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Quantitation and Isomeric Structure Analysis of Free Oligosaccharides Present in the Cytosol Fraction of Mouse Liver: Detection of a Free Disialobiantennary Oligosaccharide and Glucosylated Oligomannosides[1]

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The amounts and isomeric structures of free oligosaccharides derived from *N*-linked sugar chains present in the cytosol fraction of perfused mouse liver were analyzed by tagging the reducing end with 2-aminopyridine followed by 2-dimensional HPLC mapping with standard sugar chains. Sixteen pyridylaminated (PA-) oligomannosides terminating with a PA-GlcNAc residue (GN1-type), three glucose-containing oligomannosides, and four oligomannosides terminating with a PA-di-*N*-acetylchitobiose (GN2-type) were detected. The total contents of the GN1- and GN2-type oligomannosides were 3.4 and 0.5 nmol, respectively, per gram of wet tissue. Maltooligosaccharides (dimer to pentamer) were also detected, the total content of which was 13 nmol per gram of wet tissue. Besides these oligosaccharides, a PA-disialobiantennary sugar chain—the sole complex-type sugar chain—was also detected. All the oligomannosides identified had partial structures of $Glc_3Man_9GlNAc_2$-p-p-dolichol, revealing that they were metabolic degradation products. Manα1-2Manα1-2Manα1-3(Manα1-6)Manβ1-4GlcNAc (M5B′) was the major oligomannoside, suggesting that cytosolic endo-β-*N*-acetylglucosaminidase and neutral α-mannosidase participate in the degradation, because these enzymes have suitable substrate specificities for the production of M5B′. Degradation by these enzymes seems to be the main pathway by which oligomannosides are degraded in mouse cytosol; however, small amounts of Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4(GlcNAc)$_{1-2}$ and related oligomannosides together with parts of their structures were also detected, suggesting that there is another minor route by which cytosolic free oligomannosides are produced.

Key words: cytosol, free oligosaccharides, mouse liver, oligomannoside.

The presence of free oligomannosides in the cytosol has been demonstrated in a number of animal cells by means of metabolic radiolabeling of sugar chains (*1-11*). Several reports have indicated that endo-β-*N*-acetylglucosaminidase and neutral α-mannosidase, which are found in the cytosol of animal cells, participate in the degradation of oligomannosides (*12-18*). The contribution of these enzymes to the degradation of cytosolic oligomannosides was recently confirmed by the detection of M5B′ as the main oligomannoside in the cytosol of hen oviduct (*18*), because purified endo-β-*N*-acetylglucosaminidase (*12*) and neutral α-mannosidase (*17*) from the same source had preferential substrate specificities in producing M5B′. Moore's group explained the process of further M5B′ degradation in a vesicular compartment, probably the lysosome, after its translocation from the cytosol (*11*). Besides M5B′, the small oligomannosides $(Man)_{2-5}GlcNAc$ were also detected in the cytosol fraction of hen oviduct but $(Man)_{6-9}GlcNAc$ were not found (*18*). The oligomannosides $(Man)_{4-9}GlcNAc$ together with $GlcMan_9GlcNAc$ and $GlcMan_5GlcNAc$ were detected in the cytosol of permeabilized HepG2 cells (*7*). In addition, free oligomannosides have been identified in the cytosol of CHO cells (*9*), calf thyroid slices (*1*), mouse splenocytes (*2*), MDBK cells (*10*), and fibroblasts (*3*) using metabolic radiolabeling with ^{3}H-Man or ^{14}C-Man. However, because the amount of an oligomannoside that can be detected by metabolic radiolabeling is time-dependent, the exact proportions of the cytosolic oligomannosides found in these studies are unknown, and except for M5B′ (*7, 9, 18*) and M4D′ (*18*) their isomeric structures were not reported.

To properly understand the process by which free oligomannosides are degraded in cytosol, it is important to determine both their relative amounts and isomeric structures in detail. In the work reported here, we prepared the cytosol fraction from perfused mouse liver and analyzed the free oligomannosides derived from *N*-linked sugar chains. Twenty free oligomannosides were detected, in-

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Abbreviations: ER, endoplasmic reticulum; GN1, an oligosaccharide terminating with a single GlcNAc residue; GN2, an oligosaccharide terminating with a di-*N*-acetylchitobiose; oligomannoside, Man_n-$GlcNAc_{1-2}$; PA-, pyridylamino-; PBS, phosphate buffered saline. Abbreviations for oligosaccharides, see Table I.

cluding some that were newly identified and a sialobiantennary sugar chain.

MATERIALS AND METHODS

Materials—Eight-week-old ICR mice were purchased from Japan Clea (Tokyo). The livers of anesthetized mice were perfused with 0.9% NaCl at 1.5 ml/min for 20 min using an HPLC pump. α-Mannosidase (jack bean), β-*N*-acetylhexosaminidase (jack bean), endo-β-*N*-acetylglucosaminidase H (*Streptomyces griseus*), and cellotetraose were obtained from Seikagaku Kogyo (Tokyo), sialidase (*Arthrobacter ureafaciens*) from Nacalai Tesque (Kyoto), endoglycosidase F2 (*Flavobacterium meningosepticium*) from Sigma (St. Louis, MO), phenol reagent from Wako (Osaka). TSK-Gel HW-40F and a TSK-Gel amide-80 column (4.6×75 mm) were purchased from Tosoh (Tokyo), a Cosmosil 5C18-P column (1.5×250 mm) from Nacalai Tesque (Kyoto), a YMC-Gel Sil S-5 column (4.6×75 mm) from Yamamura Kagaku (Kyoto), Bio-Gel P-2 from Bio-Rad (Hercules, CA), and Dowex 50W-X2 (50–100 mesh) from Dow Chemicals (Midland, MI).

Preparation of Standard PA-Oligosaccharides—PA-G1M9A′ was prepared by pyridylamination (*19*) of the endo-β-*N*-acetylglucosaminidase digests of PA-G1M9A obtained from egg yolk IgY (*20*), and PA-M8A′ and PA-M8C′ were prepared similarly from PA-M8A and PA-M8C (*14*). Pyridylamino derivatives of G1M4E′, G1M5B′, G1M6′, G1M7′, and G1M8′ were isolated by size-fractionation HPLC from partial α-mannosidase digests of PA-G1M9A′ and those of G1M4E, G1M5B, G1M6, G1M7, and G1M8 were similarly isolated from PA-G1M9A. PA-BI′ was prepared by pyridylamination of the endoglycosidase F2 digest of PA-BI. Pyridylamino derivatives of M1′, M2B′, M3B′, M4D′, M6′, and M7′ were prepared by partial α-mannosidase digestion of PA-M5B′ or PA-M9A′ followed by size-fractionation HPLC. Preparation of PA-M5B′ was as previously reported (*17*). PA-M2A′ was prepared by acetolysis of PA-M5A′ according to the method described elsewhere (*21*). Preparation of pyridylamino derivatives of M3A′, M4B′, M5A′, M9A′, M1, M2B, M3A, M4B, M5A, M6, M7, and M8 was as reported (*14*). PA-BI, PA-SiaBI, and PA-trisialotriantennary sugar chains were from riboflavin-binding protein (*22*).

Isolation of Cytosol Fraction from Perfused Mouse Liver—Perfused mouse livers (2.2 g) were cut into small pieces and homogenized using a Potter-Elvehjem homogenizer (by 2–3 strokes, 550 rpm) in a 6-fold volume of 50 mM Tris-HCl buffer, pH 7.4, containing 0.25 M mannitol and 5 mM $MgCl_2$. The homogenate was centrifuged at 160,000×*g* for 60 min. The supernatant was used as the cytosol fraction and the precipitate as the microsomal fraction. All procedures were carried out below 4°C.

Preparation of Oligosaccharides from Cytosol Fraction—The cytosol fraction was heated at 100°C for 5 min to denature the proteins, then ethanol was added to the solution up to 60% to precipitate proteins. The precipitates were removed by centrifugation at 14,600×*g* for 20 min. Cellotetraose (4.4 nmol) was added to the supernatant as an internal standard, then the solution was applied onto a Bio-Gel P-2 column (2.5×140 cm) equilibrated with 20 mM ammonium acetate solution. The fraction eluted from the column between the void volume and just before the elution position of mannitol was collected, concentrated, and placed on a Dowex 50W-X2 column (H^+ form, 0.7×2.0 cm). The column was washed with 6.5 ml of water and the pass-through fraction was lyophilized. The amount of lyophilized material was 32 mg.

Preparation of PA-Oligosaccharides—Three milligrams of the lyophilized material obtained as described above was pyridylaminated with 30 μl of 2-aminopyridine reagent at 90°C for 1 h followed by reduction at 80°C for 35 min with 105 μl of borane-dimethylamine reagent according to the reported methods (*19*). After adding 300 μl of aqueous ammonia to the reaction mixture, the excess reagents were removed by extraction with 300 μl of chloroform two times. The aqueous phase was concentrated and the residue was further purified by gel filtration on a TSK-Gel HW40-F column (1.0×37 cm) equilibrated with 0.01 M ammonium acetate buffer, pH 6.0. The fraction between the elution position of the void volume and that of PA-maltose was collected and used as the PA-oligosaccharide fraction.

High-Performance Liquid Chromatography (HPLC)—Size-fractionation HPLC was performed on a TSK-Gel amide-80 column with a guard column of YMC-Gel Sil-5 to protect the amide-80 column (*19*). The column was equilibrated with Eluent A, acetonitrile:water (90:10, v/v) containing 3% (v/v) acetic acid, pH 7.3, adjusted with triethylamine. The components were eluted by increasing linearly the concentration of Eluent B (3% acetic acid solution, pH 7.3, adjusted with triethylamine) from 0 to 10% in 3 min and then to 50% in 27 min at a flow rate of 1.0 ml/min. PA-oligosaccharides were detected by fluorescence using an excitation wavelength of 310 nm and an emission wavelength of 380 nm. Reversed-phase HPLC was performed on a Cosmosil 5C18-P column (1.5×250 mm) using Eluents A and B at 25°C as follows (*14, 19*). Eluent A was 0.1 M ammonium acetate buffer, pH 6.0; Eluent B was 0.1 M ammonium acetate buffer, pH 6.0, containing 1.0% (v/v) 1-butanol. The flow rate was 150 μl/min. The column was equilibrated with 5% Eluent B. The components were eluted by increasing linearly the concentration of Eluent B to 52% in 51 min and then to 100% in 12 min. PA-oligosaccharides were detected by fluorescence using an excitation wavelength of 320 nm and an emission wavelength of 400 nm.

Anion-exchange HPLC was performed on a Mono Q HR 5/5 column (5.0×50 mm) using Eluents C and D (*19*). Eluent C was distilled water adjusted to pH 9.0 with aqueous ammonia; Eluent D was 0.5 M ammonium acetate buffer, pH 9.0, adjusted with aqueous ammonia. The flow rate was 1.0 ml/min. The column was equilibrated with Eluent C. The components were eluted by increasing linearly the concentration of Eluent D to 8% in 3 min, to 30% in 14 min, and then to 100% in 5 min. PA-oligosaccharides were detected as described for the amide-80 column.

Exoglycosidase Digestions—PA-oligosaccharides (10–500 pmol) were digested at 37°C for 15 h with an exoglycosidase; digestion with α-mannosidase (300 mU) was done in 30 μl of 0.05 M sodium acetate buffer, pH 4.5, and with sialidase (100 mU) in 50 μl of 0.1 M sodium acetate buffer, pH 5.0. Enzymatic digestions were terminated by heating at 100°C for 3 min.

Marker Enzyme Assays of Subcellular Fractions—Lysosomal β-*N*-acetylhexosaminidase was assayed by the

procedure of Borooas *et al.* (*23*), microsomal glucose-6-phosphatase by the procedure of Swanson (*24*), and cytosolic alcohol dehydrogenase by the procedure of Theorell and Yonetani (*25*).

RESULTS

Purity of Cytosol Fraction Isolated from Mouse Liver—The cytosol fraction was prepared from perfused livers of ICR mice. Cross contamination from other fractions was estimated by analyzing the marker enzymes. The amount of contaminating enzyme in the cytosol fraction from the microsomal fraction was 1.1% as determined with glucose-6-phosphatase, the microsomal marker enzyme, and that from lysosome was 11% as determined with *N*-acetylhexosaminidase, the lysosomal marker enzyme.

Separation of Neutral and Acidic PA-Oligosaccharides from Cytosol Fraction—Free oligosaccharides in the cytosol fraction were purified by heat denaturation, ethanol precipitation, and gel filtration. Heat denaturation was effective to remove contaminant peaks. Free oligosaccharides were pyridylaminated and separated by Mono Q anion-exchange chromatography into a neutral fraction (Fraction A), a

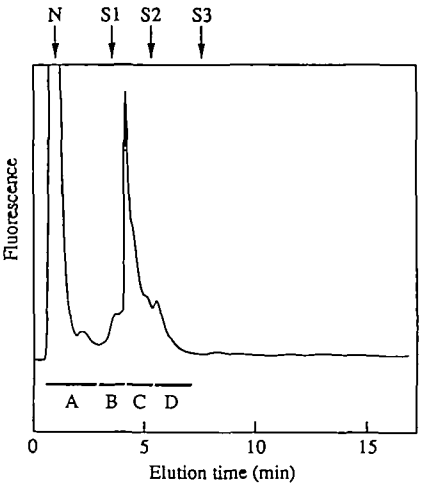

Fig. 1. **Mono Q chromatography of PA-oligosaccharides obtained from mouse liver.** Oligosaccharides in the cytosol fraction of perfused mouse liver were pyridylaminated and the products were fractionated on a Mono Q column into four fractions: A, B, C, and D. Arrows indicate the elution positions of standard PA-oligosaccharides: N, a PA-neutral sugar chain; S1, a PA-monosialo sugar chain; S2, a PA-disialo sugar chain; and S3, a PA-trisialo sugar chain.

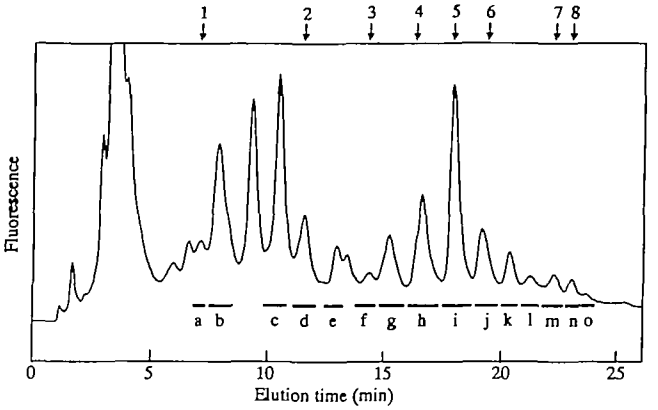

Fig. 2. **Size-fractionation HPLC of Fraction A obtained in Fig. 1.** Fractions a-o were collected as indicated by the bars considering the elution positions of standard PA-oligosaccharides. The arrows indicate the elution positions of standard PA-oligosaccharides: 1, M1′; 2, M2B′; 3, M3B′; 4, M4D′; 5, M5B′; 6, M6′; 7, M8C′; and 8, M9A′.

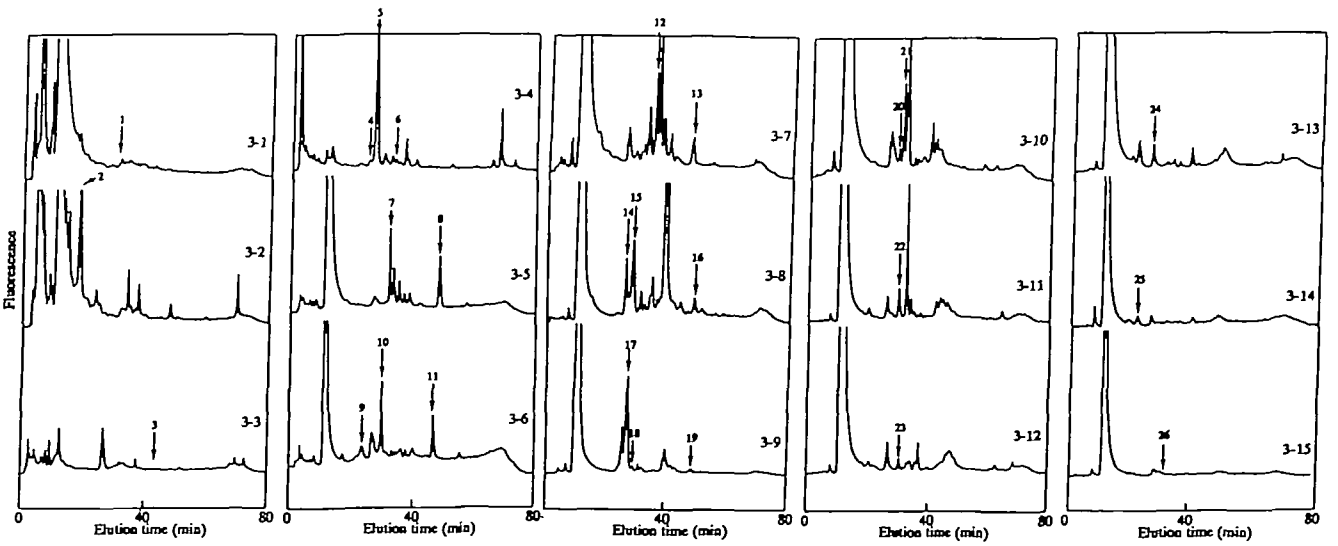

Fig. 3. **Reversed-phase HPLC of Fractions a-o separated by size-fractionation HPLC in Fig. 2.** 3-1, HPLC of Fraction a; 3-2, Fraction b; 3-3, Fraction c; 3-4, Fraction d; 3-5, Fraction e; 3-6, Fraction f; 3-7, Fraction g; 3-8, Fraction h; 3-9, Fraction i; 3-10, Fraction j; 3-11, Fraction k; 3-12, Fraction l; 3-13, Fraction m; 3-14, Fraction n; 3-15, Fraction o. The arrows show the elution positions of standard PA-oligosaccharides: 1, PA-M1′; 2, PA-maltose; 3, PA-M1; 4, PA-M2B′; 5, PA-maltotriose; 6, PA-M2A′; 7, PA-maltotetraose; 8, PA-M2B′; 9, PA-M3A′; 10, PA-M3B′; 11, PA-cellotetraose; 12, PA-maltopentaose; 13, PA-M3A; 14, PA-M4B′; 15, PA-M4D′; 16, PA-M4B; 17, PA-M5B′; 18, PA-M5A′; 19, PA-M5A; 20, PA-G1M5B′; 21, PA-M6′; 22, PA-M7′; 23, PA-M8C′; 24, PA-M8A′; 25, PA-M9A′; and 26, PA-G1M9A′.

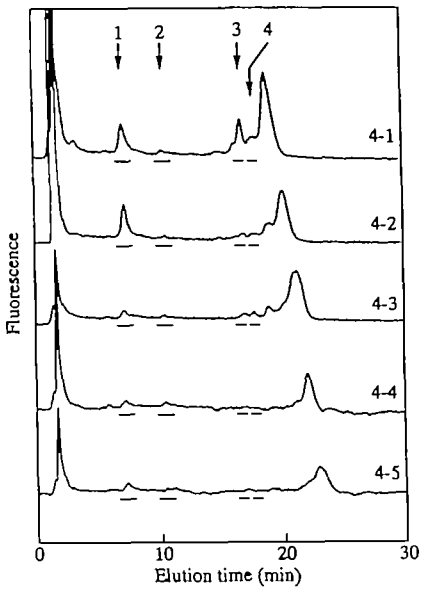

Fig. 4. **Size-fractionation HPLC of α-mannosidase digests of fractions obtained in Fig. 2.** The elution positions of standard PA-oligosaccharides are indicated by arrows: 1, PA-M1′; 2, PA-M1; 3, PA-G1M4E′; 4, PA-G1M4E. 4-1, HPLC of α-mannosidase digests of Fraction j; 4-2, Fraction k; 4-3, Fraction l; 4-4, Fraction m; and 4-5, Fraction n.

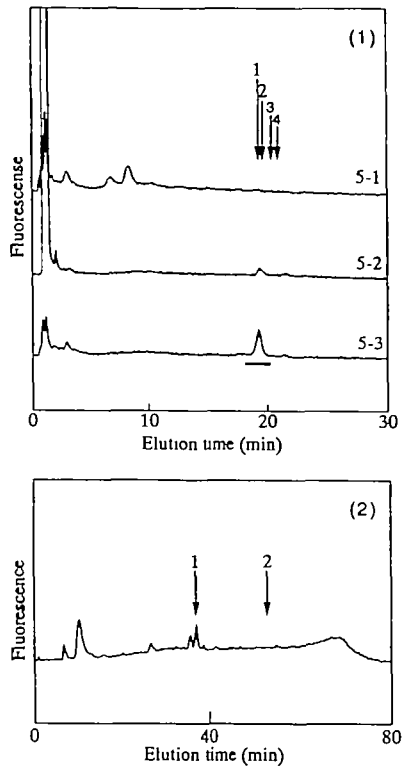

Fig. 5. **Analysis of sialooligosaccharides in acidic fractions.** The elution positions of standard PA-sugar chains are indicated by arrows: 1, BI′; 2, BI; 3, PA-triantennary sugar chain; 4, PA-tetra-antennary sugar chain. (1) Size-fractionation HPLC of the sialidase digests of Fraction B (5-1), Fraction C (5-2), and Fraction D (5-3). (2) Reversed-phase HPLC of the fraction collected in (1) as indicated by the bar.

monosialo fraction (Fraction B), a fraction between the mono- and disialo fractions (Fraction C), and disialo fraction (Fraction D), based on the elution positions of authentic PA-oligosaccharides (Fig. 1).

Analysis of Fraction A—Fraction A was separated by size-fractionation HPLC into 15 fractions (a–o) considering the elution positions of authentic PA-oligomannosides (Fig. 2). The PA-oligosaccharides in each fraction were further separated by reversed-phase HPLC (Fig. 3). Each peak was identified by comparing the elution positions with those of authentic PA-oligomannosides (*14, 19, 26*). Cellotetraose used as an internal standard was detected as PA-cellotetraose in Fraction f (Fig. 3-6, arrow 11). Fourteen GN1-type and four GN2-type oligomannosides were detected and are indicated by arrows in Fig. 3. PA-M1′ and PA-M1 were not detected at the positions where they might have appeared (Fig. 3-1 and 3-3). The large peak between fractions b and c did not contain PA-oligomannoside as analyzed by reversed-phase HPLC. The presence of glucosylated oligomannosides was further confirmed by α-mannosidase digestion followed by size-fractionation HPLC (Fig. 4). The peaks indicated by bars corresponding to the elution positions of the four standard sugar chains (PA-M1′, PA-M1, PA-G1M4E′, and PA-G1M4E) were collected, and the structure of the sugar chain in each fraction was analyzed by reversed-phase HPLC. PA-G1M4E′ was detected in the α-mannosidase digest of Fraction j together with M1′, but PA-M1 and PA-G1M4 were not (data not shown). PA-G1M4E′ might be derived from PA-G1M5B′, which was coeluted with PA-M6′ in Fraction j (Fig. 3-10). Detection of a small amount of PA-G1M4E′ in the α-mannosidase digests of Fractions k and l revealed the presence of small quantities of PA-G1M6′ and PA-G1M7′ respectively in these fractions. PA-G1M4E and PA-M1 were not detected in these fractions, showing that GN2-type oligomannosides such as PA-M6, PA-M8, and PA-M9A were not present. Besides oligomannosides, PA-maltose-PA-maltopentaose were detected as major components in Fractions b, d, e, and g (Fig. 3-2, 4, 5, and 7, respectively). Although there are unknown peaks on the reversed-phase HPLC, contamination of *O*-glycosidic sugar chain might be small since the amount of PA-GalNAc was a little as compared with those of PA-GlcNAc and PA-Glc judging from the results of the reducing end sugar analysis of total PA-sugar chains (data not shown).

The amounts of the oligosaccharides in the cytosol fraction were calculated based on the recovery of cellotetraose which was added to the cytosol fraction (Table I). The detection limit was about 5 pmol per gram of wet liver, which means that the contents of GN2-type oligomannosides such as M6-M8 and M9A were less than this limit.

Analysis of Complex-Type Oligosaccharides—The presence of asialo complex-type sugar chain in Fraction A was examined on size-fractionation HPLC of Fractions j, k, l, m, and o after sequential β-galactosidase and β-*N*-acetylhexosaminidase digestion. PA-agalacto-Bi, -tri, and -tetra-antennary sugar chains and PA-M3 were not detected in the enzyme digests (data not shown). The presence of complex-type oligosaccharides in Fractions B, C, and D was next examined. In the neutral oligosaccharides obtained by sialidase digestion of Fractions C and D, an asialobiantennary oligosaccharide (GN1-type) was detected by size-fractionation and reversed-phase HPLC (Fig. 5, 5-2 and 5-3).

TABLE I. **Abbreviation of oligosaccharides and the amount of oligosaccharides detected in the mouse cytosol fraction.**

Abbreviation	Structure	Content (pmol/g wet tissue)
M1'	Manβ1-4GlcNAc	N.D (a)*
M2A'	Manα1-3(Manβ1-4GlcNAc)	260 (d)
M2B'	Manα1-6(Manβ1-4GlcNAc)	170 (d)
M3A'	Manα1-6(Manα1-6)Manβ1-4GlcNAc	49 (f)
M3B'	Manα1-6(Manα1-3)Manβ1-4GlcNAc	110 (f)
M4A'	Manα1-6(Manα1-3)Manα1-6Manβ1-4GlcNAc	200 (h)
M4D'	Manα1-6(Manα1-2Manα1-3)Manβ1-4GlcNAc	370 (h)
M5A'	Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAc	67 (i)
M5B'	Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc	1370 (i)
M6'	(Manα1)$_5$-Manβ1-4GlcNAc	
M7'	(Manα1)$_6$-Manβ1-4GlcNAc	190 (j)
M8A'	Manα1-2Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc	71 (k)
M8C'	Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc	200 (k)
M9A'	Manα1-2Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc	150 (n)
G1M4E'	Glcα1-3(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc	N D
G1M5B'	Glcα1-3(Manα1-2Manα1-2Manα1-3)Manα1-6Manβ1-4GlcNAc	60 (j)
G1M6'	Glcα1-3(Manα1-2Manα1-2Manα1-3)(Manα1)-{6-3Manα1-6}Manβ1-4GlcNAc	9 (j)
G1M7'	Glcα1-3(Manα1-2Manα1-2Manα1-3)(Manα1)$_2$-{6-3Manα1-6}Manβ1-4GlcNAc	13 (i)
G1M8'	Glcα1-3(Manα1-2Manα1-2Manα1-3)(Manα1)$_3$-{6-3Manα1-6}Manβ1-4GlcNAc	N.D.(m)
G1M9A'	Glcα1-3(Manα1-2Manα1-2Manα1-3)Manα1-2Manα1-6(Manα1-2Manα1-3)Manβ1-4GlcNAc	150 (n)
M1	Manβ1-4GlcNAcβ1-4GlcNAc	N.D.(c)
M2B	Manα1-6(Manβ1-4GlcNAcβ1-4GlcNAc	310 (e)
M3A	Manα1-6(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc	96 (g)
M4A	Manα1-6(Manα1-3)Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc	71 (h)
M5A	Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc	58 (i)
M6	(Manα1)$_5$-Manβ1-4GlcNAcβ1-4GlcNAc	N.D. (j)
M7	(Manα1)$_6$-Manβ1-4GlcNAcβ1-4GlcNAc	N D (k)
M8	(Manα1)$_7$-Manβ1-4GlcNAcβ1-4GlcNAc	N.D. (l)
M9A	Manα1-2Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc	N.D. (n)
G1M4E	Glcα1-3(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc	N.D. (i)
G1M5B	Glcα1-3(Manα1-2Manα1-2Manα1-3)Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc	N D (j)
G1M6	Glcα1-3(Manα1-2Manα1-2Manα1-3)(Manα1)-{6-3Manα1-6}Manβ1-4GlcNAcβ1-4GlcNAc	N D. (k)
G1M7	Glcα1-3(Manα1-2Manα1-2Manα1-3)(Manα1)$_2$-{6-3Manα1-6}Manβ1-4GlcNAcβ1-4GlcNAc	N D. (l)
G1M8	Glcα1-3(Manα1-2Manα1-2Manα1-3)(Manα1)$_3$-{6-3Manα1-6}Manβ1-4GlcNAcβ1-4GlcNAc	N.D. (m)
G1M9A	Glcα1-3(Manα1-2Manα1-2Manα1-3)Manα1-2Manα1-6(Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc	N.D. (o)
BI'	Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAc	N D. (j)
SiaBI'	Siaα2-6 Galβ1-4GlcNAcβ1-2Manα1-6(Siaα2-6 Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAc	110
BI	Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc	N.D. (k)
	Maltose	4600 (b)
	Matotriose	7900 (d)
	Maltotetraose	310 (e)
	Maltopentaose	150 (f)

N.D., not detected. *Fractions on size-fractionation HPLC of Fig. 2.

DISCUSSION

Since about 10% of the lysosomal marker enzyme was observed in the cytosol fraction, it was presumably contaminated with around 10% of oligosaccharides present in lysosome. However, we have found that the amount of oligosaccharides in the microsomal fraction of hen oviduct is only one-twentieth of the amount in the cytosol fraction (unpublished data), from which it can be assumed that the total amount of lysosomal oligosaccharides was small in comparison to the amount of cytosolic oligosaccharides. We therefore consider that lysosomal oligosaccharides were probably not detected in the present analysis, and that the amounts of oligosaccharides determined (Table I) reflects the actual amount in the cytosol. Little oligosaccharide

degradation is thought to have occurred during sample preparation, because (i) perfused liver was used, (ii) cytosol was prepared at below 4°C, and (iii) cytosolic enzymes were heat-inactivated immediately after isolation of the cytosol fraction.

Sixteen kinds of GN1-type and four kinds of GN2-type oligomannosides, four maltooligosaccharides, and a GN1-type sialobiantennary oligosaccharide were detected in the cytosol fraction of perfused mouse liver. Since all the oligomannosides detected had partial structures of the precursor oligosaccharide, $Glc_3Man_9GlcNAc_2$, in glycoprotein biosynthesis, the formation processes of these oligomannosides seems to be related to the degradation pathway of glycoproteins. Glycoprotein biosynthesis studies using cultured cells labeled with [^{3}H]-Man or [^{14}C]-GlcNAc suggest the following routes for the production of free oligomannosides in cytosol. (I) Oligosaccharyltransferase, which transfers the oligosaccharide from $Glc_3Man_9GlcNAc_2$-p-p-dolichol to Asn residues of nacent polypeptides, has hydrolase activity (*1, 5*) and produces the free oligomannoside in the endoplasmic reticulum (ER). The oligomannoside is then processed by glycosidases in the ER, after which the products are translocated into the cytosol *via* a transporter dependent on ATP (*8*). (II) Pyrophosphatase in the ER membrane has the activity to produce oligomannoside-phosphate from $Man_nGlcNAc_2$-p-p-dolichol (*2-4, 9, 27*), and endo-*N*-acetylglucosaminidase can release the free oligomannoside from the oligomannoside-phosphate (*28*). (III) Enzymes having a latent ability to produce free oligosaccharides from endogeneous glycoproteins or related compounds are known to be present in the cytosol or ER. Peptide:*N*-glycanase in the ER of rat liver (*29*) and hen oviduct (*30*), cytosolic neutral chitobiase in MDBK cells (*6, 10*), and endo β-*N*-acetylglucosaminidase (*12, 31-33*) and α-mannosidases in cytosol and ER (*13-17*) are believed to participate in the formation of the oligomannosides.

Most of the oligomannosides detected seemed to be formed by the actions of the enzymes described above, but M5A, M4B, M3A, M2B, and their GN1-type oligomannosides cannot be formed without an α-1,2-mannosidase. α-1,2-Mannosidase activity is known to be distributed in the Golgi apparatus, rough ER, and lysosomes, but not in the cytosol (*13, 34, 35*); therefore, the formation routes of M5A and its related oligomannosides remain obscure. The presence of G1M5′-G1M7′ indicates that either a glucose-containing oligomannoside or a glycoprotein with G1M9 is transportable from the ER lumen to the cytosol (*8*), because the oligosaccharides transported were converted to G1M5′-G1M7′ by the cytosolic endo β-*N*-acetylglucosaminidase and α-mannosidase. The probability of this transport is supported by the observations that G1M9-G3M9 were liberated in the presence of glucosidase inhibitors in HepG2 (*7*), calf tyroid slices (*5*), and CHO cells (*9*) and that G1M9 was liberated in the cytosol of permeabilized MDBK cells (*10*).

The sialylbiantennary oligosaccharide was detected (Fig. 5) at 110 pmol/g tissue (Table I), but the formation route of this complex-type oligosaccharide is not known. Sialooligosaccharides in animal cells have been reported in fish eggs, in which BI, BI′, tri-, and tetra-antennary oligosaccharides were detected (*36*).

Maltooligosaccharides (dimer to pentamer) were detected in the cytosol fraction in relatively high quantities (Table I). These oligosaccharides might be derived from glycogen, although again the details of the formation route(s) are unknown.

In a previous study, we analyzed oligomannosides in the cytosol fraction of hen oviduct using a method similar to that employed in the present work (*18*). The analysis revealed the following features: (i) among the oligomannosides, the content of M5B′ was the highest, (ii) the ratio of GN1-type to GN2-type oligomannosides was about 2:1, and (iii) no oligomannosides larger than $Man_5GlcNAc$ were detected. The results of the present study reveal that the cytosol fraction of mouse liver also has a high M5B′ content, which at 1.4 nmol per gram of wet tissue (Table I) is nearly the same as that of HepG2 cells (unpublished data). Furthermore, M5B′ was the main free oligosaccharide observed after 2 h of metabolic labeling of CHO cells (*9*). Accordingly, we speculate that animal cells may usually contain M5B′ as a major oligomannoside in the cytosol. The ratio of GN1-type oligomannosides to the GN2-type was 7:1 in mouse liver, 8:1 in HepG2 cells (unpublished data), and 2:1 in hen oviduct (*18*). A higher content of GN1-type oligomannosides than of the GN2-type might also thus be a common feature of animal cells. A broad distribution of oligomannosides from M2′ to M9A′ was observed in mouse liver, and oligomannosides smaller than M5B′ were also detected in hen oviduct, although Saint-Pol *et al.* reported that oligosaccharides smaller than M5B′ are not present in the cytosol of permeabilized HepG2 cells because these oligomannosides are transferred to a membrane-bound compartment, probably the lysosome (*11*).

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