproteins.

Recent reports that Fuc-containing units occur as the major protein-bound oligosaccharide groups in salmon egg polysialoglycoproteins (Shimamura et al., 1983, 1984) have suggested their occurrence in trout egg polysialoglycoproteins, too. We have substantiated the presence of Fuc-containing units in the latter glycoproteins though in much less amount than in salmon egg glycoproteins (S. Inoue, M. Iwasaki, K. Kitajima, H. Nomoto, and Y. Inoue, unpublished results). We feel that the structures of long-core units are reasonable on biogenetic grounds and are closely related to those of Fuc-containing units. We assume that a tetrasaccharide unit, GalNAcβ1→3Galβ1→4Galβ1→3GalNAcα1→Ser(or Thr), could be a common precursor to both Fuc-containing units and long-core units.

Acknowledgments

We are grateful to Professor Go Matsumura of Showa University for his interest and helpful suggestions.

Registry No. GalNAcβ1→4(NeuGc2→3)GalNAcβ1→3Galβ1→4Galβ1→3GalNAcol, 87862-16-6; GalNAcβ1→4(NeuGc2→3)-GalNAcβ1→3Galβ1→4Galβ1→3[(→8NeuGcα2)$_1$→6]GalNAcol, 87869-40-7; GalNAcβ1→4(NeuGc2→3)GalNAcβ1→3Galβ1→4Galβ1→3[(→8NeuGcα2)$_2$→6]GalNAcol, 87862-17-7; GalNAcβ1→4(NeuGc2→3)GalNAcβ1→3Galβ1→4Galβ1→3[(→8NeuGcα2)$_3$→6]GalNAcol, 87862-18-8.

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Fish Egg Polysialoglycoproteins: Circular Dichroism and Proton Nuclear Magnetic Resonance Studies of Novel Oligosaccharide Units Containing One Sialidase-Resistant *N*-Glycolylneuraminic Acid Residue in Each Molecule†

Ken Kitajima, Hiroshi Nomoto,‡ Yasuo Inoue,* Mariko Iwasaki, and Sadako Inoue

ABSTRACT: Long-core units having the common sequence GalNAcβ1→4(NeuGc2→3)GalNAcβ1→3Galβ1→4Galβ1→3GalNAc are one of the major constituents of rainbow trout egg polysialoglycoproteins. The existing ambiguity regarding the anomeric configuration of the sialidase-resistant unsubstituted sialyl group present in this novel type of oligosaccharide chains has been resolved by a circular dichroism difference spectral method. The fact that the negative band originating from the carbohydrate n → π* transition for this sialyl group was observed offers conclusive proof of the α-anomeric configuration. Next particularly interesting is the fact that the chemical shifts of the sialidase-resistant sialyl H-3_{eq} and H-3_{ax} protons were respectively found at relatively higher and lower magnetic field than for the corresponding protons of other sialyl groups. A consideration of molecular models shows that the observed anomalies are all in the directions compatible with expectations on the basis of the magnetic anisotropy effect due to the carboxylate group and steric compression effects by van der Waals interactions between groups that are sterically compressed. In addition to the observed resistance to bacterial sialidases of this sialyl group, it did not behave even as a competitive inhibitor of the sialidase, *Arthrobacter ureafaciens*, indicating that inaccessibility of this unique sialyl group toward the enzyme. Finally, the analysis of the proton nuclear magnetic resonances of sialidase-sensitive mono- and oligosialyl groups present in the long-core units was based on comparisons of diagnostically important regions in the spectra of homologous oligosaccharides of *N*-glycolylneuraminic acid.

We have separated and determined the structures of a series of oligosaccharide alditols of the long-core units with mono- to tetrasialyl groups in the preceding paper (Iwasaki et al., 1984). Long-core units are particularly interesting since a sialidase-resistant *unsubstituted* NeuGc[1] residue is attached to each molecule of these units: sialidases from *Arthrobacter ureafaciens*, *Clostridium perfringens*, and *Streptococcus sp IID6646*, enzymes known to cleave α-linked sialyl residues, exhibited *absolutely no* reactivity against this NeuGc residue. There seemed to be a probability of a β-linked NeuGc residue in this class of carbohydrate chains, so that in the previous papers (Inoue et al., 1981; Iwasaki et al., 1984) the anomeric configuration of the sialidase-resistant NeuGc residue was left unspecified.

The most noteworthy observation on a series of long-core units is that the H-3 protons of the sialidase-resistant NeuGc resonate at 1.86 (H-3_{ax}) and 2.56 ppm (H-3_{eq}) downfield of DSS in the high-resolution ^{1}H NMR spectra in D_2O. Although it is known [e.g., Kamerling et al. (1982)] that H-3 chemical shifts can be correlated with the configuration of the ketosidic linkage, the above data alone are not conclusive in identifying the anomeric configuration of the anomalous NeuGc residue. However, chemical evidence on this point has been secured, and we report in this paper the results of a more extensive investigation on the CD and ^{1}H NMR spectra of the long-core units, which provide fundamental information about certain structural features of a new class of oligosaccharides containing sialidase-resistant sialic acid.

Materials and Methods

In the present work, materials and methods followed closely those described in the preceding paper (Iwasaki et al., 1984) unless otherwise stated.

Preparation of SRO and TlbLP. The following description is for a large-scale preparation procedure that can be used to obtain milligram quantities of SRO. A mixture of oligosaccharide alditols (76 mg in NeuGc) released by the alkali–borohydride treatment of polysialoglycoproteins was subjected to exhaustive digestion in 40 mL of 0.05 M acetate buffer, pH 4.7, at 37 °C for 4 days with *Arthrobacter ureafaciens* sialidase by stepwise addition of 0.5–0.25 unit every several hours (total 2.75 units). The release of free NeuGc was monitored by the thiobarbituric acid method

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[1] Abbreviations: CD, circular dichroism; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; SRO, sialidase-resistant oligosaccharide; GalNAcol, *N*-acetyl-D-galactosaminitol; Galol, D-galactitol; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; Cer, *N*-(fatty acyl)sphingosine; G_{M1}, Galβ1→3GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glcβ1→1Cer; G_{M2}, GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glcβ1→1Cer.

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