molecules per cubic centimeter ≃ molecules per milliliter. The initial rate of loss of free receptors is now linearly proportional to $\bar{N}$, and the time course of N is obtained by solving eq A9. The solution is

$$N = \bar{N}(1 - r)/(1 - re^{-\alpha t}) \qquad r \neq 1 \qquad \text{(A10)}$$

where

$$\alpha \equiv (4/3)k_1 s^3 \bar{N} c(1 - r) \qquad \text{(A11)}$$

Shortly after mixing, the rate of ligand binding is

$$\frac{dN}{dt} \simeq -(4/3)k_1 s^3 L_0 \bar{N}(1 - \alpha t)/\left(1 + \frac{r\alpha t}{1 - r}\right)^2 \qquad \text{(A12)}$$

The initial rate of binding ($t \rightarrow 0$) is therefore proportional to $\bar{N}$ (see, also, eq A9). This is in distinct contrast to behavior in the diffusion-limited regime in which the initial rate of binding is independent of $\bar{N}$. Therefore, if the reaction is not diffusion limited, we expect the reaction velocity shortly after mixing to satisfy eq A12, and the limiting velocity as $t \rightarrow 0$ should vary linearly with the initial number of free receptors per cell.

Registry No. Metrizamide, 31112-62-6; Triton X-100, 9002-93-1.

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A Novel Neutral Oligosaccharide Chain Found in Polysialoglycoproteins Isolated from Pacific Salmon Eggs. Structural Studies by Secondary Ion Mass Spectrometry, Proton Nuclear Magnetic Resonance Spectroscopy, and Chemical Methods†

Michio Shimamura, Toshiya Endo (in part), Yasuo Inoue,* and Sadako Inoue

ABSTRACT: A novel carbohydrate chain possessing a hitherto unknown disaccharide unit, α-L-Fuc→D-GalNAc, has been isolated from salmon egg polysialoglycoproteins on alkali–borohydride treatment. Salmon egg polysialoglycoproteins contain O-glycosidically linked neutral pentasaccharide chains in addition to a number of oligosialosyl group containing sugar chains. Composition analysis of the neutral pentasaccharide gave fucose, galactose, 2-acetamido-2-deoxygalactose, and 2-acetamido-2-deoxygalactitol in a molar ratio of 1:2:1:1. The structure was determined to be α-L-Fuc(1→3)-β-D-GalNAc-(1→3)-β-D-Gal(1→4)-β-D-Gal(1→3)-D-GalNAcol by the following three major procedures: First, the sequential order of the constitutional monosaccharides was determined by secondary ion mass spectrometry before and after permethylation. Second, linkages were established by methylation analysis and Smith degradation and hydrazinolysis–nitrous deamination studies. Third, anomeric configuration of the glycosidic linkages involved was deduced from 270-MHz proton nuclear magnetic resonance spectroscopy.

Polysialoglycoproteins, first discovered in rainbow trout eggs by Inoue & Iwasaki (1978), are a novel type of glycoprotein molecules, characterized by the presence of as much as about 50% *N*-glycolylneuraminic acid by weight. Similar acidic glycoproteins are also found in the eggs of Pacific salmon, *Oncorhynchus keta* (Walbaum). They are primarily localized in the cytoplasm of the eggs and can be involved in the interaction with basic proteins although the biological role of the carbohydrate in these polysialoglycoproteins still remains veiled.

Salmon egg polysialoglycoproteins differ from the corresponding trout egg glycoproteins in the existence of neutral oligosaccharide chains conjugated to the protein through

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GalNAc→Ser(Thr) [for trout egg glycoproteins, see Inoue & Iwasaki (1980), Inoue et al. (1981a,b), and Nomoto et al. (1982)]. Here, we describe the isolation of a neutral oligosaccharide from the eggs of salmon and the structural elucidation of this novel sugar chain by chemical (methylation, Smith degradation, and hydrazinolysis–nitrous deamination) and instrumental (SIMS, 270-MHz ^{1}H NMR, and GLC–MS)[1] methods.

A relatively new method, SIMS, has been used to verify further its usefulness in the rapid sequence analysis of both derivatized and underivatized carbohydrate oligomers [for a preliminary application of SIMS to a simpler carbohydrate system, see Reinhold (1979)]. This method involves the bombardment of a solid surface or complex matrices by a beam of primary ions which causes the ejection of neutral particles and both positive and negative ions from the solid or the matrices (Grade & Cooks, 1978; Benninghoven & Sichtermann, 1978; Daves, 1979; Day et al., 1980; Unger et al., 1981). In the present investigation the secondary positive ions from the glycerol matrices are mass analyzed by a mass spectrometer, resulting in SIMS spectra. The structure of the neutral pentasaccharide alditol thus determined is α-L-Fuc(1→3)-β-D-GalNAc(1→3)-β-D-Gal(1→4)-β-D-Gal(1→3)-D-GalNAcol.

Materials and Methods

Preparation of Polysialoglycoproteins. Polysialoglycoproteins were extracted from salmon eggs by a modification of the method described by Wallace et al. (1966) for phosvitin. Fresh eggs (600 g in wet weight) of Pacific salmon, *O. keta* (Walbaum), caught in off the coast of Sanriku to Hokkaido, in the Pacific Ocean, in Oct 1981, were washed with 0.9% NaCl and squashed in a porcelain mortar. The squashed material was extracted with 500 mL of 0.9% NaCl and centrifuged for 30 min at 9000 rpm. To the supernatant solution was added 2 L of distilled water and the solution was left to stand overnight at 4 °C. The supernatant liquid was decanted from the pasty pink pitch which was washed with a little water and then dissolved, with stirring, in 600 mL of 0.9% NaCl containing 1% $NaDodSO_4$. A total of 780 mL of 90% phenol was added to this solution and the mixture was stirred for 3 h at room temperature. The aqueous upper phase was centrifuged for 15 min at 4000 rpm to remove any precipitable material. Another 600 mL of 0.9% NaCl–1% $NaDodSO_4$ solution was added to the phenol phase and the extraction procedure was repeated. The combined supernatant fractions were dialyzed exhaustively for 20 h at room temperature first against tap water and then against deionized water. The aqueous solution in cellophane bags was lyophilized, yielding 5.4 g of crude material containing both polysialoglycoproteins and phosvitin. The crude glycoprotein fraction (5.4 g) was dissolved in 2 L of 0.004 M Tris-HCl (pH 8.0) and applied on a DEAE-Sephadex A-25 column (2.6 × 110 cm). The column was washed with 0.004 M Tris-HCl buffer (pH 8.0) and then eluted with 6.6 L of 0–0.975 M NaCl linear gradient in the same buffer. The results are shown in Figure 1, where the elution pattern was monitored by (a) the resorcinol method (Svennerholm, 1963) and (b) absorbance at 230 nm. This procedure yielded a good separation of polysialoglycoproteins and phosvitins. The material of peak 1 was polysialoglycoproteins and that in peak 2 was identified as phosvitin. The polysialoglycoprotein fraction was desalted by dialysis and lyophilized. The yield was 0.68 g.

[1] Abbreviations: GLC–MS, gas–liquid chromatography–mass spectrometry; NMR, nuclear magnetic resonance; SIMS, secondary ion mass spectrometry.

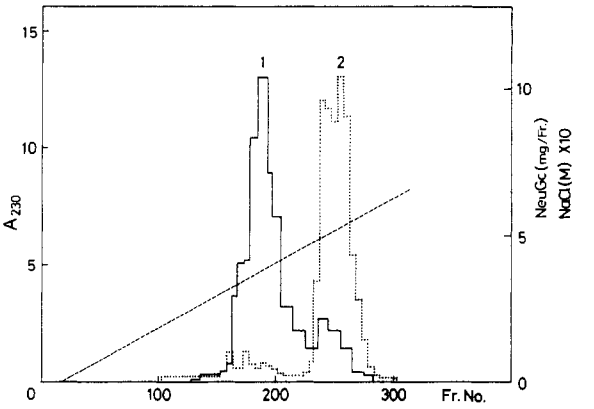

FIGURE 1: Chromatographic separation of a mixture of polysialoglycoproteins and phosvitin on DEAE-Sephadex A-25 (Cl^- form; 2.6 × 110 cm) in the presence of 0.004 M Tris-HCl, pH 8.0. The eluent was a linear gradient of NaCl as shown (broken line), from 0.0 M NaCl (3.25 L) to 0.975 M NaCl (3.25 L); the fraction volume was 17.5 mL; the flow rate was 34 mL/h. The ordinate on the left expresses absorbance at 230 nm (···) and that on the right expresses sialic acid content as estimated by the resorcinol method of Svennerholm (—).

Preparation of the Neutral Oligosaccharide Alditol from Polysialoglycoproteins. The polysialoglycoproteins (0.68 g) was dissolved in 60 mL of 0.1 N NaOH–1 M $NaBH_4$ solution and left at 37 °C for 48 h. The mixture was ice-cooled, acidified with 4 M acetic acid, and desalted by gel filtration through a Sephadex G-10 column. Oligosaccharide alditols released by the alkali–borohydride treatment were applied to a DEAE-Sephadex A-25 column, and the material not retained on the column was collected and subjected to gel filtration on a column (1.6 × 145 cm) of Sephadex G-25. The neutral compounds were eluted in the three well-separated peaks, one major and two minor fractions. The latter two seemed not to originate from O-glycosidically linked oligosaccharides as judged from the composition analysis. Finally, the material in the major peak was chromatographed on a Bio-Gel P-4 column (1.6 × 145 cm). The eluate was lyophilized to give a white powder (yield, 4.4 mg from 600 g of wet eggs). This material (S1) was pure by thin-layer chromatography in solvent systems (a) *n*-PrOH–concentrated NH_4OH–H_2O, 6:1:2, and (b) ethyl acetate–pyridine–AcOH–H_2O, 5:5:1:3.

Composition Analysis. The molar ratios of neutral monosaccharides and hexosamines were determined by GLC after methanolysis (0.57 M methanolic HCl, 65 °C, 16 h) (Reinhold, 1972) and subsequent re-N-acetylation (Kozulić et al., 1979) and trimethylsilylation (Reinhold, 1972). GLC was carried out on a glass column (3 mm × 1 m) containing 1.5% w/w of OV-17 on Chromosorb W operated with a temperature gradient of 4 °C/min from 160 to 240 °C. A Shimadzu GC-4BM gas chromatograph with flame ionization detector was used.

Methylation Analysis. Methylation of the oligosaccharides was performed according to Hakomori (1964), and the products were desalted, hydrolyzed, and reduced (Tai et al., 1975). A mixture of partially methylated alditols was acetylated (Stellner et al., 1973) and then subjected to GLC (occassionally to GLC–MS). For GLC–MS, a column (2 mm × 1 m) of 1.5% OV-17 on Chromosorb 750 was used together with a JEOL JGC-20KP gas chromatograph–JMS-D300 mass spectrometer. GLC was performed at 170–230 °C at 4 °C/min, and the mass spectra were recorded at 70 eV with an ion-source temperature of 200 °C.

Smith Degradation and Hydrazinolysis–Nitrous Deamination. Smith degradation of S1 was carried out by a pro-

cedure similar to that described by Spiro (1966). Hydrazinolysis–nitrous deamination was also carried out according to Strecker et al. (1981).

Secondary Ion Mass Spectrometry. A description of the basic operational principles of the SIMS has been described elsewhere [e.g., see Kambara & Hishida (1981)]. The SIMS method is based on the bombardment of the primary ion (Ar^+) with molecules in dry surface or in glycerol matrix, resulting in the desorption of the secondary ions from the dry surface or glycerol matrix, which enter the mass analyzer with low energy. Positive ion SIMS spectra were obtained with a Hitachi M-80 double-focusing mass spectrometer equipped with a sputtered ion MS system and an M-003 data processor. A 5-keV argon ion beam was used to bombard the sample and an acceleration voltage used was 3 kV. A solution of the sample (about 1 μg/1 μL of H_2O or H_2O–MeOH) was prepared and deposited onto a silver planchette, and then 0.5–1 μL of glycerol was placed over and admixed. If necessary, a drop of acid (0.1 M HCl) was further added in order to get an enhanced SIMS spectrum (Benninghoven & Sichtermann, 1978; Busch et al., 1982).

^1H NMR Spectroscopy. The 270-MHz ^1H NMR spectra were recorded on a Bruker WH270 spectrometer, operating in the Fourier-transform mode with a probe temperature of 23 or 60 °C. S1 was dissolved in 99.8% D_2O and lyophilized. This procedure was repeated 3 times to get a high degree of proton exchange. Chemical shifts are given in ppm relative to that of sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) and indirectly to acetone (δ 2.225 at 23 °C) in D_2O.

Digestion with α-L-Fucosidase. The oligosaccharide alditol was treated with *Charonia lampas* α-L-fucosidase (0.1 M sodium acetate, pH 3.8; 37 °C; 24 h). The quantitative release of L-fucose was confirmed by applying the reaction mixture to a thin-layer Kieselgel 60 plate, which was developed in ethyl acetate–pyridine–acetic acid–water (5:5:1:3). Thin-layer chromatograms were monitored by staining with a 5% concentrated H_2SO_4–methanol solution, 160 °C, 5 min.

Results and Discussion

Salmon egg polysialoglycoproteins, like trout egg glycoproteins (Inoue & Iwasaki, 1980), contain about 80% carbohydrate by weight. However, salmon egg glycoproteins contain considerably more fucose and galactose than trout egg glycoproteins, differences which would be consistent with a lack of any neutral fucose-containing oligosaccharide chains in the latter polysialoglycoproteins. The neutral oligosaccharide alditol, S1, obtained was highly homogeneous in thin-layer chromatography in different solvent systems and eluted as a single peak on Bio-Gel P-4.

Carbohydrate Composition. The molar ratios of the constituent sugars in the purified S1 are D-Gal:L-Fuc:D-GalNAc:D-GalNAcol = 2.0:1.1:1.1:1.0.

SIMS Spectra. The SIMS spectra, measured by depositing the intact pentasaccharide alditol (S1) in glycerol matrices onto silver, are shown in Figure 2. This underivatized sample desorbed to indicate the molecular weight and considerable structure detail: The spectrum (Figure 2A) appears to consist of $(M + H)^+$ and $(Fuc\text{-}O\text{-}GalNAc)^+$ ions at m/z 897 and 350, respectively, in addition to a series of peaks at m/z 277, 369, 461, and 553 which are ascribed to the protonated glycerol aggregates of different multiplicity, viz., $(3G + H)^+$, $(4G + H)^+$, $(5G + H)^+$, and $(6G + H)^+$, where G denotes glycerol. In Figure 2B the enhanced SIMS spectrum of the intact S1 in the m/z 800–1000 range is shown. Spectral enhancement was accomplished by addition of a drop of 0.1 M HCl. Here, the mass spectrum contains typically the peaks at m/z 919,

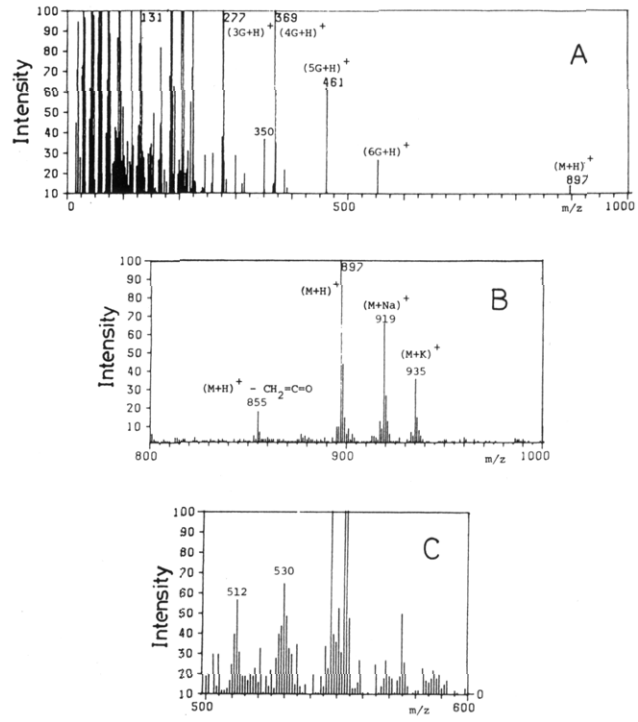

FIGURE 2: (A) Positive ion SIMS spectrum of the intact oligosaccharide, S1; (B) portion of enhanced spectrum which was obtained by the addition of acid to the sample surface; (C) enhanced spectrum (m/z 500–600) showing analytically useful fragment ions.

$(M + Na)^+$, and m/z 935, $(M + K)^+$, in addition to the usual peak, $(M + H)^+$. These stable ions corresponding to metal cation attachment can be formed in desorption ionization mass spectrometry by addition of a minute amount of alkali salt to the sample matrix, and these species are often observed in the SIMS spectra (Kambara & Hishida, 1981; Kushi & Handa, 1982; McNeal et al., 1982; Busch et al., 1982). The weak peak at m/z 855 is due to loss of ketene from the parent molecule, $(M + H - CH_2{=}C{=}O)^+$. The enhanced SIMS spectrum of S1 in the region of m/z 500–600 is reproduced in Figure 2C, which displays peaks at m/z 512 and 530 corresponding to Fuc-O-GalNAc-O-Gal and Gal-O-Gal-O-GalNAcol, respectively. These fragment ions are formed by the rupture of the labile glycosidic linkage producing diagnostic sequence ions. Consequently, the sequence of S1 appears to be most likely as follows: Fuc-O-GalNAc-O-Gal-O-Gal-O-GalNAcol

As a second example we shall give the SIMS spectrum of the permethylated derivative of S1 in the region above m/z 200 in Figure 3. Sites of bond cleavages leading to the production of the sequence-determining ions are also depicted in Figure 3. Mass ions of analytical importance were observed at m/z 276, 435, and 638 which, respectively, correspond to the masses of the mono-, di-, and trisaccharide sequence ions. The molecular ions $(M + H)^+$ and $(M + Na)^+$ are observed at m/z 1135 and 1157, respectively. Loss of ketene or methanol from $(M + H)^+$ is also detected as evidenced by the peaks of low intensity at m/z 1093 and 1103, respectively. It should be noted that a prominent peak at m/z 435 corresponds to $(perMeFuc\text{-}O\text{-}perMeGalNAc + H)^+$, which is presumably generated from the cleavage at the glycosidic bond between perMeGalNAc and perMeGal residues accompanied by the concomitant addition of hydrogen to give the singly charged positive ion. The SIMS spectrum shown in Figure 3 was obtained after addition of a drop of 0.1 M HCl on the sample deposited on the silver in order to suppress a series of cluster ions due to desorption of glycerol matrix and to enhance the

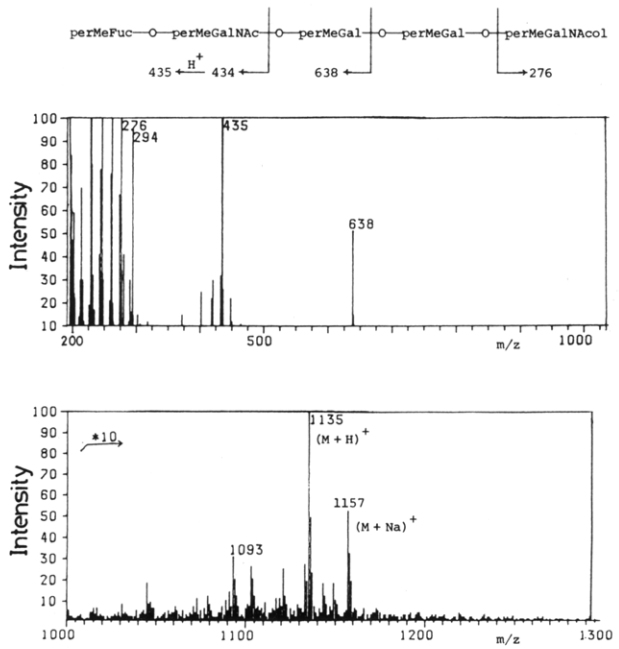

FIGURE 3: SIMS spectrum of the permethylated derivative of S1 above m/z 200. The structure and masses of the proposed fragments are shown in the inset.

Table I: Permethylation of the Neutral Pentasaccharide, S1, from Salmon Egg Polysialoglycoproteins

alditol acetate derived from	molar ratios	substituted in position
2,3,4-Me_3-Fuc	1.1	1
2,3,6-Me_3-Gal	0.7	1, 4
2,4,6-Me_3-Gal	0.9	1, 3
4,6-Me_2-GalNAc	1.1	1, 3
1,4,5,6-Me_4-GalNAcol	1.0	1, 3

intensities of the molecular ion and the diagnostic sequence ions. In SIMS spectral enhancement or simplification can often be accomplished for many other compounds by acid treatment.

Permethylation Studies. Figure 4 shows a GLC pattern obtained for the mixture of partially methylated alditol acetates derived from S1. The identities of these peaks were confirmed by GLC–MS and are given in Table I. The values in Table I have been corrected for the response factor of the different monosaccharides by using the standard oligosaccharides. The results of methylation analysis are compatible with the sequence deduced from the analysis of SIMS and demonstrate that S1 contains a nonreducing terminal Fuc residue and the rest of the other constituent monosaccharides are monosubstituted. This finding in combination with the SIMS spectra of both the intact sample and permethylated derivative reveals the following structural features: Fuc(1→3)GalNAc(1→3 or 4)Gal(1→4 or 3)Gal(1→3)GalNAcol.

Smith Degradation and Hydrazinolysis–Nitrous Deamination. So that one of the two possible linkage isomers shown above could be eliminated, S1 was subjected to Smith degradation and hydrazinolysis–nitrous deamination reactions. After Smith degradation, a disaccharide was found, and it was identified as GalNAc(1 → 3)Gal. This indicates that the penultimate GalNAc residue is 1→3 linked to a Gal residue. Hydrazinolysis–nitrous deamination followed by methylation analysis resulted in the formation of equimolar proportions of 2,3,4-tri-*O*-methylfucose, 2,3,4,6-tetra-*O*-methylgalactose, and 2,3,6-tri-*O*-methylgalactose, which were identified by GLC–MS as their alditol acetates. These findings are compatible with the structure Fuc(1→3)GalNAc(1→3)Gal(1→4)Gal(1→3)GalNAcol.

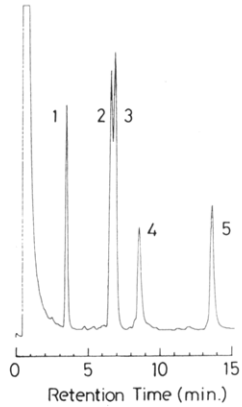

FIGURE 4: Gas–liquid chromatogram of partially methylated alditol acetates obtained by hydrolysis of permethylated S1, followed by $NaBH_4$ reduction and acetylation. The designations of the peaks 1–5 refer to the following compounds: peak 1, 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-L-fucitol; peak 2, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol; peak 3, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galactitol; peak 4, 3-*O*-acetyl-1,4,5,6-tetra-*O*-methyl-*N*-acetyl-*N*-methyl-D-galactosaminitol; peak 5, 1,3,5-tri-*O*-acetyl-4,6-di-*O*-methyl-*N*-acetyl-*N*-methyl-D-galactosaminitol. Glass column (0.3 × 200 cm). Stationary phase: Gas-Chrom Q, with 2% OV-101; gas carrier, N_2; temperature, 175–260 °C (4 °C/min).

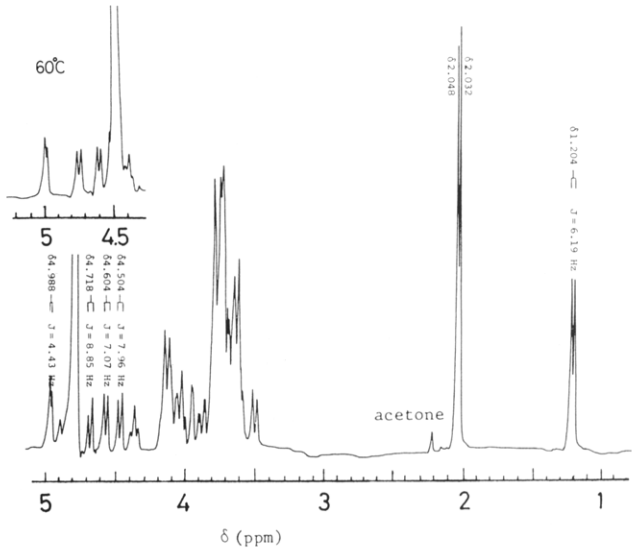

FIGURE 5: 270-MHz ^1H NMR spectrum of the intact oligosaccharide alditol, S1, in D_2O at 23 °C. Anomeric region of the spectrum at 60 °C is also shown in order to make sure that no proton resonance is partly or totally hidden under the HDO line of the 23 °C spectrum.

270-MHz ^1H NMR Spectra. The ^1H NMR spectra of the intact S1, measured in D_2O at 23 and 60 °C, are reproduced in Figure 5. The chemical shifts and coupling constants of the selected protons are included in Figure 5. The signals of all four anomeric protons are well resolved individually between 4.5 and 5.0 ppm. The signals can be assigned to specific residues on the basis of information accumulated by previous workers on ^1H NMR of carbohydrates [e.g., see Montreuil & Vliegenthart (1979) and Vliegenthart et al. (1981)], and the configurations of the glycosidic linkages involved can be determined unequivocally. A signal at δ 4.988 with $J_{1,2}$ = 4.43 Hz is assignable to the α-fucose residue. The coupling constants $J_{1,2}$ = ~8 Hz observed for other three sugar monomers in S1 correspond to an axial–axial coupling between H-1 and H-2, i.e., H-1 is in an axial position. It therefore follows that the monomer units, one D-GalNAc and two D-Gal residues,

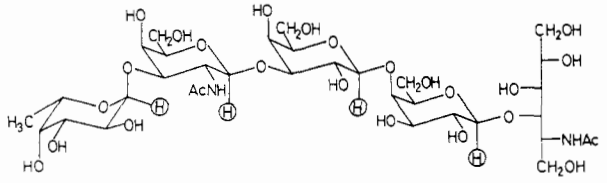

FIGURE 6: The complete structure of the neutral pentasaccharide alditol, S1, obtained from salmon egg polysialoglycoproteins.

are joined by β-glycosidic linkages. The proximal sugar, GalNAcol, shows the H-2 signal at δ 4.391. At 2.048 and 2.032 ppm the two *N*-acetyl methyl signals are found in a 1:1 ratio, the former represents GalNAc and the latter GalNAcol. The doublet at δ 1.204 with J = 6.19 Hz stems from the methyl group of the fucose residue.

From the combined data presented above the complete structure of S1 was established as shown in Figure 6.

Conclusive Remarks. In contrast to conventional electron bombardment mass spectrometry, a recently developed field desorption mass spectrometry allows study of underivatized polar molecules of considerably high molecular weight and is therefore most attractive for carbohydrate chemistry and biochemistry. Although the present SIMS measurements are preliminary and must be supported by further additional examples, SIMS method has proved to be a useful tool for the rapid structural elucidation of unknown oligosaccharide moieties of glycoconjugates [for glycolipid studies, see Hanfland et al. (1981) and Kushi & Handa (1982)].

The most noteworthy feature of the present results is that the neutral pentasaccharide chain present in polysialoglycoproteins from salmon eggs possesses the terminal sequence of α-L-Fuc(1→3)-β-D-GalNAc.... The presence of this structural element was suggested for the first time in the sialooligosaccharides isolated from rainbow trout eggs (Inoue et al., 1981b). L-Fucose is known to occur commonly in a terminal, nonreducing position, and the known typical positions of linkage of fucose in glycosphingolipids and protein-bound oligosaccharide prosthetic groups are L-Fuc[(1→2 or 6) to Gal] and L-Fuc[(1→3, 4, or 6) to GlcNAc] (Dawson, 1978). Hitherto unknown fucosyltransferase [GDP-L-fucose:2-acetamido-2-deoxy-β-D-galactoside (α-L-Fuc→D-GalNAc) 3-α-L-fucosyltransferase] should be responsible for the incorporation of L-Fuc into the novel linkage found in this study.

Acknowledgments

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Registry No. α-L-Fuc(1→3)-β-D-GalNAc(1→3)-β-D-Gal(1→4)-β-D-Gal(1→3)-D-GalNAcol, 84012-04-4.

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