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Structures of the Carbohydrate Units of Polysialoglycoproteins Isolated from the Eggs of Four Species of Salmonid Fishes

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Fish egg polysialoglycoprotein (PSGP) is a novel type of 200 kDa-glycoprotein containing more than 50 % sialic acid by weight and about 90 *O*-glycosidically-linked sialoglycan units per molecule. Of about 100 different molecular species assumed to be present in a sialoglycan mixture obtained by alkaline borohydride treatment of *Salvelinus leucomaenis pluvius* PSGP, 23 mono- to tetrasialylglycans were isolated by anion-exchange chromatography and preparative column chromatography on porous silica, and their structures were determined. Core asialo-oligosaccharides were obtained from PSGP of four species of salmonid fishes by exhaustive enzymatic desialylation of sialoglycan mixtures, and the structures of purified compounds were determined. Two complete types of asialopentasaccharide core structures, Fucα1-3GalNAcβ1-3Galβ1-4Galβ1-3GalNAcOL, and GalNAcβ1-4GalNAcβ1-3Galβ1-4Galβ1-3GalNAcOL, and all of the possible biosynthetic precursors of these pentasaccharide cores were found in every PSGP examined. All types of oligosaccharide chains, both complete and incomplete, were found to occur in highly sialylated forms in PSGP.

Fish egg polysialoglycoprotein (PSGP) is a novel type of glycoprotein first isolated by us from the eggs of rainbow trout [1]. The outstanding structural feature of PSGP is the high sialic acid content (50% by weight) most of which occurs in poly(oligo)sialyl groups linked to *O*-glycosidic carbohydrate units [2]. We have also shown that PSGP is a ubiquitous component of salmonid fish eggs [3]. Although its biological function is still unknown, our preliminary experiments showed that it is closely associated with the cortical vesicles of the eggs and undergoes fertilization-induced specific depolymerization (Inoue *et al.* unpublished results). PSGP from the unfertilized eggs of any salmonid fish has a molecular weight of 150 000 - 250 000 and its protein core consists of seven different amino

Abbreviations. NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; GalNAcOL, *N*-acetylgalactosaminitol; PSGP, polysialoglycoprotein; SRO, sialidase-resistant oligosaccharide.

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Table 1. Data on fish eggs and PSGP.

	Salvelinus leucomaenis pluvius[a]			*Salmo gairdneri*	*Oncorhynchus masou ishikawai*	*Oncorhynchus nerka adonis*
	group 1	group 2	group 3			
Age of fish (yr)	3—4	3—4	3—4	3—4	2	3
Number of fish	1	3	3	8	20	6
Weight of eggs (g)	375	555	559	2 300	1 500	500
Yield of PSGP (mg/100 g egg)	65	75	70	70	65	40
Composition of PSGP (% w/w)[b]						
Total amino acid	20.9	17.4	17.3	13.8	12.4	12.0
Total carbohydrate	79.2	82.6	82.7	86.2	87.6	88.0
Fuc	0.57	0.90	0.70	0.90	1.8	2.0
Gal	13.4	14.7	13.0	11.9	10.5	11.5
GalNAc	12.8	14.0	13.6	11.8	11.7	9.6
GlcNAc	0	0	0	0	1.4	0
Total sialic acid[c]	52.4	53.0	55.3	61.6	62.1	64.8
NeuAc/NeuGc	20/80	7/93	15/85	0	0	0

[a] The yield and composition of PSGP were compared between groups of fish for this species.
[b] Expressed relative to the sum of the weight of each analyzed component that accounted for 100 ± 10% of the weight of lyophilized material.
[c] Determined by the resorcinol method and calculated as NeuGc.

acids; Asp, Thr, Ser, Glu, Pro, Gly, and Ala. Three types of homologous oligosialyl carbohydrate structures, Galβ1-3[(-8NeuGcα2)$_n$-6]GalNAcOL, Fucα1-3GalNAcβ1-3Galβ1-4-Galβ1-3[(-8NeuGcα2)$_n$-6]GalNAcOL, and GalNAcβ1-4(NeuGcα2-3)GalNAcβ1-3Galβ1-4-Galβ1-3[(-8NeuGcα2)$_n$-6]GalNAcOL, have been found in PSGP from both rainbow trout and chum salmon eggs [4-8].

Difficulties encountered in our study were in the separation of so many (abot 100) oligosialylglycans differing in asialo-core structures and the number of sialic acid residues. Isolation of asialo-oligosaccharides by enzymatic desialylation was also difficult because of the slow rate of hydrolysis of *N*-glycolylneuraminyl linkages: prolonged incubation with a large amount of enzyme is necessary for the removal of polysialyl groups.

We have now established a procedure for the satisfactory separation of oligosialylglycans having different asialo-core structures, and also a procedure for the enzymatic desialylation of poly(oligo)sialylglycans and methods of fractionation of asialo-oligosaccharides. This paper describes the isolation and sturctures of 23 sialo-oligosaccharides from PSGP of *Salvelinus leucomaenis pluvius* (Japanese common char), and also the structures of core asialo-oligosaccharides and a sialidase-resistant oligosaccharide obtained from this and three other species of salmonid fishes, *Salmo gairdneri* (rainbow trout), *Oncorhynchus masou ishikawai* (yamame) and *Oncorhynchus nerka adonis* (kokanee).

Materials and Methods

Fish Eggs

Unfertilized ovulated eggs were supplied by courtesy of the Gunma Prefectural Fisheries Experimental Stations at Kawaba (*Salvelinus leucomaenis pluvius*) and Hakoshima (*Oncorhynchus masou ishikawai*); the National Institute of Aquaculture, Nikko Branch (*Oncorhynchus nerka adonis*); and the Okutama Fish Farm, Department of Fishery, Tokyo Metropolitan Government (*Salmo gairdneri*). Eggs were washed with 0.8% NaCl and stored at -30°C until use.

Isolation of PSGP

PSGP was isolated from the soluble fraction of fish egg extracts as described previously [9]. DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden) chromatography resulted in partial separation of PSGP and phosvitin. Final purification of PSGP was made by gel chromatography on Sephacryl S-200 (Pharmacia). Data relevant to fish eggs and PSGP used in the present study are given in Table 1.

Isolation and Fractionation of Sialo-oligosaccharide Alditols

Sialo-oligosaccharides were released from PSGP by alkaline borohydride treatment [4]. The fractionation and purification of sialo-oligosaccharide alditols were made using the following chromatographic methods: (i) DEAE-Sephadex A-25. Column size, 1.5 × 50 cm. Elution, NaCl gradient in 0.01 M Tris-HCl (pH 8.0). (ii) Bio-Rad AG 1-X2 (Bio-Rad, Rich-

mond, CA, USA). Column size, 1.5 × 55 cm. Elution, NaCl gradient. (iii) Iatrobeads. Column size, 1.4 × 20 cm. Elution, *n*-propanol/conc. NH_4OH/H_2O, 6/1/1.5 or 6/1/2 by vol. Iatrobeads 6RS-8060 were purchased from Iatron Chemical Products Co., Tokyo, Japan.

Desialylation of Oligosaccharides

A mixture of sialo-oligosaccharide alditols (containing 80 mg of NeuGc) was digested with *Arthrobacter ureafaciens* sialidase (Nakarai Chemicals, Kyoto, Japan) in 0.1 M sodium acetate buffer (pH 5.5) for 72 h at 37°C under toluene. The enzyme (total 2.5 U) was added at 24 h intervals. After the reaction, asialo-oligosaccharides were separated from the sialo-oligosaccharides by passing through a DEAE-Sephadex A-25 column. The sialo-oligosaccharides were desalted and subjected to a second round of sialidase digestion. Sialo-oligosaccharides still remaining were subjected to a third digestion, after which about 76% of the sialic acid residues was released from the original sialo-oligosaccharide mixture.

Fractionation of Asialo-oligosaccharides

Two column chromatographic systems were used: (i) Bio-Rad Bio-Gel P-4 (-400 mesh) or P-2 (-400 mesh); column size, 1.5 × 120 cm; elution, 0.5 M pyridine-acetic acid (pH 5.0): (ii) Iatrobeads; column size, 1.4 × 20 cm; elution, *n*-propanol/conc. NH_4OH/H_2O, 6/1/2 by vol.

Thin Layer Chromatography

Purity of oligosaccharides was monitored by TLC on silica gel 60 plates (Merck, Darmstadt, W. Germany) developed with *n*-propanol/conc. NH_4OH/H_2O, 6/1/2 or 6/1/2.5 by vol. This system was also used for identification of sialic acid released by hydrolysis. Sialo-oligosaccharides were visualized with the resorcinol or orcinol spray [10] and asialo-oligosaccharides with the orcinol spray.

Carbohydrate Analysis

The resorcinol [11], thiobarbituric acid [12] and phenol-sulfuric acid [13] methods were used for monitoring sialo- and asialo-oligosaccharides in column chromatography. Carbohydrate composition was determined by GLC of trimethylsilyl (TMS) derivatives after methanolysis with 0.5 M HCl in methanol for 16 h at 65°C [4]. Quantitative determination of sialic acid was also made by the thiobarbituric acid and resorcinol methods. Sialic acids were identified by TLC after hydrolysis or by GLC after methanolysis [14].

Methylation Analysis

For methylation of oligosaccharides we used a simple and time-saving method [15] with a slight modification. To a dried sample (50-100 μg carbohydrate) were added dimethylsulfoxide (0.1 ml) and NaH (approx. 5 mg). The mixture was agitated with nitrogen and left for 15 min at room temperature. Methyl iodide (0.1 ml) was added and the mixture

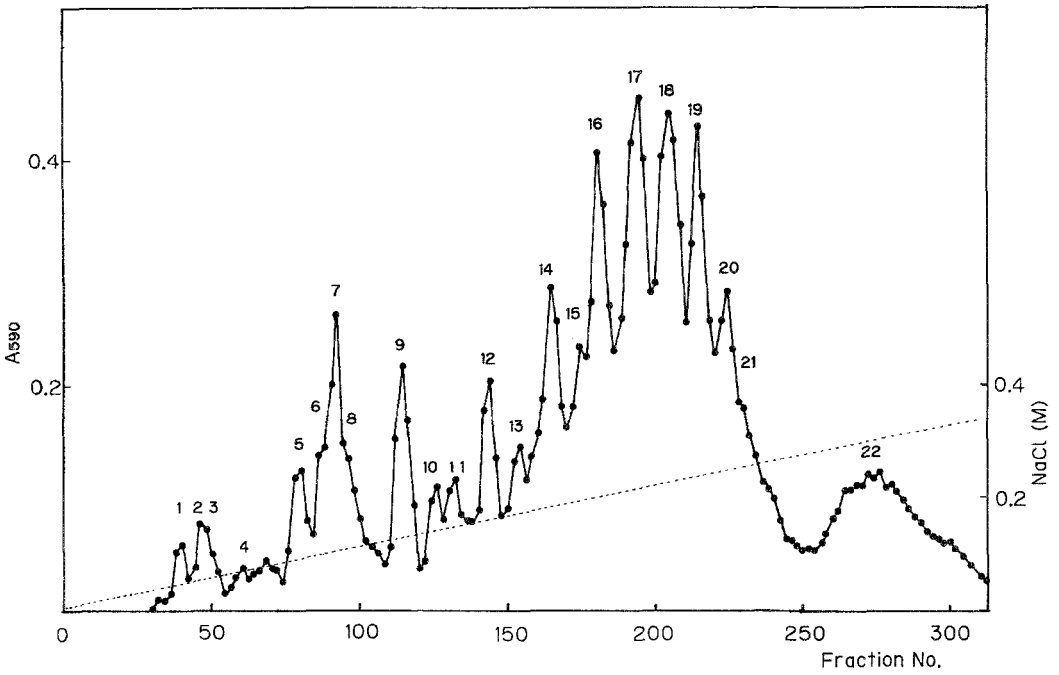


Figure 1. DEAE-Sephadex A-25 chromatography of sialo-oligosaccharide alditols isolated by alkaline borohydride treatment of *Salvelinus leucomaenis pluvius* PSGP. Fractions (3 ml) were assayed for sialic acid by the resorcinol method.

was allowed to stand for 15 min at room temperature. Other procedures were as previously described [4]. Dimethylsulfoxide and methyl iodide were products of Wako Pure Chemical Industries (Osaka, Japan) and were used after distillation. NaH was a product of Metal Hydride Inc. (U.S.A.).

Mass Spectrometry

GLC-MS and direct probe MS analyses were performed with a JEOL JMC-300 mass spectrometer equipped with a JGC-20KP gas chromatograph as described previously [4, 7]. A glass column (2 mm × 1 m) of 1.5% OV-17 on Chromosorb 750 (80 - 100 mesh) was used in GLC.

Results and Discussion

Isolation and Purification of Sialo-oligosaccharides from PSGP of Salvelinus leucomaenis pluvius

Sialo-oligosaccharide alditols isolated by alkaline borohydride treatment of PSGP (300 mg) were first subjected to DEAE-Sephadex A-25 column chromatography (Fig. 1), where

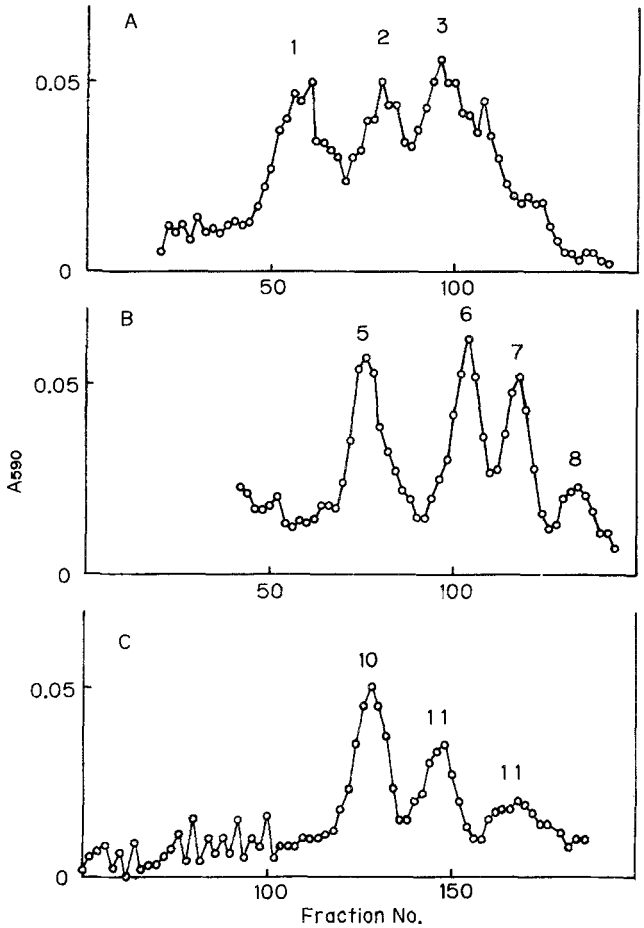


Figure 2. Chromatography on Bio-Rad AG1-X2 of DEAE-Sephadex fractions. A: Fractions 1 - 3; NaCl gradient, 0.02 - 0.06 M. B: Fractions 5 - 8; NaCl gradient, 0.07 - 0.11 M. C: Fractions 10 - 11; NaCl gradient, 0.12 - 0.16 M.Fractions (5.5 ml) were assayed for sialic acid by the resorcinol method.

oligosaccharides were separated not only by the number of sialic acid residues but also by the difference in the structure of the core asialo-oligosaccharide. Better separation of each peak was obtained by rechromatography of combined peaks on Bio-Rad AG 1-X2 column as shown, for example, in Fig. 2. Most of the peaks were still found to be mixtures of more than two sialo-oligosaccharides when examined by TLC. The separation of the mixtures was achieved by column chromatography on Iatrobeads (Fig. 3). Fig. 4 shows TLC of purified fractions. Those fractions giving a single spot on TLC were subjected to structural analysis.

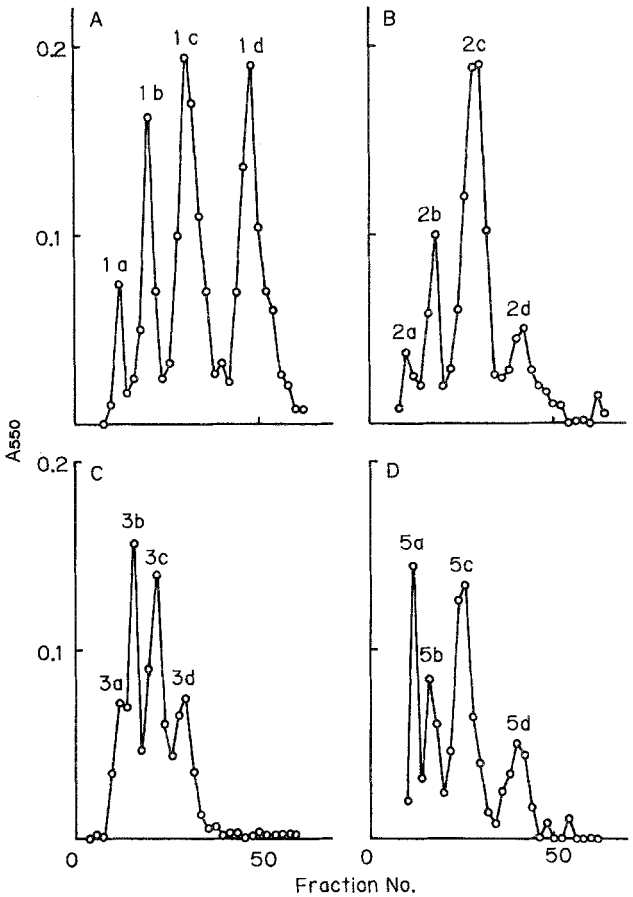


Figure 3. Fractionation on an Iatrobeads column of sialo-oligosaccharide fractions obtained by Bio-Rad AG 1-X2 chromatography. A, fraction 1; B, fraction 2; C, fraction 3; and D, fraction 5. Fractions (2.6 ml) were assayed for sialic acid by the thiobarbituric acid method.

Structure Determination of Sialo-oligosaccharides from S. leucomaenis pluvius PSGP

Since we have already determined the structures of three types of homologous sialo-oligosaccharides (short core units, long core units, and fucose-containing units) isolated from rainbow trout eggs [4, 5, 7], the determination of the structures of these types of oligosaccharides was based on composition analysis only (Table 2) and TLC (Fig. 4). The structures of the tetrasaccharide core and the trisaccharide core were determined in this study as described in the section on asialo-oligosaccharide structures. Sialo-oligosaccharide structures identified in this study are listed in Table 3. Anomeric configurations were based on our earlier investigations [4-9]. In summary, carbohydrate units of

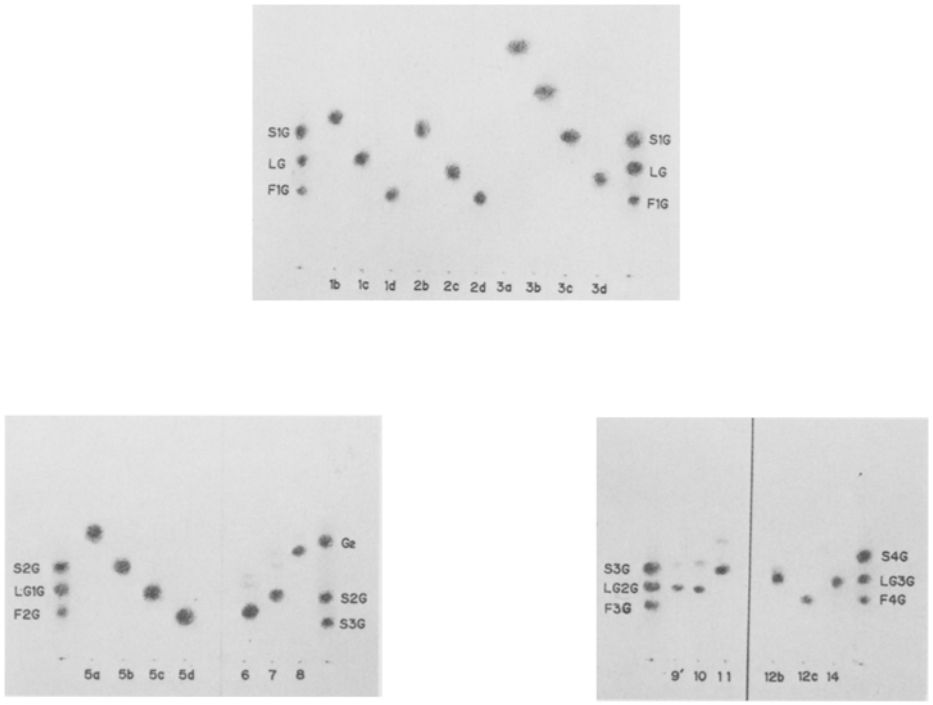


Figure 4. TLC of purified sialo-oligosaccharides obtained from *S. leucomenis pluvius* PSGP. Visualized with resorcinol. G_2, NeuGcα2-8NeuGc; S4G, Galβ1-3(NeuGcα2-8NeuGcα2-8NeuGcα2-8NeuGcα2-6)GalNAcOL [4]; F3G, Fucα1-3GalNAcβ1-3Galβ1-4Galβ1-3(NeuGcα2-8NeuGcα2-8NeuGcα2-6)GalNAcOL [7]. For other structures see Table 3.

PSGP contained two distinct types of complete units (long core unit and fucose-containing unit) and all the possible intermediate types of biosynthetically incomplete core units (mono- to tetrasaccharide core units).

Oligosialyl groups are linked to the proximal *N*-acetylgalactosamine residues in all types of carbohydrate units. A distinct feature of *Salvelinus* PSGP is the presence of *N*-acetylneuraminic acid in contrast to the fact that PSGP from *Salmo* and *Oncorhynchus* so far examined contained only NeuGc (Table 1). The presence of *N*-acetylneuraminic acid apparently resulted in further complexity in the fractionation profiles. Thus in the case of long core units and short core units, isomers containing either *N*-acetyl- or *N*-glycolylneuraminic acid were separated on the Iatrobeads column (**1b** and **1c**, **5a** and **5c**, and **3b** and **3c**). However, isomers having both *N*-acetyl- and *N*-glycolylneuraminic acid at the different sites of attachment could not be separated from each other in the present study. Since the *N*-acetylneuraminic acid-containing oligosaccharides were newly

Table 2. Composition of sialo-oligosaccharide fractions isolated from *Salvelinus leucomaenis pluvius* PSGP.

Number of sialic acid residues[a]	Fraction[b]	mol/mol GalNAcOL						
		Sialic acid[c]			Fuc	Gal	GalNAc	GalNAcOL
		GLC	TBA	RCH				
Monosialo	1b	1.1	1.4	0.92	0	2.1	1.9	1.0
	1c	1.1	0.96	1.3	0	2.0	1.6	1.0
	1d	0.90	0.88	0.97	0.93	2.0	1.1	1.0
	2b	1.2	1.4	1.3	0	2.0	1.0	1.0
	2c	0.97	0.97	1.0	0	2.0	0.65	1.0
	2d	1.3	1.1	1.4	0.74	2.0	1.1	1.0
	3a	0.95	0.87	1.1	0	0	0	1.0
	3b	1.1	1.3	0.94	0	0.88	0	1.0
	3c	1.1	1.0	1.2	0	0.91	0	1.0
	3d	0.93	1.0	1.2	0	1.9	0	1.0
Disialo	5a	2.3	2.6	1.9	0	2.0	1.9	1.0
	5b	2.1	2.3	2.3	0	2.0	1.9	1.0
	5c	2.0	1.5	2.0	0	1.8	1.5	1.0
	5d	1.7	1.8	2.4	0.89	1.8	1.1	1.0
	6	1.6	2.1	2.5	0	2.0	0	1.0
	7	1.6	2.0	2.3	0	0.92	0	1.0
	8	1.5	n.d.[d]	2.2	0	0.17	0	1.0
Trisialo	9′	3.0	3.7	3.7	0	1.9	2.0	1.0
	10	2.0	2.9	3.7	0	1.5	0	1.0
	11	1.8	2.7	4.1	0	0.96	0	1.0
Tetrasialo	12b	2.7	3.8	5.1	0	2.0	1.6	1.0
	12c	2.4	3.5	5.9	0.53	1.9	1.1	1.0
	14	2.0	3.5	4.5	0	1.5	0	1.0

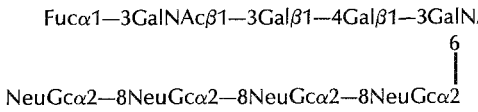
[a] Estimated from the position of the peak in anion-exchange chromaography.
[b] Fractions 1b to 5d, 12b and 12c were obtained by Iatrobeads chromatography. 6, 7, 8, 9′, 10, 11 and 14 were Bio-Rad AG fractions.
[c] All values were obtained using NeuGc as the standard. TBA, thiobarbituric acid method; RCH, resorcinol method. The thiobarbituric acid assay was performed following hydrolysis with 0.1 M trifluoroacetic acid for 2h at 80°C.
[d] n.d., not determined.

isolated, the structural determination of **1b** and **3b** will be described. Results of composition analysis suggested that **1b** has the same asialo-core structure as **1c**, the only difference between **1b** and **1c** being the sialic acid residue (*N*-acetylneuraminic acid in **1b** and *N*-glycolylneuraminic acid in **1c**). The analysis by GLC-MS of partially methylated alditol acetates derived from both **1b** and **1c** revealed the presence of 1 mol each of nonreducing terminal *N*-acetylgalactosamine, 3,4-di-*O*-substituted *N*-acetylgalactosamine, 3- and 4-*O*-substituted galactose and 3-*O*-substituted *N*-acetylgalactosaminitol. The direct-probe mass spectrum of permethylated **1c** was similar to that reported for GalNAcβ1-4(NeuGcα2-3)GalNAcβ1-3Galβ1-4Galβ1-3GalNAcOL (the sialidase-resistant oligosaccharide, SRO) [5, 8], whereas the spectrum of **1b** only differed in the appearance of the peaks originating from the *N*-acetylneuraminic acid residue in place of the peaks

Table 3. Proposed structures of sialo-oligosaccharides isolated from *Salvelinus leucomaenis pluvius* PSGP.

Abbreviation	Assigned structure	Fraction
	I. Long-core units	
LA	GalNAcβ1–4GalNAcβ1–3Galβ1–4Galβ1–3GalNAcOL 3 NeuAcα2	1b
LG	GalNAcβ1–4GalNAcβ1–3Galβ1–4Galβ1–3GalNAcOL 3 NeuGcα2	1c
LA1A	GalNAcβ1–4GalNAcβ1–3Galβ1–4Galβ1–3GalNAcOL 3 6 NeuAcα2 NeuAcα2	5a
LA1G	GalNAcβ1–4GalNAcβ1–3Galβ1–4Galβ1–3GalNAcOL 3 6 NeuAcα2 NeuGcα2	5b
LG1A	GalNAcβ1–4GalNAcβ1–3Galβ1–4Galβ1–3GalNAcOL 3 6 NeuGcα2 NeuAcα2	
LG1G	GalNAcβ1–4GalNAcβ1–3Galβ1–4Galβ1–3GalNAcOL 3 6 NeuGcα2 NeuGcα2	5c
LG2G	GalNAcβ1–4GalNAcβ1–3Galβ1–4Galβ1–3GalNAcOL 3 6 NeuGcα2 NeuGcα2–8NeuGcα2	9′[a]
LG3G	GalNAcβ1–4GalNAcβ1–3Galβ1–4Galβ1–3GalNAcOL 3 6 NeuGcα2 NeuGc[α2-8NeuGc]$_2$α2	12b
	II. Fucose-containing units	
F1G	Fucα1–3GalNAcβ1–3Galβ1–4Galβ1–3GalNAcOL 6 NeuGcα2	1d, 2d
F2G	Fucα1–3GalNAcβ1–3Galβ1–4Gal1–3GalNAcOL 6 NeuGcα2–8NeuGcα2	5d

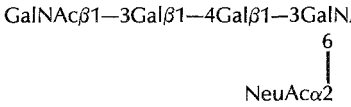
F4G



12c[b]

III. Tetrasaccharide-core units

Q1A



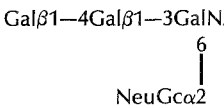
2b

Q1G

2c

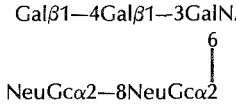
IV. Trisaccharide-core units

T1G



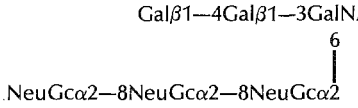
3d

T2G



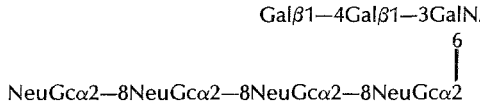
6[a]

T3G



10[a]

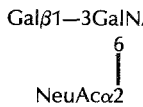
T4G



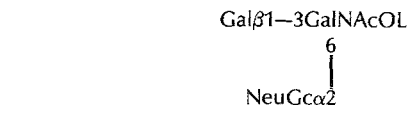
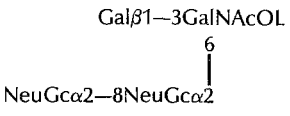
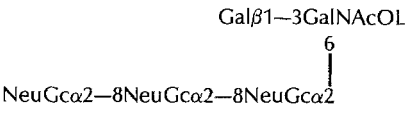
14[a]

V. Short-core units

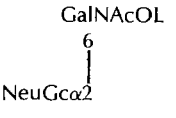
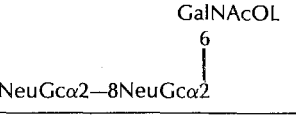
S1A



3b

S1G		3c
S2G		7[a]
S3G		11[a]

VI. Monosaccharide-core units

M1G		3a
M2G		8[a]

[a] NeuAc was detected as a minor component in these fractions.

[b] Evidence for identification of this fraction as tetrasialyl- and not trisialylfucopentasaccharide was provided by: (i) the elution order in the anion-exchange chromatography, since trisialylfucopentasaccharide must be eluted in fraction 9, though it could not be detected in the present work (Fig. 1); (ii) TLC, see Fig. 4C and [7].

originating from the *N*-glycolylneuraminic acid residue [m/z 376 and 344 (376 - 32) compared to m/z 406 and 374 (406 - 32)]. The determination of the sequence -3Gal(1-4)Gal- is tentative, but the ^{1}H-NMR spectrum of **1b** strongly supports this structure and the proposed anomeric configurations (data not shown). The *N*-acetylneuraminic acid residue linked (2-3) to the internal *N*-acetylgalactosamine was hydrolyzed by *Arthrobacter ureafaciens* sialidase, albeit at a slow rate, and thus its linkage is α (Iwasaki *et al.* unpublished results).

Methylation analysis of **3b** and **3c** showed the presence of only terminal galactose and 3,6-di-*O*-substituted *N*-acetylgalactosaminitol. Fragment ions appeared in the direct-probe mass spectrum of permethylated **3b** (m/z 637, originating from NeuAc-GalNAcOL; 480, from Gal-GalNAcOL; and 376, from NeuAc) support the proposed structure.

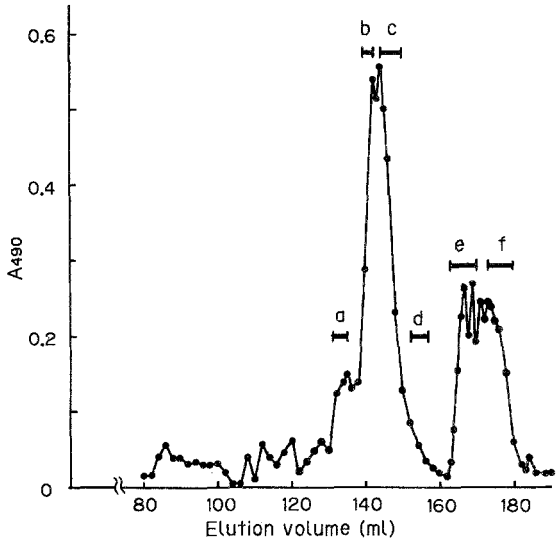


Figure 5. Bio-Gel P-4 chromatography of asialo-oligosaccharides obtained by desialylation of sialo-oligosaccharides isolated from *S. leucomaenis pluvius* PSGP. Monitored by the phenol-sulfuric acid method. Solid bars indicate the fractions pooled.

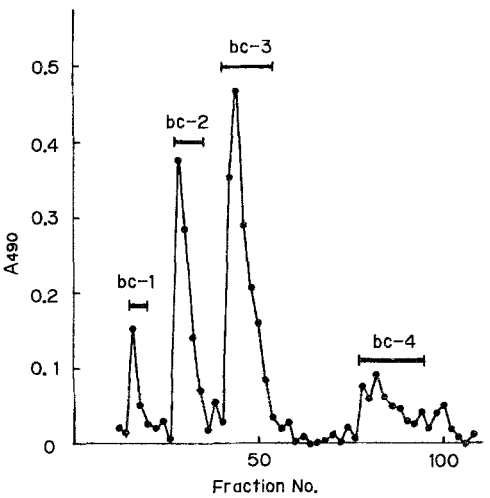


Figure 6. Fractionation of asialo-oligosaccharides (fractions **b** - **c** in Fig. 5) on an Iatrobeads column. Elution with *n*-propanol/conc. NH_4OH/H_2O, 6/1/2 by vol. Monitored by the phenol-sulfuric acid method. Solid bars indicate the fractions pooled.

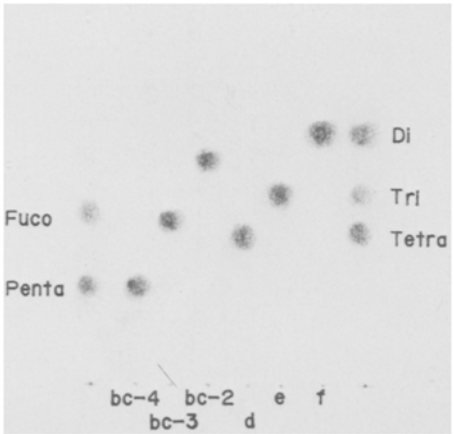


Figure 7. TLC of purified asialo-oligosaccharides obtained from *S. leucomaenis pluvius* PSGP. Visualized with orcinol reagent. Di, Galβ1-3GalNAcOL; Tri, Galβ1-4Galβ1-3GalNAcOL; Tetra, GalNAcβ1-3Galβ1-4Galβ1-3GalNAcOL; Penta, GalNAcβ1-4GalNAcβ1-3Galβ1-4Galβ1-3GalNAcOL; Fuco, Fucα1-3GalNAcβ1-3Galβ1-4Galβ1-3-GalNAcOL.

Isolation and Fractionation of Asialo-oligosaccharides and the Sialidase-resistant Oligosaccharide from S. leucomaenis pluvius PSGP

The asialo-oligosaccharide fraction obtained by exhaustive sialidase digestion of the mixture of sialo-oligosaccharide alditols (from 200 mg PSGP) was fractionated by Bio-Gel P-4 chromatography (Fig. 5). Fractions **b**, **c** and **d** were further purified by Iatrobeads column chromatography (Fig. 6). Fraction **e** appeared to consist of one component as judged from TLC, whereas fraction **f** contained, in addition to the major component, a minor component which migrated similarly to **e**. These two components in fraction **f** were separated from each other by Bio-Gel P-2 chromatography. The purity of the materials thus obtained was examined by TLC (Fig. 7).

The major components of fraction **a** and fraction **bc-1** appeared to be the same oligosaccharide as judged from composition and TLC. These fractions were combined and purified by rechromatography on the Iatrobeads column.

Structure Determination of Asialo-oligosaccharides and the Sialidase-resistant Oligosaccharide from S. leucomaenis pluvius PSGP

The proposed structures of the asialo-oligosaccharides and the sialidase-resistant oligosaccharide deduced from composition analysis (Table 4), methylation analysis (Table 5), and TLC (Fig. 7) are shown in Table 6 together with the relative yield of each oligosaccharide. Standard oligosaccharides used in TLC were those isolated from PSGP of other salmonid fishes and their structures have been established as described in the next section. Asialo-SRO was obtained by mild acid hydrolysis of SRO [5]. Identification of **bc-4**

Table 4. Composition of asialo-oligosaccharides and sialidase-resistant oligosaccharide obtained from *Salvelinus leucomaenis pluvius* PSGP.

Fraction	mol/mol GalNAcOL				
	NeuGc	Fuc	Gal	GalNAc	GalNAcOL
a+bc-1	0.75	0	2.1	1.7	1.0
bc-4	0	0	2.2	2.0	1.0
bc-3	0	1.2	2.1	1.0	1.0
bc-2[a]	0	0	2.1	1.2	1.0
d	0	0	1.9	1.1	1.0
e	0	0	1.6	0	1.0
f	0	0	0.73	0	1.0

[a] This fraction contains unidentified deoxyhexose X. See text.

Table 5. Methylation analysis of asialo-oligosaccharides and sialidase-resistant oligosaccharide obtained from *Salvelinus leucomaenis pluvius* PSGP.

Methylated sugar	R_t[a]	Relative peak area (uncorrected)						
		a+bc-1	bc-4	bc-3	bc-2	d	e	f
X[b]-	0.36				0.5			
Fuc-	0.40			0.9				
Gal-	0.64						1.1	1.0
-4Gal-	0.81	1.0	1.0	1.0	1.0	1.0	1.0	
-3Gal-	0.85	1.0	1.0	1.0	1.0	1.0		
-3GalNAcOL	1.0	0.5	0.5	0.3	0.5	0.4	0.5	0.8
GalNAc-	1.37	1.1	0.8			1.1		
-4GalNAc-	1.51		0.6					
-3GalNAc-	1.57			0.6	0.9			
>3,4GalNAc-	1.66	0.7						

[a] Retention time relative to -3GalNAcOL on GLC.
[b] Unidentified deoxyhexose. See text.

as asialo-SRO was also supported by direct-probe mass spectrometry of permethylated **bc-4**. Thus five types of asialo-oligosaccharides (**bc-4, bc-3, d, e** and **f**) each of which was also obtained as the sialylated form (Table 3) were isolated. Anomeric configurations were not determined in the present study but assumed to be the same as those previously determined for three types of sialo-oligosaccharides of PSGP from *Salmo gairdneri* [4, 5, 7, 8]. In addition to these five, a new pentasaccharide (**bc-2**) was isolated, in which an unidentified sugar (X) replaced the fucose residue at the non-reducing end of the fucopentasaccharide **bc-3**.

Sugar X was tentatively assigned as a deoxyhexose based on the following data: (i) **bc-2** migrated faster than **bc-3** on TLC, (ii) X migrated faster than fucose on TLC, (iii) both the

Table 6. Structures and relative yields of asialo-oligosaccharides and sialidase-resistant oligosaccharide obtained from *Salvelinus leucomaenis pluvius* PSGP.

Fraction	Structure	Yield[a]
a + bc-1	GalNAcβ1—4GalNAcβ1—3Galβ1—4Galβ1—3GalNAcOL 3 NeuGcα2	0.26
bc-4	GalNAcβ1—4GalNAcβ1—3Galβ1—4Galβ1—3GalNAcOL	0.20
bc-3	Fucα1—3GalNAcβ1—3Galβ1—4Galβ1—3GalNAcOL	0.60
bc-2	X[b]1—3GalNAcβ1—3Galβ1—4Galβ1—3GalNAcOL	0.30
d	GalNAcβ1—3Galβ1—4Galβ1—3GalNAcOL	0.04
e	Galβ1—4Galβ1—3GalNAcOL	0.79
f	Galβ1—3GalNAcOL	1.0

[a] Molar ratio relative to disaccharide **f**.
[b] Unidentified doxyhexose. See text.

trimethylsilylated methyl glycoside of X and methylated X were eluted ahead of the corresponding derivatives of fucose on GLC, (iv) the direct probe mass spectrum of methylated **bc-2** was essentially the same as that of **bc-3** (a prominent ion at m/z 189, originating from deoxyhexose at the non-reducing terminal, was noted), and (v) the mass spectrum of methylated X showed a fragmentation pattern similar to that for methylated fucose, except that a difference in relative intensities was noted between some ions. X migrated even faster than rhamnose on TLC and eluted earlier than rhamnose on GLC of the trimethylsilyl derivatives of the methyl glycosides. The yield being low, full characterization of X could not be accomplished. However, it could be represented by an isomeric structure of fucose, and no such observation was made in other fish egg PSGP so far analyzed. A combined fraction **a** + **bc-1** consisted of a sialo-oligosaccharide and the results of composition analysis (Table 4), methylation analysis (Table 5), and TLC suggested that this sialo-oligosaccharide is SRO. For unknown reasons, this particular sialo-oligosaccharide had a very weak affinity for the anion-exchanger and eluted in the non-retarded fraction with the bulk of the asialo-oligosaccharides (see Materials and Methods). No asialo-oligosaccharide larger than pentasaccharide (fraction **bc**) was found in PSGP.

Isolation and Structures of Asialo-oligosaccharides and a Sialidase-resistant Oligosaccharide from PSGP of Three Other Species of Salmonid Fishes

Asialo-oligosaccharides and the sialidase-resistant oligosaccharide were obtained by exhaustive sialidase digestion of sialo-oligosaccharides from PSGP of three different species of fishes, fractionated and purified as described for *Salvelinus* PSGP. The proposed structures are given in Table 7 with relative yields. Data for determination of the structure of oligosaccharides isolated from *Salmo gairdneri* and *Oncorhynchus masou*

Table 7. Structures and relative yields of asialo-oligosaccharides and sialidase-resistant oligosaccharide (SRO) obtained from PSGP of three species of salmonid fishes.

Notation	Structure	Yield[a]		
		Salmo gairdneri	*O.masou ishikawai*	*O.nerka adonis*
SRO	GalNAcβ1—4GalNAcβ1—3Galβ1—4Galβ1—3GalNAcOL 3 NeuGcα2	0.26	1.0	1.0
Asialo-SRO	GalNAcβ1—4GalNAcβ1—3Galβ1—4Galβ1—3GalNAcOL	0.024	0.040	0
Fucopenta-saccharide	Fucα1—3GalNAcβ1—3Galβ1—4Galβ1—3GalNAcOL	0.22	0.34	0.45
Tetrasaccharide	GalNAcβ1—3Galβ1—4Galβ1—3GalNAcOL	0.025	0.060	0.10
Trisaccharide	Galβ1—4Galβ1—3GalNAcOL	0.02	0.66	0.10
Disaccharide	Galβ1—3GalNAcOL	1.0	1.0	1.0

[a] Molar ratio relative to disaccharide.

ishikawai are given below. Structures of oligosaccharides from *O. nerka adonis* were proposed only from TLC data because of the limited amounts of material isolated. All anomeric configurations were proposed from those established for the series of sialo-oligosaccharides obtained from *Salmo gairdneri* PSGP [4, 5, 7, 8].

Disaccharide. Composition analysis gave 1 mol each of galactose and *N*-acetylgalactosaminitol. Methylation analysis revealed 1 mol each of terminal galactose and 3-*O*-substituted *N*-acetylgalactosaminitol.

Trisaccharide. Composition analysis gave 2 mol of galactose and 1 mol of *N*-acetylgalactosaminitol. Methylation analysis revealed terminal galactose, 4-*O*-substituted galactose and 3-*O*-substituted *N*-acetylgalactosaminitol in about equal proportions.

Tetrasaccharide. Galactose, *N*-acetylgalactosamine and *N*-acetylgalactosaminitol were found in the ratio 2:1:1. Methylation analysis revealed 1 mol each of terminal *N*-acetylgalactosamine, 4- and 3-*O*-substituted galactose and 3-*O*-substituted *N*-acetylgalactosaminitol. The terminal *N*-acetylgalactosamine was removed by digestion with ascidian β-*N*-acetylhexosaminidase [16] and methylation analysis of the trisaccharide obtained after digestion revealed terminal galactose, 4-*O*-substituted galactose and 3-*O*-substituted *N*-acetylgalactosaminitol in the ratio 1.0 : 0.9 : 0.7. The direct-probe mass spectrum of the permethylated tetrasaccharide is given in Fig. 8.

Fucopentasaccharide. Fucose, galactose, *N*-acetylgalactosamine and *N*-acetylgalactosaminitol were present in the ratio 1:2:1:1. Methylation analysis revealed terminal fucose, 4-*O*-substituted galactose, 3-*O*-substituted galactose, 3-*O*-substituted *N*-acetylgalactosamine and 3-*O*-substituted *N*-acetylgalactosaminitol in the ratio 0.9 : 1.2 : 1.3 : 1.0 : 1.0. Methylation analysis of the products obtained after Smith degradation of the parent oligosaccharide revealed the presence of 1 mol each of terminal *N*-acetylgalactosamine

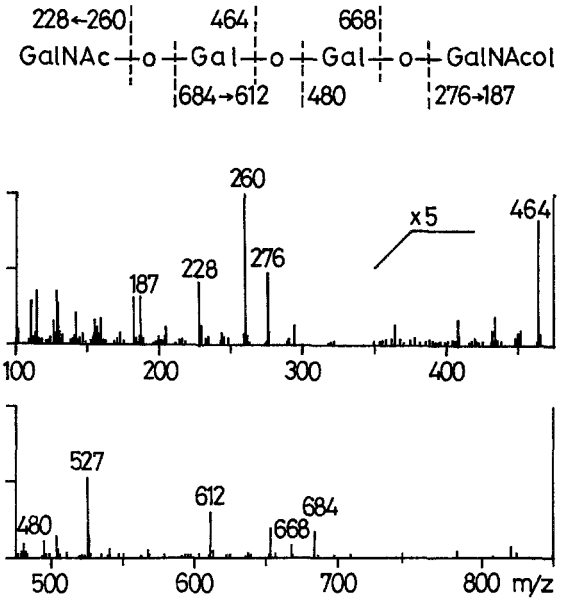


Figure 8. Direct-probe mass spectrum of the permethylated tetrasaccharide obtained from *Salmo gairdneri* PSGP.

and 3-*O*-substituted galactose. The direct-probe mass spectrum obtained for the permethylation product was similar to that of the permethylated asialofucopentasaccharide reported previously [7].

Conclusion and General Discussion

The present work showed that in PSGP from three species of salmonid fishes, in addition to two species studied earlier two different types of asialopentasaccharide cores, Fucα1-3GalNAcβ1-3Galβ1-4Galβ1-3GalNAcOL (fucopentasaccharide) and GalNAcβ1-4-GalNAcβ1-3Galβ1-4Galβ1-3GalNAcOL (asialo-SRO) occur as the largest chains. In most species of fish, asialo-SRO was isolated only in minute amounts presumably because of the sialidase-resistant nature of the *N*-glycolylneuraminic acid residue attached α(2-3) to the penultimate *N*-acetylgalactosamine residue [5, 8]. Thus, Galβ1-4(NeuGcα2-3)Gal-NAcβ1-3Galβ1-4Galβ1-3GalNAcOL (SRO) was the product obtained after exhaustive sialidase treatment of the poly(oligo)sialylglycans. In contrast, much larger amounts of asialo-SRO were isolated from *Salvelinus* PSGP (**bc-4** in Table 6) and this is apparently due to the presence of NeuAc-type SRO (LA, LA1A, LA1G in Table 3). The *N*-acetylneuraminic acid residue in GalNAcβ1-4(NeuAcα2-3)GalNAcβ1-3Galβ1-4Galβ1-3GalNAcOL was hydrolysed by *Arthrobacter* sialidase, although at a slow rate (Iwasaki *et al.*, unpublished results). In spite of the fact that very small amounts of asialo-SRO were detected for PSGP from *Salmo gairdneri* and *Oncorhynchus masou ishikawai*, no direct evidence was obtained to show the occurrence of NeuAc-type SRO in the PSGP molecules from these species.

All of the possible biosynthetic precursors of these pentasaccharide cores were found in every PSGP examined. Relative yields of these core oligosaccharides given in Table 6 and 7 show only a rough estimate of their proportion in PSGP molecules, since the values are based on the final yields after many steps of purification. In any case, the disaccharide Galβ1-3GalNAcOL seems to be the most abundant core structure. SRO (+ asialo-SRO in the case of *Salvelinus*) was usually isolated in an amount comparable to the disaccharide while, in PSGP from *Oncorhynchus keta*, the yield of fucopentasaccharide was comparable to that of the disaccharide and eight times more than SRO [6].

The tri- and tetrasaccharide cores are structurally related to each other in a manner to be expected for a stepwise biosynthetic pathway, but the difference in their yields between fish species may be less significant. The result perhaps more significant from both biosynthetic and functional viewpoints of PSGP is that all the carbohydrate units of PSGP in the intact forms are sialylated and not only the complete chains but all the intermediary precursor chains are highly sialylated. We have shown the presence of $(\text{-8NeuGc}\alpha2)_n\text{-6GalNAcOL}$ in this study. No difference has so far been detected in the degree of sialylation between the types of core oligosaccharides. Thus, we consider that sialylation of the proximal *N*-acetylgalactosamine, the elongation of polysialyl chains and the growth of core oligosaccharides occur simultaneously and independent of each other in the biosynthesis of PSGP. Although sialo-oligosaccharides purified in the present study were those containing mono- to tetrasialyl groups, evidence for the presence of carbohydrate units with higher oligosialyl groups (10 - 20 *N*-glycolylneuraminic acid residues) was given for PSGP from *Salmo Gairdneri* [2, 4] and is apparent from the elution profile shown in DEAE-Sephadex A-25 chromatography of sialo-oligosaccharides (Fig. 1).

It is worthwhile at this stage to discuss briefly the applicability and usefulness of the Iatrobeads (porous silica) column chromatography used here. Considerable success has been achieved in earlier reports in the separation of neutral glycolipids [17] and gangliosides [18] with very similar characteristics by using this method. In the present study, we showed that Iatrobeads column chromatography is also effective in separation of both asialo- and sialo-oligosaccharides. The great advantages of this method over others are that the same or a slightly modified solvent system can be used in column chromatography on silica gel as in TLC, and the order of elution from the Iatrobeads column is much the same as migration rate in TLC. Thus Iatrobeads column chromatography can be used in place of preparative TLC with the advantage that larger amounts of material can be separated with better yields.

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