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Novel Tri- and Tetrasialosylpoly-*N*-acetyllactosaminyl Gangliosides of Human Placenta: Structure Determination of Pentadeca- and Eicosaglycosylceramides by Methylation Analysis, Fast Atom Bombardment Mass Spectrometry, and ^{1}H NMR Spectroscopy†

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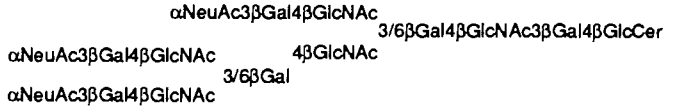
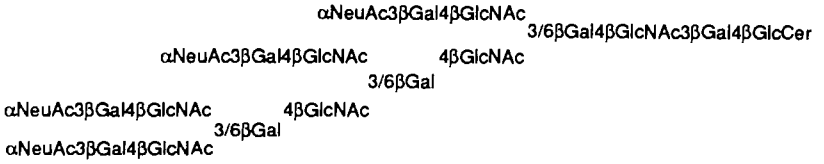
ABSTRACT: A series of highly polar neolacto series (poly-*N*-acetyllactosaminyl) gangliosides were isolated from human placenta tissue and purified by HPLC and preparative HPTLC. Two of these ganglioside fractions (G-12 and G-13) were analyzed by 500-MHz ^{1}H NMR spectroscopy, GC–EIMS, $^+$FAB-MS, and sequential exoglycosidase treatments. Their structures have been identified as being of the repeating *N*-acetyllactosamine type, multiply branched through GlcNAcβ1→6/3 linkages, with every nonreducing Gal terminal α2→3-sialosylated, as shown below. These are among the highest molecular weight glycosphingolipids whose detailed oligosaccharide structures are presently known.





The presence of glycoconjugates having repeating *N*-acetyllactosamine units in certain types of cells and tissues has been known since they were initially found in highly polar glycolipids (Hakomori et al., 1972), as well as in long-chain glycosaminoglycans bound to transmembrane proteins of erythrocytes (Järnefelt et al., 1978; Fukuda, M., et al., 1979). Such glycoconjugates were originally identified as carriers of blood group ABH determinants and were found to be present in either branched or unbranched form (Hakomori et al., 1972, 1977). The unbranched species are present predominantly in fetal erythrocytes, while the branched forms are found mainly in adult erythrocytes (Watanabe & Hakomori, 1976). Subsequently, the unbranched form was identified as the classically known i antigen (Niemann et al., 1978) and the branched form as I antigen (Watanabe et al., 1979b). Thus, the ontogenic development of erythrocytes and erythroid cells is associated

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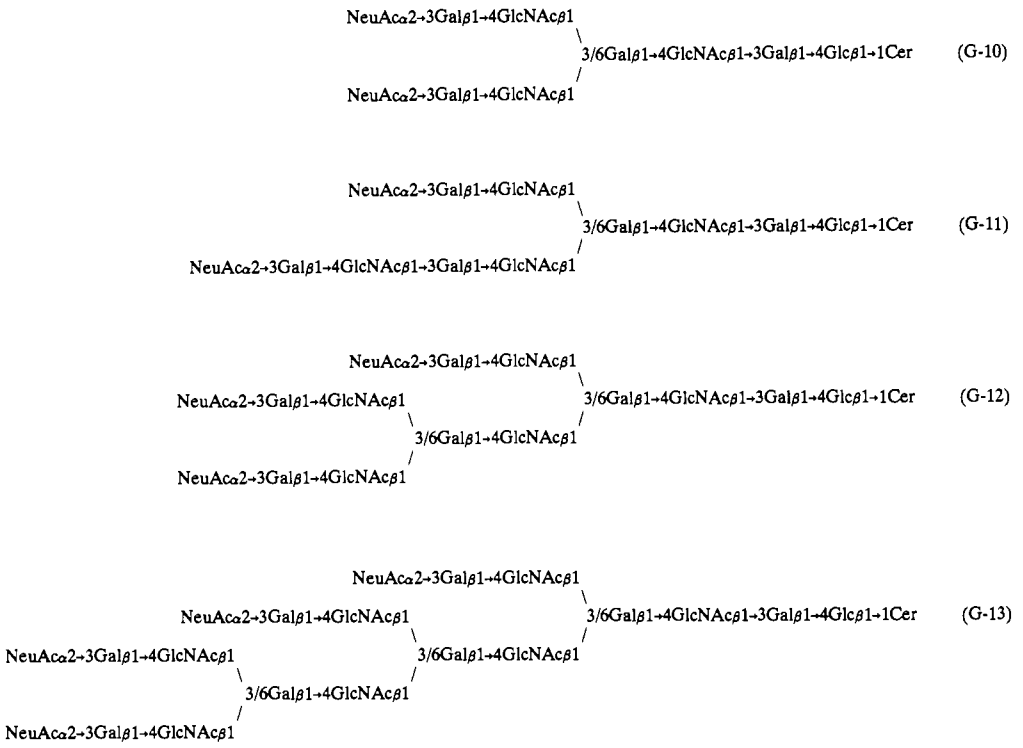
Chart I



with the transition from unbranched to branched forms of repeating *N*-acetyllactosamine chains, for which the term "polylactosaminoglycan" or "polylactosamine" has been proposed (Fukuda, M., et al., 1980). Polylactosamine glycans were also found as the major glycoconjugate in placenta, and some of them are associated with the collagen-binding domain of fibronectin (Zhu & Laine, 1985). Since polylactosamine is present in both glycoproteins and glycolipids (Fukuda, M. N., et al., 1979), it was thought that placenta might also contain high molecular weight polylactosamine glycolipid. A recent study by Taki et al. (1988) indicated the presence of both unbranched and branched forms of polylactosamine structure having *N*-acetylneuraminic acid linked to the terminal Gal residues. So far, four types of ganglioside in placenta have been characterized. These are II^{3}NeuAcLacCer (GM_3), IV^{3}NeuAcnLc$_4$, VI^{3}NeuAcnLc$_6$, and VI^{3}NeuAcIV^6[NeuAc2→3Galβ1→4GlcNAc]nLc$_6$ (G-10 ganglioside)[1] (Taki et al., 1988). The latter two compounds are known to possess i and I blood group activities, respectively (Niemann et al., 1978; Okada et al., 1984; Hakomori, 1981). In view of the long-standing interest, in this laboratory, in elucidating the relationship of Ii antigenic expression to mammalian development, differentiation, cellular processes, and carcinogenesis, we have undertaken the isolation and characterization of more complex gangliosides from human placenta. Recently, we elucidated the structure of a novel dodecaosylceramide from placenta, which is an extension of G-10 possessing both i and I epitopes (G-11; see Chart I) (Nudelman et al., 1989). In this paper, we describe the isolation and characterization of two more novel gangliosides having complex, GlcNAcβ1→6-branched, repeating poly-*N*-acetyllactosamine core structures.

Experimental Procedures

Thin-Layer Chromatography and Immunostaining. Thin-layer chromatography (TLC)[2] was performed on HPTLC plates. Silica gel 60 (E. Merck, Darmstadt, West Germany) was used for analytical and preparative-scale TLC. HP-KF (Whatman, Maidstone, U.K.) was used for immunostaining. The solvent systems used contained the following: solvent system A, CMW (30:60:8 v/v/v); solvent system B, CMW (50:40:10) (0.05% $CaCl_2$); solvent system C, CMW (50:47:14) (0.05% $CaCl_2$); solvent system D, CMW (50:55:19) (0.05% $CaCl_2$). Analytical plates were visualized by spraying with 0.5% orcinol (Sigma) in 10% H_2SO_4 and baking for 3 min at 250 °C. Preparative plates were visualized with primulin (Sigma, 0.01% in acetone–H_2O, 8:2 v/v).

TLC immunostaining was performed with a modified version (Kannagi et al., 1982a,b) of the method originally described by Magnani et al. (1980). MoAbs used were 1B2 (Young et al., 1981), C6 (Fenderson et al., 1988), and NUH2 (Nudelman et al., 1989).

Homogenization and Extraction of Tissue. Seven freshly collected placentas were washed and sliced into 50-g pieces. A total of 3.5 kg wet weight of placenta tissue was homogenized in a Waring blender in 10 volumes of IHW (55:25:20 v/v/v, upper phase discarded), and the suspension was filtered on a large Büchner funnel with Whatman No. 3 filter paper. The residue was rehomogenized in IHW as above and filtered, and both the first and second filtrates were pooled and evap-

[1] This fraction has been alternatively designated DG-6, when first characterized from human erythrocytes (Kundu et al., 1983); G-10 (Okada et al., 1984), on the basis of its slower TLC migration compared with that of other branched erythrocyte ganglioside components, which were called G-8 and G-9 (Watanabe et al., 1978, 1979a; Kannagi et al., 1983); and G-4, when characterized from human placenta (Taki et al., 1988). We tentatively refer to this component as G-10, to be consistent with our own previous work, without intending to dispute the priority of other workers in establishing its structure.

[2] Abbreviations: CM, chloroform–methanol; CMW, chloroform–methanol–water; $^+$FAB-MS, positive ion fast atom bombardment mass spectrometery; GC–EIMS, gas chromatography–electron-impact mass spectrometry; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; IHW, 2-propanol–hexane–water; MoAb, monoclonal antibody; NBA, 3-nitrobenzyl alcohol; ^{1}H NMR, proton nuclear magnetic resonance.

orated to dryness under reduced pressure in a rotary evaporator. The dried organic extract was taken up in 9 L of CM (2:1) and divided into three 3-L portions.

Folch Partition. Polar lipids were separated from the glycolipid fraction in CM (2:1) as above by a modification of Folch partition (Folch-Pi et al., 1951), as follows: 500 mL of deionized water was added to each 3-L portion and the solution mixed by being inverted 20 times. The phases were allowed to separate overnight and the upper phases removed by suction filtration. The exact volume removed was replaced in each portion with ideal upper phase [CMW, 1:10:10 (0.1% KCl)] and inverted as before. After separation, the second upper phase was drawn off, combined with the first, and evaporated to dryness under reduced pressure with addition of absolute ethanol to facilitate evaporation. The dried combined upper phases were transferred to a Spectra/Por No. 3 dialysis bag (MW cutoff 3500) in a 200-mL final volume of H_2O, and dialyzed exhaustively against distilled water.

The resultant dialyzed upper phase was again reduced to dryness in a rotary evaporator with addition of absolute ethanol and 2-propanol to prevent bumping at high concentration of ganglioside. The upper-phase lipids were solubilized in 500 mL of solvent A.

DEAE-Sephadex Chromatography. DEAE-Sephadex chromatography was performed according to the method of Ledeen et al. (1973). DEAE-Sephadex A-25 (400 mL) was activated overnight in 3 L of CM–0.8 M sodium acetate (30:60:8). Excess sodium acetate was removed by washing Sephadex extensively with solvent A in a Büchner funnel. The upper-phase lipids in solvent A were applied to a column (6 cm × 30 cm, 300-mL bed volume) of activated Sephadex and eluted as follows: solvent A (1500 mL) to remove neutral lipids; methanol wash (1000 mL); then a stepwise gradient of ammonium acetate in methanol with concentrations of 0.04 M (1500 mL), 0.15 M (1500 mL), and 0.45 M (1000 mL). The last three fractions were evaporated to dryness, taken up in a small volume of distilled water, and dialyzed exhaustively against distilled water. The fraction which eluted at 0.15 M was further purified by HPLC and HPTLC as described below.

HPLC I: First-Step Purification of "0.15 M Fraction". Ninety percent of the total fraction was evaporated to dryness several times with addition of ethanol and then solubilized in 8 mL of CMW (2:1:0.1), with warming and sonication. The gangliosides were applied to an HPLC column (1 cm × 50 cm) of 10-μm Iatrobeads (6RS-8010, Iatron Chemical Co., Tokyo) previously equilibrated in the following gradient: IHW (55:25:20 to 55:40:5) over 60 min at 2.0 mL/min. The column was washed an additional 20 min at 55:40:5. The gangliosides were applied to the column in a 2-mL sample loop, with four separate injections at 10-min intervals. A gradient of IHW was applied from 55:40:5 to 55:25:20 over the first 350 min, followed by isocratic elution at 55:25:20 from 350 to 400 min. The flow rate was 1.0 mL/min with fractions collected over 200 tubes (2 mL/fraction). Fractions were checked for ganglioside content by HPTLC (solvent system B) and pooled on the basis of similar migrations into three groups: 65–84, which contained very pure GM_3 [NeuAcα(2→3)Galβ(1→4)Glcβ(1→1)Cer]; 85–100, which contained $IV^3NeuAcnLc_4Cer$, $VI^3NeuAcnLc_6Cer$, and $VI^3,VIII^3NeuAc_2$*iso*-nLc_8Cer (G-10); 101–200, which contained G-10 along with the polar, slower migrating gangliosides (Figures 1 and 2).

HPLC II: Second-Step Purification of "Fraction 101–200 Polar Gangliosides". Fractions 101–200, pooled from HPLC I, were applied in CMW (2:1:0.1) to a column (0.5 × 60 cm) of 10-μm Iatrobeads (equilibrated and washed as above), and a gradient of IHW was applied as follows: 55:40:5 to 55:33:12 over 90 min; 55:33:12 isocratic from 90 to 170 min; 55:33:12 to 55:25:20 from 170 to 280 min; 55:25:20 isocratic from 280 to 330 min. The flow rate was 0.5 mL/min, and 165 fractions of 1.0 mL each were collected. Fractions were analyzed by HPTLC (solvent system C), and bands of similar migration were pooled as follows: 53–67, which contained very pure G-10; 68–71, which contained impure G-10; 72–77; 90–102; 121–126 (Figure 2). The minor fraction pooled as 72–77, due to its somewhat different antigenicity, was analyzed separately, and its structure elucidation will be reported elsewhere (Nudelman et al., 1989).

Preparative HPTLC. Fractions 90–102 and 121–126 were further purified to homogeneity by preparative HPTLC as follows. Each fraction, after evaporation to dryness under N_2 stream, was taken up in 600 μL of IHW (55:25:20) and streaked lengthwise onto silica gel HPTLC plates (EM Science) (10 × 20 cm). These were chromatographed with solvent system D. Aftery drying, the glycolipid bands were detected by spraying with 0.01% primulin and viewing with a hand-held UV light. Clearly resolved major bands were carefully marked with a soft pencil, and the bands were scraped and transferred into screw-cap test tubes (16 × 150 mm). The silica was then sonicated in 10 mL of IHW (55:25:20) for 30 min, the tubes were centrifuged at 3000 rpm on a Beckman Acuspin for 10 min, and the supernatant was drawn off and evaporated to dryness under N_2 stream.

The primulin was removed by HPLC on a 0.5 × 30 cm 10-μm Iatrobead column using a short "clean-up" program consisting of a linear gradient of IHW (55:40:5 to 55:25:20) at 1 mL/min over 60 min. The primulin eluted in the first few tubes, while gangliosides were retained on the column until two-thirds of the way through the program. The major purified bands of 90–102 and 121–126 were subjected to sequential exoglycosidase cleavages, NMR, permethylation analysis, and $^+$FAB-MS as described below.

^1H NMR Spectroscopy. Samples were prepared for ^1H NMR analysis by lyophilization from D_2O (two times) to remove all exchangeable protons. They were then dissolved in 0.4 mL of dimethyl sulfoxide-d_6 containing 2% D_2O (Dabrowski et al., 1980), and spectra were recorded at 308 ± 2 K on a Bruker AM-500 spectrometer equipped with an Aspect 3000 computer and pulse programmer. Further conditions and data treatment have been previously described (Levery et al., 1988).

Methylation Analysis. A portion (≈200 μg) of each glycosphingolipid sample was permethylated (Hakomori, 1964), and 20% of this material was hydrolyzed in 0.5 N H_2SO_4/90% acetic acid, reduced with $NaBH_4$, and acetylated according to published procedures (Levery & Hakomori, 1987).

Partially O-methylated, N-methylated deoxyhexitol, hexitol, and hexosaminitol acetates were analyzed by GC–EIMS on a Hewlett-Packard 5890A gas chromatograph interfaced to a 5970B mass selective detector. Separation was on a 30M DB-5 (J&W Scientific, Rancho Cordova, CA) bonded-phase fused silica capillary column (temperature program, 140–250 °C at 4 °C/min) using splitless injection. Electron-impact mass spectra were acquired from 50 to 500 atomic mass units at 0.95 s/scan. Derivatives were identified by characteristic fragmentation patterns and retention times (Björndal et al., 1970; Jansson et al., 1976; Stellner et al., 1973b; Stoffel & Hanfland, 1973; Tai et al., 1975), verified by coinjection with standards when necessary.

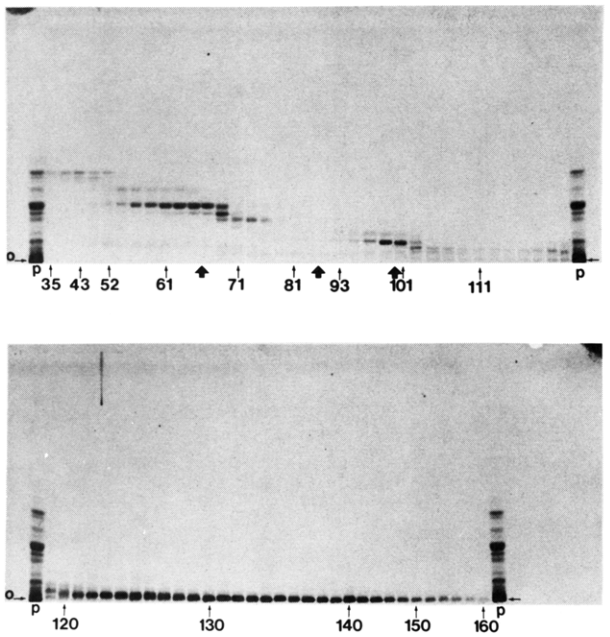


FIGURE 1: HPTLC pattern of placenta ganglioside fractions generated by first-step HPLC. Developed with solvent system A. Lane p, placenta total ganglioside fraction. Arrows marked by o indicate origin; boldface arrows indicate cutoff limits for pooling of fractions (see text, Figure 2).

Positive Ion Fast Atom Bombardment Mass Spectrometry. The remainder of the permethylated glycolipids were analyzed by $^{+}$FAB-MS on a JEOL HX-110 mass spectrometer/DA-5000 data system. A total of 20–30 μg of permethylated ganglioside in thioglycerol–glycerol–15-crown-5 (Isobe et al., 1987) or NBA (Meili & Seibl, 1984; Barber et al., 1988) matrix was bombarded with a xenon beam source. Acceleration voltage was 10 kV, and resolution was 1000 or 3000. Cesium iodide/potassium iodide was used as the mass calibration standard. Further conditions are described in the legends to Figures 4 and 5.

Enzyme Hydrolysis. Desialosylation was effected with sialidase from *Clostridium perfringens* (Sigma); gangliosides were mixed with a CM solution of sodium deoxytaurocholate (Sigma) in 1:5 (w/w) ratio, evaporated to dryness under nitrogen stream, dissolved in 100 mM sodium acetate buffer (pH 4.5), mixed with an equal volume of sialidase (1 unit/mL activity), and incubated at 37 °C for 18 h. The incubation mixture was passed through a column of C18 alkylated porous silica gel (Bond-Elut, Analytichem International, Harbor City, CA) and washed with 5 volumes of H_2O, and the glycosphingolipids were recovered by elution with 5 volumes of CM (2:1 v/v).

Further degradations were performed with jackbean β-galactosidase (Sigma) in sodium citrate buffer (200 mM, pH 4.5) and jackbean β-*N*-acetylhexosaminidase (Sigma) in the same buffer. Cleanup procedures were the same as those with sialidase. Samples to be analyzed by methylation analysis had deoxytaurocholate removed by passage of the lipid mixture through a short column of DEAE-Sephadex (Ac^{-} form) in solvent A.

Results

Isolation and Purification of Placenta Ganglioside Fractions. Through sequential application of preparative HPLC and HPTLC techniques, a number of components more polar than G-10 were isolated from the ganglioside fraction of human placenta (see Figures 1 and 2). In addition to G-10 (fractions 53–67), more polar components, including fractions

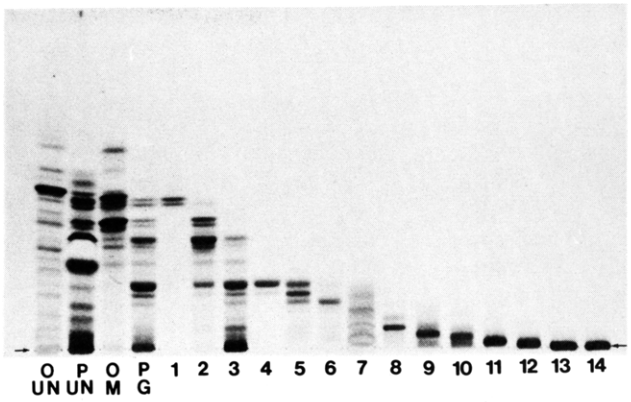


FIGURE 2: HPTLC pattern of placenta ganglioside fractions pooled from first-step HPLC and semipurified by second-step HPLC of pooled fractions 101–200. Developed with solvent system B. Lane O/UN, O erythrocyte upper neutral glycosphingolipid standard; lane P/UN, placenta upper neutral glycosphingolipids; lane O/M, O erythrocyte monosialoganglioside standard; lane P/G, placenta total gangliosides. From pooling of first-step HPLC: lane 1, fractions 65–84; lane 2, fractions 85–100; lane 3, fractions 101–200. Pooled from second-step HPLC of fractions 101–200: lanes 4–14, fractions 53–67 (G-10), 68–71, 72–77 (G-11), 78–89, 90–102 (G-12), 103–117, 118–120, 121–126 (G-13), 127–130, 131–140, and 141–160, respectively. Arrows mark position of origin.

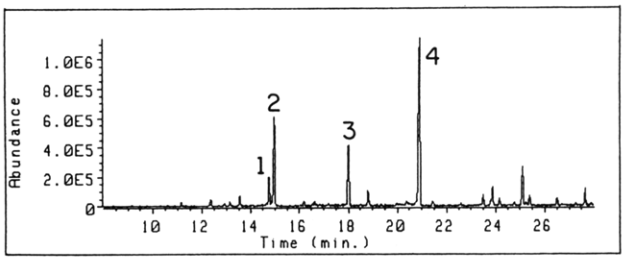


FIGURE 3: Electron-impact limited mass chromatogram of monodeuterated partially O-methylated hexitol and hexosaminitol acetates obtained from the hydrolysis of permethylated placenta ganglioside G-12 (fractions 90–102). Separation was on a 30M DB-5 bonded-phase fused silica capillary column (see Experimental Procedures for conditions). The GC–EIMS is plotted as a composite of relevant structural ions: ordinate, summation of selected ion intensities; abscissa, retention time in minutes. Peaks identified were as follows: 1, 2,3,6-tri-*O*-Me-Glc; 2, 2,4,6-tri-*O*-Me-Gal; 3, 2,4-di-*O*-Me-Gal; 4, 3,6-di-*O*-Me-GlcNAcMe.

90–102 (called G-12) and 121–126 (called G-13), were found to be reactive with MoAb NUH2, which appears to recognize a binary α2→3 sialosyl type 2 chain, i.e., a branching structure with two terminal neuraminic acid residues, each linked to Galβ1→4GlcNAcβ1→6 and -β1→3 chains, which are part of the structure of G-10 but not of G-11 (Nudelman et al., 1989). The purified gangliosides, G-12 and G-13, each gave a single band on HPTLC in various polar solvent systems and were characterized by a positive immunostaining with MoAb NUH2 (see Enzymatic Degradations; lane 2, Figures 6B and 7B). These fractions were therefore subjected to detailed structural analysis as described below.

Methylation Analysis. In order to analyze the positions of glycosidic linkages in the gangliosides, an aliquot of each fraction was permethylated, and a portion thereof was hydrolyzed, reduced, and acetylated to produce partially methylated alditol acetates (PMAAs). GC–EIMS of these PMAAs (Figure 3) indicated in each case the following pattern: 2,3,6-tri-*O*-Me-Glc (→4Glc); 2,4,6-tri-*O*-Me-Gal (→3Gal); 2,4-di-*O*-Me-Gal [→3(→6)Gal]; and 3,6-di-*O*-Me-GlcNAcMe (→4GlcNAc). This simple pattern is consistent with repeating Galβ1→4GlcNAcβ1→poly-*N*-acetyllactosamine structures, branched GlcNAcβ1→3 and GlcNAcβ1→6 to Gal and ter-

Table I: Molar Proportions of Partially Methylated Alditol Acetates Produced by Placenta Ganglioside Fractions

fraction	2,3,6-Glc[a]	2,4,6-Gal	2,4-Gal	3,6-GlcNAcMe
eryth (G-10)[b]	1.00 (1)	3.00 (3)	1.00 (1)	3.00 (3)
72–77 (G-11)[c]	1.00 (1)	4.28 (4)	1.18 (1)	3.88 (4)
90–102 (G-12)	1.00 (1)	4.21 (4)	2.01 (2)	4.62 (5)
121–126 (G-13)	1.00 (1)	4.62 (5)	2.51 (3)	7.33 (7)

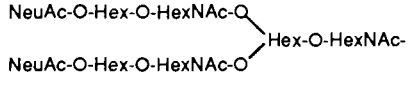
[a] All results normalized to 2,3,6-Glc = 1.00. [b] Results from erythrocyte G-10 taken as standard values given, and all other values calculated with response factors derived from each derivative from this ganglioside. [c] Nudelman, Mandel, Levery, and Hakomori, unpublished data.

minated at the nonreducing end exclusively with NeuAcα2→3Gal groups. In view of the previous findings of Taki et al. (1988) and Nudelman et al. (1989), this was adopted as a working hypothesis.

The relative molar proportions obtained for each fraction are listed in Table I. The previous result for fractions 72–77 (G-11) shows that the structure of that ganglioside differs from that of G-10 by the additional presence of one →3Gal and one →4GlcNAc, which suggested the insertion of a single →3Galβ1→4GlcNAcβ1→3 unit somewhere in the sequence for G-10 (Nudelman et al., 1989). The result for fractions 90–102 (G-12) differs from that for G-11 by the addition of one →4GlcNAc and one →3(→6)Gal, consistent with the addition of a branching NeuAcα2→3Galβ1→4GlcNAcβ1→6 unit to G-11. The result for fractions 121–126 (G-13) differs further by one additional →3Gal, one additional →3(→6)Gal, and two additional →4GlcNAc. This suggests the elaboration of G-12 by both insertion of →3Galβ1→4GlcNAcβ1→3 and addition of another branching unit. In the subsequent analysis using mass spectrometry, these results were confirmed, and in addition, the precise arrangements of the branch points could be determined.

Positive Ion Fast Atom Bombardment Mass Spectrometry. The remainder of each permethylated fraction was subjected to sequence analysis using $^+$FAB-MS. The spectra produced in this way provided clear information on branching patterns, which could be deduced from the saccharide fragment ions, and on the overall sugar and ceramide compositions, from the pseudomolecular ions and ceramide fragments.

In the mass spectrum of permethylated G-12 (Figure 4), the predominant pseudomolecular ions (nominal masses 4400 and 4428 amu) are consistent with the sugar composition $NeuAc_3{\cdot}Hex_7{\cdot}HexNAc_5$ plus ceramides consisting of d18:1 sphingosine in combination with 22:0 and 24:0 fatty acids. The corresponding ceramide ions are found at m/z 632 and 660, respectively. The remainder of the spectrum clearly displays fragments produced by cleavage preferentially at *N*-acetylneuraminosyl and *N*-acetylhexosaminyl linkages, with charge retention on the nonreducing portions (Egge & Peter-Katalinic, 1987; Fukuda, M. N., et al., 1985, 1986a,b; Hanfland et al., 1984). The abundant fragments at m/z 376 and 344 (NeuAc), 580 (NeuAc·Hex), and 825 and 793 (NeuAc·Hex·HexNAc) demonstrate the presence of the linear sequence NeuAc-O-Hex-O-HexNAc- in the glycosphingolipid. A fragment at m/z 2084 ($NeuAc_2{\cdot}Hex_3{\cdot}HexNAc_3$) is abundantly produced, representing the structure



There is no fragment at m/z 1274 ($NeuAc{\cdot}Hex_2{\cdot}HexNAc_2$), indicating the absence of the linear sequence NeuAc-O-Hex-O-HexNAc-O-Hex-O-HexNAc. After this, the next

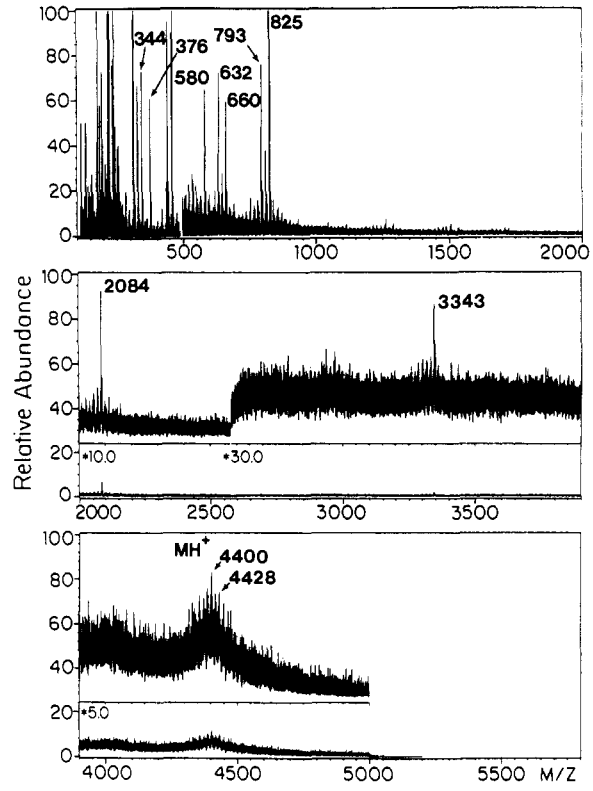
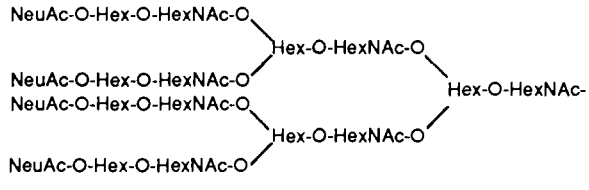


FIGURE 4: $^+$FAB-MS spectrum of permethylated placenta ganglioside G-12. For simplicity, nominal, monoisotopic masses are indicated; actual masses measured were larger, due to accumulation of proton mass defect. Resolution, 3000. Figure is a composite of two separate determinations, one with scan range 100–4400 amu and scan slope 4′30″ and the other with scan range 3000–5200 amu and scan slope 10′. Glycerol/thioglycerol matrix was doped with the cyclic polyether 15-crown-5 to increase yield of pseudomolecular ions, while decreasing chemical noise and eliminating contribution of sodium adducts in pseudomolecular ion region.

fragment clearly produced is at m/z 3343 ($NeuAc_3{\cdot}Hex_5{\cdot}HexNAc_5$), which must represent a structure with two branch points as shown in Figure 6A. This is consistent with the detection of two branching →3(→6)Gal units in the methylation analysis.

The mass spectrum of permethylated G-13 (Figure 5) exhibits predominant pseudomolecular ions (nominal masses 5659 and 5687 amu) which are consistent with the sugar composition $NeuAc_4{\cdot}Hex_9{\cdot}HexNAc_7$ plus the same ceramides as found for G-12. In this spectrum, the fragments at m/z 376, 344, 825, 793, 2084, and 3343 are again abundantly produced. The next fragment which can be detected is at m/z 4602 ($NeuAc_4{\cdot}Hex_7{\cdot}HexNAc_7$), which must be produced by a structure having three sequential branch points, as shown in Figure 6B. This is again consistent with the results of methylation analysis. The possibility of a significant contribution from the alternative structure, possessing a "symmetrical" arrangement of the branches, is excluded by the abundant m/z 3343 fragment, which could not be produced by this structure:



Enzymatic Degradations. The sequence and anomeric configuration of sugar units in the oligosaccharide branches

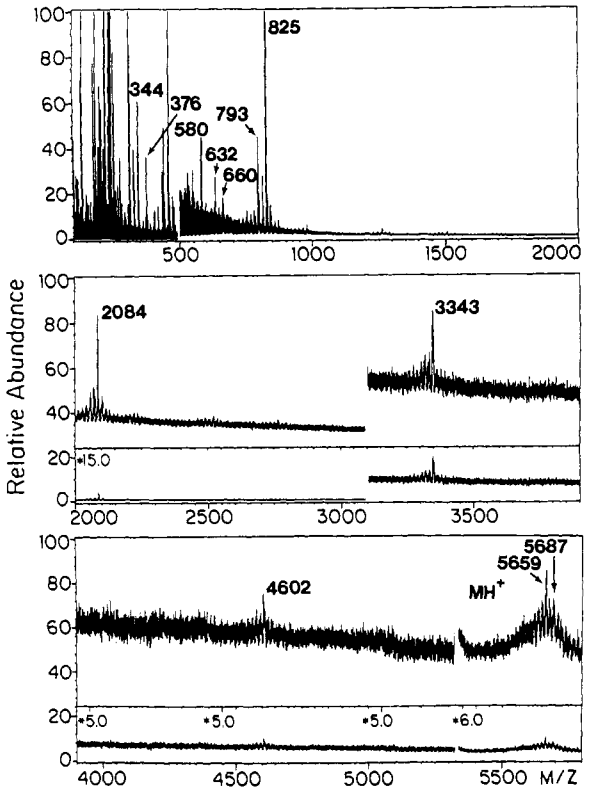


FIGURE 5: $^{+}$FAB-MS spectrum of permethylated placenta ganglioside G-13. Resolution, 1000. Figure is a composite of four separate determinations: scan range 100–3000 amu, scan slope 4′, matrix glycerol/thioglycerol/15-crown-5; scan range 100–3600 amu, scan slope 4′30″, matrix NBA; scan range 3000–5400 amu, scan slope 7′, matrix glycerol/thioglycerol/15-crown-5; scan range 4000–6200 amu, scan slope 7′45″, matrix glycerol/thioglycerol/15-crown-5.

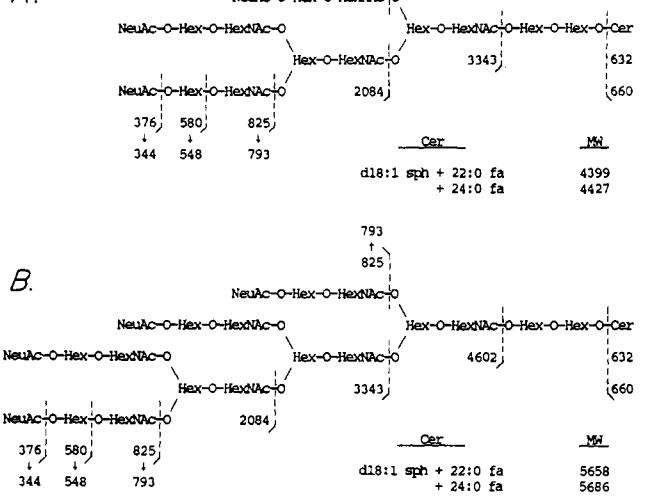


FIGURE 6: Proposed fragmentation scheme for $^{+}$FAB-MS of permethylated gangliosides G-12 (A) and G-13 (B).

were studied by sequential reaction with specific exoglycosidases. Changes in mobility and structure after removal of sugar units was followed by HPTLC with both orcinol and immunostaining (Figures 7 and 8). Both glycosphingolipids were found to be susceptible to sequential treatment with *C. perfringens* sialidase, jackbean β-galactosidase, and β-*N*-acetylglucosaminidase from bovine epididymis. HPTLC immunostaining clearly showed the reactivity of the intact gangliosides G-12 and G-13 with MoAb NUH2 (lane 2 in Figures 7B and 8B). Following treatment with sialidase, a mobility change in G-12 corresponding to loss of three neuraminic acid residues (Figure 7A, lane 3) was accompanied by loss of NUH2 activity (Figure 7B, lane 3), with a concomitant uncovering of binding activity with the antibody C6 (Figure 7C, lane 3), which recognizes only the terminally unsubstituted branching I epitope (Fenderson et al., 1988), and with the antibody 1B2 (Figure 7D, lane 3), which recognizes more generally the terminally unsubstituted Galβ1→4GlcNAcβ1→R sequence (Young et al., 1981). Treatment with β-galactosidase caused a further mobility change corresponding to three sugar residues (Figure 7A, lane 4), with a loss of C6 and 1B2 reactivity (Figure 7C,D, lane 4). Treatment of this product with β-*N*-acetylhexosaminidase caused a loss of three more sugar residues (Figure 7A, lane 5), yielding a product with mobility similar to that of nLc_6Cer. The product reacted strongly with 1B2 (Figure 7D, lane 5) and weakly with C6 (Figure 7C, lane 5). The reason for this latter result must be the cross-reactivity of C6 with the monoantennary I sequence Galβ1→4GlcNAcβ1→6Galβ1→R, the presence of which can be explained by heterogeneity of branching structure at β-Gal IV (see following section). Together with the methylation data, this confirms the sequence NeuAcα2→3Galβ1→4GlcNAcβ1→ on three nonreducing branches of the G-12 ganglioside. Further sequential treatment of this product with β-galactosidase and β-*N*-acetylhexosaminidase yielded a product migrating as nLc_4 (Figure 7A, lane 7) and reacting strongly with 1B2 (Figure 7D, lane 7), confirming the interior sugar sequence and linkages as well.

Similar treatment of G-13, followed with antibodies NUH2 and 1B2, gave analogous, though somewhat more complex, results. Clearly, the major 1B2-active product, following one cycle of sialidase, β-galactosidase, and β-*N*-acetylhexosaminidase treatments, comigrated with nLc_8 (Figure 8A,D, lane 5). The residual NUH2 activity exhibited following sialidase treatment (Figure 8B, lane 3) can be explained by components having incomplete removal of NeuAcα2→3 termini. This is supported by detection of a rather diffuse band of orcinol-positive and 1B2-positive glycosphingolipid in Figure 8A,C (lane 3), while the NUH2-reactive area is confined to a sharper band within it.

Determination of Branch-Point Asymmetry. Because more than one possible isomeric branching arrangement exists for each compound, a complete structure determination would require that these arrangements also be elucidated unambiguously. For example, enzymatic degradation of G-12 to nLc_6Cer, as described in the previous section, could lead to two possible products, depending on whether the GlcNAcβ1→6 or the GlcNAcβ1→3 branch is further elaborated. Similar possibilities exist for G-13 being degraded to nLc_8Cer. In order to determine which arrangement exists in the placenta gangliosides, small aliquots (50–100 μg) of the hexaglycosylceramides resulting from enzymatic degradation of G-12 were subjected to permethylation, hydrolysis, reduction, acetylation, and GC–EIMS, as described for the intact gangliosides. The somewhat unexpected result was the detection of 2,3,4-tri-*O*-Me-Gal (→6Gal) as well as 2,4,6-tri-*O*-Me-Gal (→3Gal) among the final products. This implies that the ganglioside fraction is a mixture, wherein one component has the extra I unit on the GlcNAcβ1→6 branch and the other is extended on the GlcNAcβ1→3 branch. Integration of the two tri-*O*-Me-Gal peaks yielded the following approximate percentages for Gal IV, allowing for one full →3Gal unit at Gal II: 65/35 →3Gal/→6Gal. A similar analysis of the octaglycosylceramides from enzymatic degradation of G-13 yielded the following approximate combined

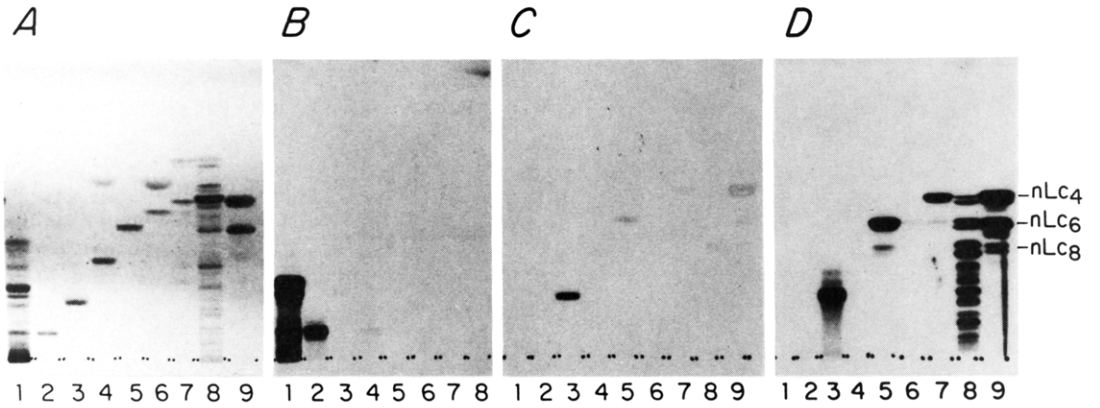


FIGURE 7: HPTLC of the products of sequential exoglycosidase hydrolysis of fractions 90–102 with detection by (A) orcinol staining and by immunostaining with MoAbs: (B) NUH2, (C) C6, and (D) 1B2. Lanes: 1, placenta total gangliosides; 2, fractions 90–102; 3, fractions 90–102 + sialidase; 4, fractions 90–102 + sialidase, β-galactosidase; 5, fractions 90–102 + sialidase, β-galactosidase, β-*N*-acetylhexosaminidase; 6, fractions 90–102 + sialidase, β-galactosidase, β-*N*-acetylhexosaminidase, β-galactosidase; 7, fractions 90–102 + sialidase, β-galactosidase, β-*N*-acetylhexosaminidase, β-galactosidase, β-*N*-acetylhexosaminidase; 8, O upper neutral standard; 9, standard nLc_4Cer, nLc_6Cer, and nLc_8Cer. Developed with solvent system C.

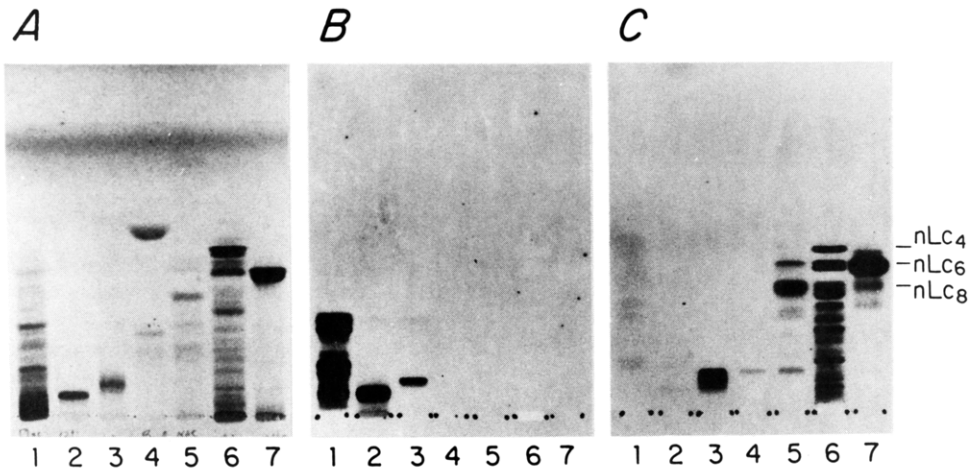


FIGURE 8: HPTLC of the products of sequential exoglycosidase hydrolysis of fractions 121–126 with detection by (A) orcinol staining and by immunostaining with MoAbs: (B) NUH2 and (C) 1B2. Lanes: 1, placenta total gangliosides; 2, fractions 121–126; 3, fractions 121–126 + sialidase; 4, fractions 121–126 + sialidase, β-galactosidase; 5, fractions 121–126 + sialidase, β-galactosidase, β-*N*-acetylhexosaminiase; 6, O upper neutral standard; 7, standard nLc_6Cer. Developed with solvent system C.

percentages for Gal IV and VI, allowing for one full →3Gal unit at Gal II: 42/58 →3Gal/→6Gal.

^1H NMR Spectroscopy. Obtaining satisfactory 1-D ^1H NMR spectra for the placenta gangliosides proved to be difficult, due at least in part to the presence of isomeric branching structures in each fraction (see previous section). In spite of this problem, a number of prominent features, shared by spectra of all of the branched placenta gangliosides, could be used to confirm some of the predicted structural features. This could be done by referring to "structural reporter groups" (Vliegenthart et al., 1983) previously noted in spectra of branched and unbranched neolacto series gangliosides (Levery et al., 1988).

Reproduced in Figure 9 are portions of the spectrum of fractions 90–102 (G-12), accompanied by a partial assignment of key resonances. The following resonances, found in this spectrum by inspection, are diagnostic for NeuAcα2→3Galβ1→4 terminal structures: ≈2.74 ppm (3 overlapping α-NeuAc H-3_{eq}); 1.368 ppm (3 α-NeuAc H-3_{ax}); 1.891 ppm (3 α-NeuAc NAc methyl); 3.702 ppm (3 β-Gal H-4); 3.953 ppm (3 β-Gal H-3); 4.205 ($^3J_{1,2}$ = 7.3 Hz) ppm (3 β-Gal H-1) (Levery et al., 1988). H-1 resonances characteristic of the internal →3Galβ1→4Glcβ1→1Cer are found at 4.271 ($^3J_{1,2}$ = 7.3 Hz) and 4.165 ppm ($^3J_{1,2}$ = 7.9 Hz) (Levery et al., 1988). Overlapping branching →3(→6)Galβ1→4 H-1 resonances are found at ≈4.31 ppm, slightly downfield from the normal 4.30 ppm found consistently for these signals. Overlapping →4GlcNAcβ1→6 H-1 resonances can be seen at ≈ 4.39 ppm [compared with 4.392 ppm for this resonance in G-10 ganglioside (Levery et al., 1988)]. The poor definition of these last two signals (4.39 and 4.30 ppm) may be an indication of the presence of the minor isomeric component with GlcNAc V and GlcNAc V′(6) switched. Three overlapping →4GlcNAcβ1→3 resonances are found at 4.67 (2 H) and 4.65 ppm (1 H). These δ values are comparable to those found for the same units in G-10 ganglioside (Levery et al., 1988), although it is not clear at this time which of GlcNAcs III, V, and VII should be assigned to which particular signal. NAc methyl resonances for the GlcNAcs were found at the characteristic locations 1.817 and 1.836 ppm, the same values observed for G-10 ganglioside (Levery et al., 1988).

Although the occurrence of remote shift effects in these highly branched structures cannot be ruled out, it is clear that every H-1 resonance in the spectrum could be accounted for on the basis of the proposed structure of G-12. The diagnostic structural reporter groups for NeuAcα2→3Galβ1→4 are unambiguous. Similar features were found in the spectrum of G-13 (not shown), although the envelopes for the →4GlcNAcβ1→3/6Galβ1→4 unit H-1 resonances were much more poorly defined.

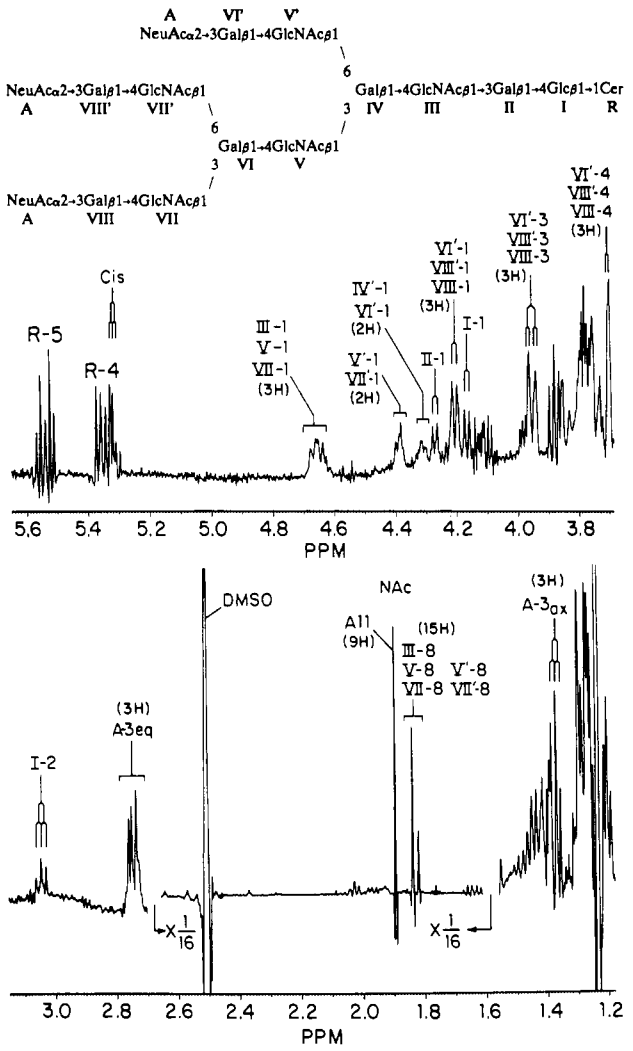
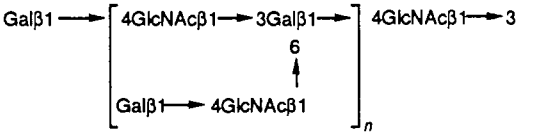


FIGURE 9: Resolution-enhanced 500-MHz ^{1}H NMR spectrum of placenta ganglioside G-12 in DMSO-d_6/D_2O (98:2 v/v) at 308 ± 2 K. Arabic numerals refer to ring protons of residues designated by Roman numerals or capital letters in the corresponding structure. R refers to protons of sphingosine backbone only; Cis refers to vinyl protons of unsaturated fatty acids. For simplicity, the structure of the major branching isomer only is drawn above the spectrum.

Discussion

The results of this study and that of Taki et al. (1988) clearly indicate that gangliosides of human placenta consist exclusively of unbranched and branched molecular species of polylactosamine structures having NeuAcα2→3 linked to the terminal Gal residues. Four of these were previously identified by Taki et al. (1988) as II^{3}NeuAcLacCer (GM_3) [see also Svennerholm (1965)], IV^{3}NeuAcnLc$_4$Cer, VI^{3}NeuAcnLc$_6$Cer, and VIII^{3}NeuAcIV^6(NeuAc2→3Galβ1→4GlcNAc)Lc$_8$ (G-10, Chart I). Recently, the structure designated G-11 in Chart I was isolated and characterized (Nudelman et al., 1989). This structure would be expected to exhibit both i- and I-antigenic activity; however, it was found in only small quantities in human placenta. By contrast, the two larger gangliosides characterized in this paper (G-12 and G-13; Chart I), selected on the basis of their reactivity with MoAb NUH2, are of the I antigen family only. Compared quantitatively, G-12 is present in much more significant amounts than G-11, just as there is more G-10 than III^{3}NeuAcnLc$_6$Cer (Taki et al., 1988). Thus, there would appear to be considerable bias toward I over i expression, due to the activity of an efficient branching GlcNAcβ1→6 transferase.

The series of human placenta iI ganglioside antigens can be compared with the extensive array of type 2 neutral blood group glycosphingolipids which have been isolated from erythrocytes of human A (Hakomori et al., 1972; Fukuda, M. N., & Hakomori, 1982; Clausen et al., 1986), B (Hanfland, 1975; Koscielak et al., 1973; Hanfland & Egge, 1975; Hanfland et al., 1984), and O (Koscielak et al., 1973; Stellner et al., 1973a; Watanabe et al., 1975) blood groups and of rabbit (Hanfland et al., 1981, 1988; Dabrowski et al., 1984, 1988; Dabrowski & Hanfland, 1982; Egge et al., 1985). In the latter series, structures of branched polyglycosylceramides with up to 40 sugar residues have been elucidated (Dabrowski et al., 1988). From this work it is apparent that, while the core type 2 structures in human erythrocyte glycosphingolipids are elongated in such a way as to produce i- and I-antigenic activities on single molecular species, the core structures of rabbit erythrocyte glycosphingolipids are extended exclusively as regular, repeating I-antigenic domains, with the general formula



with no apparent i-active species (Hanfland et al., 1988; Dabrowski et al., 1988). These conclusions have been corroborated by immunochemical methods (Uemura et al., 1983).

Because the gangliosides studied in this work were selected by their reactivity with MoAb NUH2, which recognizes an epitope overlapping with, but not identical with, the I blood group, the results for placenta obtained so far must be generalized cautiously. A number of NUH2-negative (and larger NUH2-positive) components remain to be characterized. The presence of several more components with mixed i and I determinants appears likely. However, the *major* polar components were those which were NUH2 active.

Thus, the situation with human placenta gangliosides in certain respects resembles that of rabbit, more than that of human, erythrocytes. That is, with the exception of the minor component G-11, the larger major components (G-10, -12, and -13) exhibit a high degree of branching, with no i-antigenic domains. However, they appear to differ in one important aspect, which is that their branching patterns do not fit into the regular formula exhibited by homogeneous molecular species isolated from rabbit. Instead, a single fraction is made up of multiple components with isomeric branching structures. This complicated the structure elucidations as well as their descriptions. In particular, the quality and usefulness of the ^{1}H NMR spectra obtained were considerably less than could be hoped for, such that they could be used only in a limited, confirmatory role. Despite their high molecular weight, if the fractions had been completely homogeneous, they would no doubt have been amenable to elucidation by two-dimensional NMR techniques, as demonstrated recently for the octaantennary ceramide tetracontasaccharide from rabbit erythrocytes (Dabrowski et al., 1988). Mixtures of oligosaccharides have also been analyzed by 2-D NMR techniques [see, for example, Bhattacharyya et al. (1984)]; however, this requires that at least a few resonances from different constituents be better resolved than are apparent in this case.

In the absence of more definitive NMR data, the value of obtaining high-quality $^+$FAB mass spectra on the permethylated gangliosides cannot be overestimated. Such spectra produced for rabbit erythrocyte neutral glycosphingolipids with pseudomolecular ions appearing as high as 6181 amu (per-

methylated, Na adduct, nominal mass) show that the useful upper limit for application of this technique has not yet been reached (Hanfland et al., 1988). In this connection, it has been pointed out that the selection of matrix can contribute significantly to the success or failure of obtaining spectra into the higher mass range.

In this work, considerable improvement in the yield of pseudomolecular ions, along with a reduction in chemical noise, was obtained by addition of the cyclic polyether 15-crown-5 (Isobe et al., 1987) to the more traditional glycerol/thioglycerol matrix. This is in fact opposite to the recommendation of Egge and Peter-Katalinic (1987) to add sodium acetate to the matrix, producing a high yield of Na adduct ions. In our hands, we have seen, in some cases, an improvement in spectral quality when sodium adduct formation is completely suppressed. High-quality spectra of permethylated glycosphingolipids were also obtained with NBA (Meili & Seibl, 1984; Barber et al., 1988) as matrix. Most recently, we have had excellent results with the combination of NBA and 15-crown-5 (Salyan and Levery, unpublished experiments). We suggest that these may be useful alternatives.

Since it is not yet possible to differentiate by mass spectrometric techniques the isomeric branching structures encountered, it was still necessary to use the laborious enzyme degradation–methylation analysis to probe this feature. In future studies, a more detailed analysis of fractions containing such branching isomeric components may be obtained by a sequence of steps including endoglycoceramidase-catalyzed removal of ceramide (Ito & Yamagata, 1986; Li et al., 1986) and HPLC using newer column technologies. This can be expected to simplify the MS and NMR analysis by eliminating heterogeneity and to help keep to a minimum the molecular weight of compounds subjected to MS. An extensive library of placenta oligosaccharide structures purified in this way would greatly facilitate studies of Ii blood group immunochemistry, especially providing core structures not available in human or rabbit erythrocytes.

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Release of the σ Subunit from *Escherichia coli* RNA Polymerase Transcription Complexes Is Dependent on the Promoter Sequence†

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ABSTRACT: The σ subunit of bacterial RNA polymerase is required for the specific initiation of transcription at promoter sites. However, σ is released from the transcription complex shortly after transcription is initiated, and elongation proceeds in the absence of σ. In order to study the position of σ release, we have developed a method to quantify the photoaffinity labeling produced by an aryl azide positioned at the leading (5′-) end of nascent RNA, as a function of the transcript length [Stackhouse, T. M., & Meares, C. F. (1988) *Biochemistry 27*, 3038–3045]. Here we compare photoaffinity labeling of transcription complexes containing three natural bacteriophage promoters (λ P_R, λ P_L, and T7 A1) and two recombinant constructs, A1/P_R (T7 A1 promoter with the λ P_R transcribed region) and P_R/A1 (λ P_R promoter with the T7 A1 transcribed region). Significant photoaffinity labeling of the σ subunit was observed only on the templates containing the λ P_R promoter region, regardless of the sequence of the transcribed region. These results indicate the molecular interactions responsible for the position of σ release from the transcription complex mainly involve the nucleotide sequence of the promoter region—rather than the transcribed region—of the DNA template. Further studies on transcription complexes containing the A1/P_R and the P_R/A1 templates were performed, using polyclonal antibodies against the holoenzyme or against the σ subunit. These experiments corroborate the promoter dependence of σ release. They also show a correlation between the release of σ and stable binding of the transcript by the transcription complex.

Much of our understanding of the control of gene expression at the level of transcription has evolved from studies of *Escherichia coli* RNA polymerase (Burgess, 1976; Lewin, 1983). RNA polymerase from *E. coli* contains five major subunits, with a total molecular weight of 449 068. The primary structures of all the subunits have been determined: α (M_r 36 512; Ovchinnikov et al., 1977); β (M_r 150 619; Ovchinnikov et al., 1981); β' (M_r 155 162; Ovchinnikov et al., 1982); and σ (M_r 70 263; Burton et al., 1981). The *core enzyme* contains four subunits ($\alpha_2\beta\beta'$) and is capable of transcriptional elongation. However, specific initiation of a transcript at a promoter site requires the *holoenzyme*, which contains the core enzyme and the σ subunit. In addition to the predominant σ, other σ factors have been discovered in *E. coli* and other bacteria, all of which use the same core enzyme but require unique promoter DNA sequences to initiate transcription. The presence of another subunit (ω) has also been observed [Gentry & Burgess (1986) and references cited therein]; the function of ω is not yet established.

In vivo, only one-fourth to one-half of bacterial RNA polymerase is found as the holoenzyme. This observation, as well

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