Isolation and Characterization of an I-Active Ceramide Decasaccharide from Rabbit Erythrocyte Membranes†

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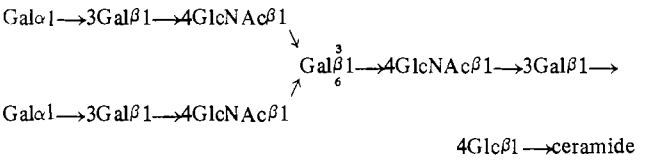
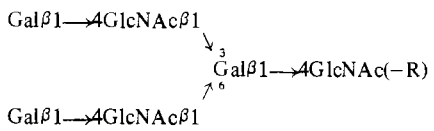
ABSTRACT: The major complex glycolipid from rabbit erythrocyte membranes was isolated in a high yield (40 mg from 5 L of packed erythrocytes). By means of silica gel high-performance thin-layer chromatography, gas–liquid chromatographical composition analysis, hemagglutination inhibition tests, and two-dimensional immunodiffusion, it was identified as a homogeneous I-active and blood group B active ceramide decasaccharide. Its structure was completely elucidated by the analysis of its partially methylated alditol acetates with the aid of combined gas chromatography–mass spectrometry in order to establish the glycosidic linkages, direct inlet mass spectrometry of the permethylated glycolipid for sequence analysis, enzymatic partial degradation by α- and β-galactosidases from coffee and jack beans, respectively, and 360- and 500-MHz ^1H nuclear magnetic resonance spectroscopy in Me_2SO-d_6 of the deuterium exchanged glycosphingolipid. The latter method established the anomeric nature of glycosidic linkages and corroborated additional structural features concerning sugar linkage, sequence, branching pattern, and ceramide moiety. All data firmly establish the structure:



The predominant constituents of the ceramide residue are nervonic acid and C-18 sphingosine. Due to the importance of the I/i antigen complex with regard to autoimmune hemolytic anemia and malignant degeneration, it can be anticipated that this glycolipid, obtainable in high yield and purity, will be used successfully as a reference I antigen and a favorable starting material for further detailed immunochemical investigations on the I/i antigen system.

I/i antigens as cryptic structures of ABH blood group substances (Feizi et al., 1971a,b; Feizi & Kabat, 1972) are of particular interest due to their reaction with corresponding autoantibodies resulting in the cold agglutinin disease (Dacie, 1947; Wiener et al., 1956; Marsh, 1961). I/i antigens are found on oligosaccharides of glycoconjugates of different origin. Their reactivities have been investigated by using several autoantisera of differing fine specificities (Feizi & Marsh, 1970; Feizi et al., 1971a,b, 1978a,b; Watanabe et al., 1975, 1978, 1979a,b; Koscielak et al., 1976; Feizi, 1977; Childs et al., 1978, 1979; Lisowska et al., 1978; Gardas, 1976; Hounsell et al., 1980). Thus far, the different I antigenic determinants can be reduced to various domains within a branched hexasaccharide region



(Watanabe et al., 1979a; Feizi et al., 1979). The reactivity of this hexasaccharide with various anti-I sera may be reduced, destroyed, or retained, when substitution with or step-by-step removal of saccharide units of one or two of its branches occurs, depending on the specificity of the corresponding anti-I sera. The majority of the I antibodies reacts well, when no substitution or degradation of the terminal galactoses of the above-mentioned hexasaccharide can be found (Feizi et al., 1979; Watanabe et al., 1979a; Koscielak et al., 1979; Gardas, 1976; Kabat et al., 1978). The majority of i antibodies recognizes the linear oligosaccharide sequence Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Gal-residue (Niemann et al., 1978; Koscielak et al., 1979). The importance of the I/i antigen complex is underlined by its structural alteration during fetal or postnatal development and during malignant degeneration of certain epithelial tissues (Feizi et al., 1975; Watanabe & Hakomori, 1976; Picard et al., 1978; Koscielak et al., 1979). However, due to the complexity of the I/i antigen–antibody system many questions on its molecular basis still remain unresolved. Therefore, in order to have enough starting material and/or a reference standard for further immunochemical investigations, it is important to be able to obtain an I substance, which is easily isolated in a homogeneous form and high yield. Because rabbit erythrocytes are particularly rich in I antigens (Doinel et al., 1976), we chose this material as a source for the isolation. This paper describes the isolation, purification, immunological identification, and complete characterization of the major complex glycosphingolipid of rabbit erythrocyte membranes. In addition to its I antigenicity the glycolipid also displays some B blood group activity.

Materials and Methods

Materials. Glycosphingolipids as reference standards were essentially those described previously (Hanfland, 1975, 1978; Hanfland & Egli, 1975). α-Galactosidase from coffee beans (~10 units/mg) was purchased from Boehringer, Mannheim, West Germany, and *Bandeirea simplicifolia* seeds were supplied by Calbiochem, Frankfurt, West Germany. *B. simplicifolia* lectin was prepared according to Hayes & Goldstein (1974). *Dolichos biflorus* seeds were a gift from Merz & Dade, Bern, Switzerland. The corresponding crude lectin fraction was prepared by simple extraction of the ground seeds with phosphate-buffered saline and ammonium sulfate frac-

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0006-2960/81/0420-5310$01.25/0

tionation. The fraction of 50–75% saturation was taken for immunodiffusion. Anti-I/i sera were from patients suffering from cold agglutinin disease.

Preparation of Crude Glycolipid Fraction. Blood was collected from 150 rabbits (Ostrop-Kaninchen-Farm, 4005 Meerbusch, West Germany) during slaughtering. During collection, each 400 mL of blood was immediately and thoroughly mixed with 100 mL of an aqueous solution containing 1.32 g of sodium citrate, 0.44 g of citric acid, 13.4 g of glucose, and 2000 IU of heparin (Liquemin, Hoffmann-La Roche, Grenzach-Wyhlen, West Germany). Erythrocyte membranes were prepared according to Dodge et al. (1963) as modified by Hanfland & Egli (1975). Hemolysis and washing of the ghosts was performed with 24 mosM sodium phosphate buffer, pH 7.4. Lyophilized, hemoglobin-free ghosts (54 g) obtained from 5 L of packed erythrocytes, were extracted as described previously (Hanfland & Egli, 1975). The whole lipid extract (14.2 g) was subjected to a modified phase partition (Folch et al., 1957; Hanfland & Egli, 1975). The upper-phase lipids (3.2 g) were distributed again in 0.8 L of theoretical upper phase and 0.2 L of theoretical lower Folch phase, and the upper phase was extracted a second time with 0.2 L of theoretical lower phase. The remaining upper phase was concentrated, freed from organic solvents, dialyzed, and lyophilized (yield, 650 mg).

Purification of the I Glycolipid. The lyophilized upper-phase substances were acetylated with 200 mL of pyridine–acetic anhydride (2:1) for 24 h at room temperature. The mixture was evaporated in a rotatory still with an excess of toluene and dried in a vacuum desiccator over phosphorus pentoxide. The mixture (890 mg) was partitioned again in chloroform–methanol–water (8:4:3). The lower-phase substances were brought to dryness and applied to a 800-g silicic acid (Bio-Sil A, Bio-Rad, München, West Germany) column (4.6 × 95 cm) equilibrated with chloroform–methanol (99:1). The column was eluted with the following chloroform–methanol mixtures: 1 L of 99:1 and 98:2, 2 L of 97:3, 3 L of 96:4, 4 L of 95:5, and finally 2 L of 90:10. Fractions (40 mL) were collected by a fraction collector. Every fifth tube was examined by 10 × 10 cm analytical silica gel HPTLC[1] (nanoplates, Macherey & Nagel, Düren, West Germany) using solvent system A [chloroform–methanol–water (50:3:0.01)]. Fractions containing the acetylated major glycosphingolipid were combined and further purified by preparative 20 × 20 cm silica gel HPTLC. Preparative HPTLC was performed as described previously in detail (Hanfland, 1978). Solvent system A was used throughout. Homogeneity of the peracetylated glycolipid was controlled by 10 × 10 cm analytical HPTLC using solvent system A, solvent system B [chloroform–methanol–water (92.5:7.5:0.02)], solvent system C [1,2-dichloroethane–methanol–water (88:12:0,2)], solvent system D [1,2-dichloroethane–acetone–water (55:45:1)], and solvent system E (ethyl acetate–acetone–water (60:40:0,5)].

Deacetylation. Dried substance (10 mg), homogeneous in all solvent systems, was dissolved in 75 mL of freshly distilled anhydrous pyridine. Under stirring, 25 mL of 0.6% sodium methoxide in anhydrous methanol was added, and the solution was kept for 30 min in the dark at 22 °C. Finally an equivalent amount of aqueous HCl (0.1 N) was added. Organic solvents were removed by evaporation under repeated addition of distilled water. Care was taken to avoid complete dryness of the glycolipid. The concentrate was dialyzed against water and lyophilized. In order to achieve complete deacetylation for NMR analysis, the procedure had to be repeated once. Completeness of deacetylation was checked by HPTLC using solvent system F [chloroform–methanol–water (55:45:12)]. Homogeneity of the deacetylated glycolipid was investigated further by HPTLC using solvent system G [chloroform–methanol–water (60:35:8)], solvent system H [1-propanol–water–concentrated ammonia (60:35:5)], and solvent system I [chloroform–methanol–acetic acid–water (40:60:5:10)].

Compositional Analysis. All derivatized components were analyzed on a Packard-Becker gas chromatograph, Model 419, equipped with a flame ionization detector. For carbohydrate analysis, the lyophilized glycolipid sample (40–100 μg) was hydrolyzed in 200 μL of 0.7 N sulfuric acid in 85% aqueous acetic acid for 20 h at 80 °C. Further derivatization to peracetylated alditols and their analysis on a 1-m 3% ECNSS-M column on gaschrom Q (100–200 mesh, Applied Science Laboratories, Inc.) were performed as already described (Hanfland et al., 1978a). For the analysis of the ceramide residue, 200 μg of the glycolipid was hydrolyzed in 300 μL of 1 N aqueous methanolic HCl (18% H_2O v/v) at 80 °C for 20 h according to Gaver & Sweeley (1965). Preparation of fatty acid methyl esters and of Me_3Si-sphingosine bases was performed as described (Hanfland, 1975). GC analysis of fatty acid methyl esters was carried out on a 11-m WCOT AR-AFFP capillary column (Macherey & Nagel, Düren, West Germany). The starting temperature was 180 °C with a temperature program of 2 °C/min up to 225 °C. Me_3Si-sphingosine bases were identified on 1% SE 30 on Chromosorb W AW DMCS at 200 °C (80–100 mesh; column length, 2 m).

Enzymatic Degradation. The lyophilized glycolipid (3 mg) was dissolved in 2.4 mL of distilled water and carefully sonicated for a few seconds. Under stirring, 300 μL of 1 M potassium phosphate buffer, pH 6.5, and finally 300 μL of an α-galactosidase suspension from coffee beans (15 units) were added. After being stirred for 24 h at 37 °C the mixture was dialyzed against distilled water and lyophilized. The residue was dissolved in 100 μL of water, and 0.5 mL of methanol and 0.5 mL of chloroform were added. After the mixture was stirred overnight, precipitated protein was removed by filtration through a disposable pipet containing a small sample of silicic acid, which was previously washed several times with water. Organic solvents were removed from the filtrate by gradual concentration and replaced by a gradual addition of water. After final concentration to ~300 μL the sample was lyophilized and ready for further analysis. Subsequent degradation by β-galactosidase from jack beans in the presence of apolipoproteins was carried out as described previously (Hanfland et al., 1978b).

Methylation. It was performed according to Hakomori (1964), as adapted for the analysis of amino sugar containing glycolipids (Stoffel & Hanfland, 1973). The purification included chromatography on a Sephadex LH-20 column with

[1] Abbreviations used: HPTLC, high-performance thin-layer chromatography; Me_3Si, trimethylsilyl; GC, gas–liquid chromatography; MS, mass spectrometry; FD, field desorption; B_{rab}, blood group B active ceramide pentasaccharide from rabbit erythrocyte membranes (IV^3Gal-α-neolactotetraosylceramide); BI_{rab}, blood group B active and I-active ceramide decasaccharide from rabbit erythrocyte membranes; R_{PG} value, ratio between thin-layer chromatographical migration distance of individual glycolipid and thin-layer chromatographical migration distance of human erythrocyte membrane paragloboside, i.e., neolactotetraosylceramide; Cer, ceramide; NMR, nuclear magnetic resonance; SDDS, spin-decoupling difference spectroscopy; NOE, nuclear Overhauser enhancement or effect; ppm, parts per million; Me_2SO, dimethyl sulfoxide. Abbreviations of sugars are those which are commonly used; in addition, Hex and HexNAc mean hexose and *N*-acetylhexosamine, respectively. All ratios of solvents indicated are volume/volume ratios.

chloroform–acetone (1:1) as eluent and preparative HPTLC using solvent system A.

Carbohydrate Linkage Analysis by Combined Gas Chromatography–Mass Spectrometry. Partially methylated alditol acetates were obtained after hydrolysis of 100–200 μg of permethylated glycolipid in 100 μL of 0.7 N sulfuric acid in 80% aqueous acetic acid at 80 °C for 20 h as described before (Stoffel & Hanfland, 1973). Due to the high complexity of the glycolipid, particular care was taken to obtain an easy and rapid solubility of the permethylated glycolipid in the acetolysis reagent: the sample, previously dissolved in benzene, was transferred into the analysis tube and dried briefly in a stream of nitrogen. Before addition of the acetolysis reagent the sample was dissolved again in a minimum of benzene (2–4 μL). Partially methylated neutral and 2-acetamido-2-deoxyalditol acetates were analyzed by GC on a 1-m column of 3% ECNSS-M on gaschrom Q (100–200 mesh) (Hanfland, 1975). Reference standards were prepared from methylated ceramide pentasaccharide of rabbit erythrocyte membranes and gangliotetraosylceramide derived from human brain gangliosides. The alditol acetates were also identified by a combined GC–MS system, Model 3100 D (Finnigan), at 70 eV according to the data given by Björndal et al. (1970), Stoffel & Hanfland (1973), and Stellner et al. (1973). GC conditions and reference substances were the same as described above.

Direct Inlet Mass Spectrometry of Permethylated Glycolipid. Mass spectra were obtained on a LKB 9000 instrument as formerly described (Hanfland & Egge, 1976): probe temperature 300 °C, ion source temperature 350 °C, ionization energy 20 eV, trap current 60 μA, and acceleration voltage 2.33 kV. Scanning time was ~50 s per mass decade.

Field Desorption Mass Spectrometry of Permethylated Glycolipid. FD measurements (Beckey, 1977) were performed on a Kratos MS 50 system equipped with a high-field magnet. Fomblin pump oil (Montedison) was used as the reference compound. The acceleration voltage was 8 kV, the emitter current was 8–10 mA.

High-Resolution ^1H Nuclear Magnetic Resonance Spectroscopy of Deuterium-Exchanged Glycolipid. All technical details have been described previously (Dabrowski et al., 1980b). The operating frequency was 360 MHz with the Bruker HX-360 and 500 MHz with the Bruker WM-500 spectrometer. The accuracy of integration was better than 3% for separate signals. The overlapping signals were singled out with the aid of SDDS. NOE was measured in the manner described by Wagner & Wüthrich (1979). Reference substances were those, which have been analyzed previously (Dabrowski et al., 1980b).

Immunological Identification. Mixed liposomes of the glycolipid and auxiliary lipid were prepared as follows: 10 mg of sphingomyelin was sonicated extensively with a Branson sonifier in 800 μL of distilled water until the solution became clear and transparent. Glycolipid, 1 mg dissolved in 100 μL of water, was added, and the whole mixture was stirred and slightly sonicated for several seconds. After addition of 100 μL of a 10-fold concentrate of isotonic phosphate-buffered saline, pH 7.4, and thorough mixing, the solution was ready for the hemagglutination inhibition test. Further details have been described previously (Hanfland & Egli, 1975; Hanfland 1978). Hemagglutination inhibition against each 50 μL of 4 hemagglutinating units of different anti-I/i sera was tested at 0 °C and against anti-B serum of human origin (Merz & Dade, München, West Germany) at room temperature.

Two-dimensional immunodiffusion was performed in 1% Agar-Noble (Difco, Detroit, MI) in isotonic phosphate-buffered saline containing 0.05% sodium azide on 75 × 25 mm glass slides. For this purpose the glycolipid–auxiliary reagent mixture was prepared as follows: 1.2 mg of sphingomyelin and 0.6 mg of sodium dodecyl sulfate were sonicated extensively in 250 μL of water–ethanol (4:1). Glycolipid (300–600 μg) dissolved in 50 μL of water was added and the whole mixture carefully sonicated for several seconds. This mixture was tested against different anti-I sera (at 0 °C) and *B. simplicifolia* and *D. biflorus* lectin (at room temperature).

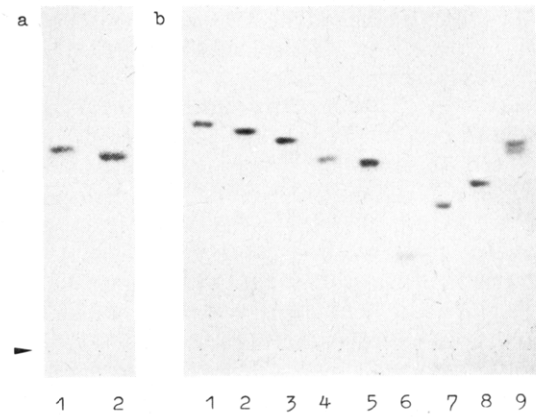


FIGURE 1: Analytical silica gel HPTLC of ceramide decasaccharide (BI_{rab}) from rabbit erythrocytes and reference glycosphingolipids (solvent system F). (a) Lane 1, ceramide pentasaccharide (B_{rab}) from rabbit erythrocytes; 2, gangliotetraosylceramide derived from human brain gangliosides. (b) Lane 1, globoside; 2, neolactotetraosylceramide; 3, H blood group active ceramide pentasaccharide; 4, P_1 blood group active glycolipid fraction; 5, B blood group active ceramide hexasaccharide (B-I); 6, BI_{rab}; 7, B blood group active ceramide octasaccharide (B-II); 8, sialoparagloboside; 9, hematosides from human plasma (lanes 1–5, 7, and 8 were isolated from human erythrocytes).

Results

Purification and Yield. The peracetylated major glycolipid could be separated easily from the bulk of other glycolipid acetates on a Bio-Sil-A silicic acid column by elution with chloroform–methanol (96:4 and 95:5). The fractions containing the major glycolipid (150 mg) were contaminated with small amounts of closely slower and faster migrating bands as identified by analytical HPTLC. One to three runs on preparative HPTLC finally yielded the pure acetylated glycolipid (~70 mg). This fraction was homogeneous in HPTLC with solvent systems A–D. Twofold deacetylation with 0.15% sodium methoxide in pyridine–methanol (3:1) finally yielded ~40 mg of a completely deacetylated glycolipid homogeneous in analytical HPTLC with solvent systems F–I. Figure 1 demonstrates the HPTLC behavior in solvent system F of the major complex glycolipid in relation to other glycolipids.

Compositional Analysis. GC analysis of the peracetylated alditols showed glucose, galactose, and *N*-acetylglucosamine in molar ratios of 1.00:6.09:2.62 in accordance with a ceramide decasaccharide. Neither fucose nor neuraminic acid could be detected. The long-chain base moiety consisted of sphingosine, with only traces of octadecasphinganine. The fatty acid composition, summarized in Table I, shows a striking similarity to that of the ceramide pentasaccharide from rabbit erythrocytes with nervonic acid as the main fatty acid constituent.

Biological Identification. As measured by the hemagglutination inhibition test at 0 °C (Table II), different anti-I sera (anti-I Hir, Vog, Sat) were strongly inhibited by the ceramide decasaccharide (Table II). Others, however (anti-I Hör, Schü, Scha, San, Lin), showed only weak specificity against the glycolipid. A third group of antisera (anti-I Sche and Thi) finally could not be inhibited by at least 50 μg of

Table I: Fatty Acid Composition of Rabbit Erythrocyte Ceramide Penta- and Decasaccharides (%)

fatty acid	B_{rab}	BI_{rab}
C16	1.2	0.3
C16:1	0.3	0.1
C18	1.3	1.3
C18:1	0.4	0.6
C19	0.3	0.1
C20	0.6	0.7
C22	6.1	7.0
C22:1	0.6	0.7
C23	3.5	3.8
C23:1	0.6	0.6
C24	8.3	9.2
C24:1	63.6	62.1
C24:2	9.7	8.5
C25	1.2	1.5
C26	2.3	3.5

Table II: I and B Blood Group Activities of the Major Complex Glycolipid from Rabbit Erythrocyte Membranes[a]

antisera				
no.	donor's name (abbr)	specificity	type	min amount of glycolipid (μg)[b]
1	Lin	anti-I	auto monotyp (IgM, λ)	50
2	Vog	anti-I	auto	0.8
3	Hör	anti-I	auto monotyp (IgM, κ)	25
4	Hir	anti-I	auto monotyp (IgM, κ)	0.2
5	Schü	anti-I	auto	25
6	Sche	anti-I	auto monotyp (IgM, κ)	>50
7	Sat	anti-I	auto monotyp (IgM, κ)	3.1
8	Sau	anti-I	auto	50
9	Thi	anti-I	auto monotyp (IgM, κ)	>50
10	Scha	anti-I/i (?)	auto monotyp (IgM, λ)	32
11		anti-B	iso	6

[a] Determined by the hemagglutination inhibition test with different human antisera. [b] Numbers are the minimum amounts in micrograms of substance which completely inhibit the specific hemagglutination by 50 μL of 4 hemagglutinating units of antiserum.

the substance. This reaction pattern, typical for other I blood group substances (Feizi et al., 1979; Gardas, 1976; Watanabe et al., 1979a) also could be confirmed by two-dimensional immunodiffusion of the glycolipid against different I antisera. The sera Hir and Vog, for example, formed distinct precipitates with the ceramide decasaccharide, whereas others (anti-I Hör) reacted only very weakly and others finally failed to react. Precipitation was formed only at 0 °C; upon warming of the gels to room temperature the precipitates disappeared. Furthermore, in the presence of sphingomyelin as auxiliary lipid, 6 μg of the substance completely inhibited the agglutination of human B erythrocytes by 50 μL of 4 hemagglutinating units of human anti-B serum. By two-dimensional immunodiffusion the ceramide decasaccharide formed strong precipitates with the purified *B. simplicifolia* lectin. No reaction was observed with *D. biflorus* lectin (Figure 2).

Enzymatic Degradation by α- and β-Galactosidases. In contrast to the incubation of the native glycolipid with jack bean β-galactosidase, coffee bean α-galactosidase appreciably altered the thin-layer chromatographical behavior of the substance (R_{PG} = 0.38 in solvent system G) in relation to the

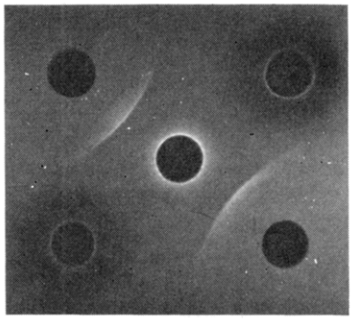


FIGURE 2: Two-dimensional immunodiffusion of BI_{rab} (center well) against *B. simplicifolia* lectin (upper left and lower right well) and *D. biflorus* lectin (lower left and upper right well).

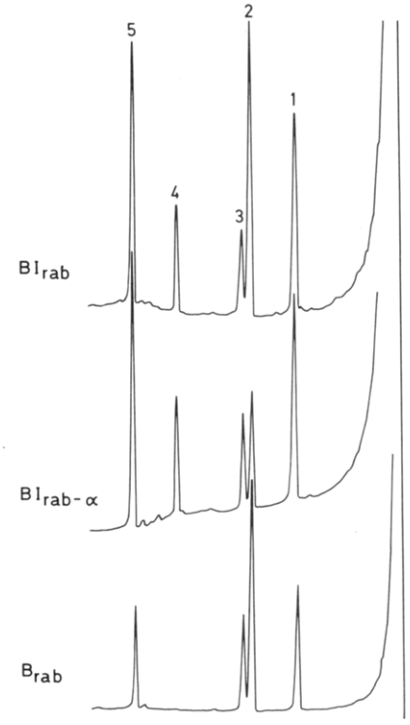


FIGURE 3: Gas liquid chromatography on 3% ECNSS-M of the alditol acetates of partially methylated neutral and amino sugars released from BI_{rab}, from its α-galactosidase-degraded product ($BI_{rab-\alpha}$), and from B_{rab} after permethylation. For conditions see Materials and Methods. (1) 2,3,4,6-Tetra-*O*-methyl-1,5-di-*O*-acetylgalactitol; (2) 2,4,6-Tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitol; (3) 2,3,6-Tri-*O*-methyl-1,4,5-tri-*O*-acetylglucitol; (4) 2,4-Di-*O*-methyl-1,3,5,6-tetra-*O*-acetylgalactitol; (5) 2-(*N*-methylacetamido)-2-deoxy-3,6-di-*O*-methyl-1,4,5-tri-*O*-acetylglucitol (see Figure 4).

R_{PG} value (0.18) of the native substance. Further degradation by β-galactosidase produced a substance with the R_{PG} value of 0.70 in solvent system G. For further details see the following section and Figure 3.

Linkage Analysis. Combined GC–MS of the methylated ceramide decasaccharide after acetolysis, reduction, and acetylation yielded each 2,3,4,6-tetra-*O*-methylgalactose, 2,4,6-tri-*O*-methylgalactose, 2,3,6-tri-*O*-methylglucose, 2,4-di-*O*-methylhexose, and 2-(*N*-methylacetamido)-2-deoxy-3,6-di-*O*-methylglucose as their alditol acetates in molar ratios of 2.1:3.0:0.9:0.9:2.5 (Figure 3). Peaks 1, 2, 3, and 5 were identified by their GC retention times and their MS fragmentation patterns (Hanfland, 1975). The alditol acetate as peak 4 (Figure 3) was identified as 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylgalactitol (Figure 4) (Björndal et al., 1970). Treatment of the native glycolipid with α-galactosidase before methylation did not qualitatively alter the pattern of partially methylated alditol acetates. Their molar ratios, however, were

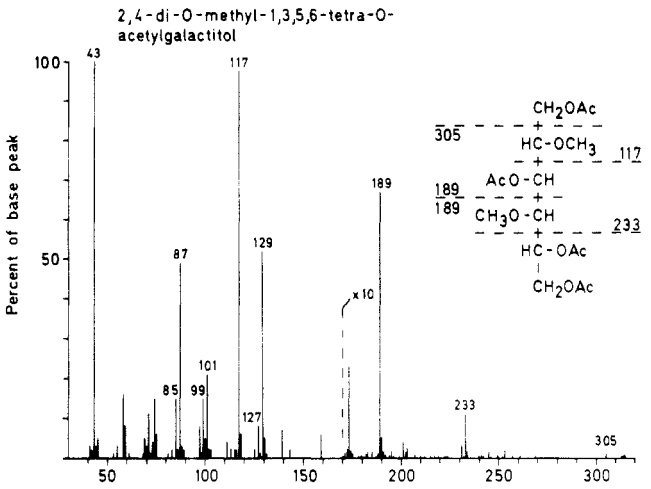


FIGURE 4: Mass spectrum of the alditol acetate of the partially methylated 3- and 6-linked galactose obtained from BI_{rab} and $BI_{rab-\alpha}$ after permethylation, acetolysis, reduction, acetylation, and GC separation (see Figure 3, peak 4).

changed to 2.1:1.0:0.9:0.9:2.6 in the above-mentioned order (Figure 3).

Mass Spectrometric Sequence Analysis. The mass spectra and some of the major fragmentation pathways are shown in Figure 5. The experimental conditions were essentially those described earlier (Hanfland & Egge, 1976). Although the whole molecule could be evaporized at 280 °C into the ion source, only a partial mass spectrum was obtained due to the low sensitivity of the instrument in the higher mass range.

Since, however, fragment ions are present that are derived from the carbohydrate as well as from the ceramide residue, valuable data supporting the proposed structure could be obtained. The carbohydrate moiety up to a nonasaccharide ion *m/e* 1974 is represented by series of ions like *m/e* 219, 423, 668, 1525, 1770, and 1974 and their daughter ions *m/e* 187, 391, 636, 1493, 1738, and 1942 produced by loss of 32 amu (CH_3OH). The attribution of these ions is corroborated by the presence of the metastable ions at *m** 159.6, 361.4, 605.5. The absence of *m/e* 228 and the intense ion at *m/e* 182 indicate the 1→4 substituted hexosamine of a type 2 chain (Egge, 1978). Other ions probably derived from the carbohydrate moiety are *m/e* 1103 and 1348 produced by the elimination of two hexoses from the hepta- and octasaccharide ions *m/e* 1525 and 1770. In a similar way *m/e* 1086 can be derived from *m/e* 1770 by elimination of a trisaccharide moiety. No ions attributable to a tetra-, penta-, or hexasaccharide moiety could be observed. The ceramide moiety gives rise to several series of ions as outlined before (Egge, 1978): a strong ion at *m/e* 364 is indicative of a normal sphingosine moiety. The variation in fatty acid chain length (C_{16}–C_{26}) and desaturation is reflected by a series of ions (B type) ranging from *m/e* 294 to *m/e* 434 with the highest intensity of *m/e* 404 for the C24:1 and *m/e* 402 for the C24:2 fatty acid.

The whole ceramide residue (A type) furnishes an analogous series of fragments with appropriate mass increments between *m/e* 548 and 688. These ions are accompanied by daughter ions that are produced by the elimination of CH_3OH thus producing a very complex pattern of fragments. An additional series of ions *m/e* 606/608, 810/812, and 1055/1057 can be explained by the combination of the B fragment of the ceramide residue and part of the carbohydrate chain.

Field Desorption Mass Spectrometry. Due to the "soft ionization", directly from the solid phase without prior volatilization, the FD technique is especially suitable to the analysis of thermally labile or high molecular weight substances (Beckey, 1977). FD-MS has recently been introduced successfully to the analysis of glycosphingolipids (Costello et al., 1980). Due to the high electric field, ions besides M^+ are produced predominantly by the addition of cations like H^+, Na^+, or K^+, thus allowing the determination of molecular weights (Beckey, 1977).

On the basis of the formula shown in Figure 5 with nervonic acid as constituent, the major permethylated ceramide decasaccharide corresponds to a calculated molecular weight of 2855.631. From the masses of the nuclides relative to ^{12}C = 12.000, the lowest isotope peak calculated for $^{12}C_{141}{}^{1}H_{256}{}^{14}N_4{}^{16}O_{53}$ = 2853.74. By use of FD mass spectrometry, a group of molecular ions between *m/e* 2850 and *m/e* 2950 was obtained 14 or 16 mass units apart and centered aroung the base peak *m/e* 2893 produced by the potassium salt ($M + K^+$) of the major molecular species shown in Figure 5. Ions $(M + H)^+$ or $(M + 23)^+$ produced by the addition of sodium ion were of minor prominence. No other ions could be observed in the mass range between *m/e* 2500 and 3100. These data are in perfect agreement with the proposed structure of a ceramide decasaccharide.

High-Resolution ^1H NMR Spectroscopy. The detailed NMR analysis of BI_{rab} was performed at 360 MHz. The overlapping signals of H-2–H-5 were found by SDDS and NOE. The 500-MHz spectrum was taken in order to see whether the two slightly differentiated branches of BI_{rab} exhibit separate signals. This was not the case, in contrast to the branched mannose/*N*-acetylglucosamine/galactose oligosaccharides examined on the same machine by van Halbeek et al. (1980). However, a few details were better resolved, among them the now completely separated H-1 signal of one of the galactose units at 4.27 ppm (Figure 6a,b).

The sugar proton region of the NMR spectrum of the ceramide decasaccharide (BI_{rab}) is shown in Figure 6. The assignments of the signals shown in the Table III are based on the results of the systematic study of the ceramide pentasaccharide (B_{rab}) from rabbit erythrocytes and its precursors and analogues (Dabrowski et al., 1980b).

The integration of the well-separated spectral region of the anomeric protons showed the presence of 10 sugar residues in groups of 1 + 1 + 3 + 1 + 2 + 2 (see also Figure 6). The one-proton H-1 resonances at 4.17 and 4.27 ppm coupled to H-2 resonances at 3.05 ppm ($J_{1,2}$ = 7.7 Hz) and 3.43 ppm ($J_{1,2}$ = 7.2 Hz), respectively, can unequivocally be ascribed to β-glucose-1 and β-galactose-2 of the head group. Their sequence follows from the combined data of our recent works. As a matter of fact, for a reverse sequence of that shown in the Table III, one would expect Gal δ 4.10 (H-1) (instead of 4.27 experimentally found), which is composed of the values 4.05 ppm for galactosylceramide (Dabrowski et al., 1980a), plus 0.04–0.06 ppm for the shift induced by glycosidic substitution (Dabrowski et al., 1980b). For glucose in the same reverse sequence, a value of δ ~4.33 (H-1) instead of the experimentally found 4.17 would be expected, the former being composed of 4.10 ppm for glucosylceramide, plus ~0.17 ppm for the linkage to a glycosyl vs. ceramide aglycon, plus 0.06–0.07 ppm for a glycosidic substitution. Hence, the two hexoses of the head group visible in the mass spectrum should be placed in the order indicated in Table III.

The two-proton H-1 resonance at 4.84 ppm coupled to the H-2 one at 3.59 ppm ($J_{1,2}$ = 3.6 Hz) can be attributed with the same degree of certainty to two terminal α-galactose-7 and -7′, each of which is 1→3 glycosidically linked to a β-galactose unit. A 1→4 glycosidic linkage would require δ 4.81 (H-1) (loc. cit.). The alternative linear order of these two α-galactose

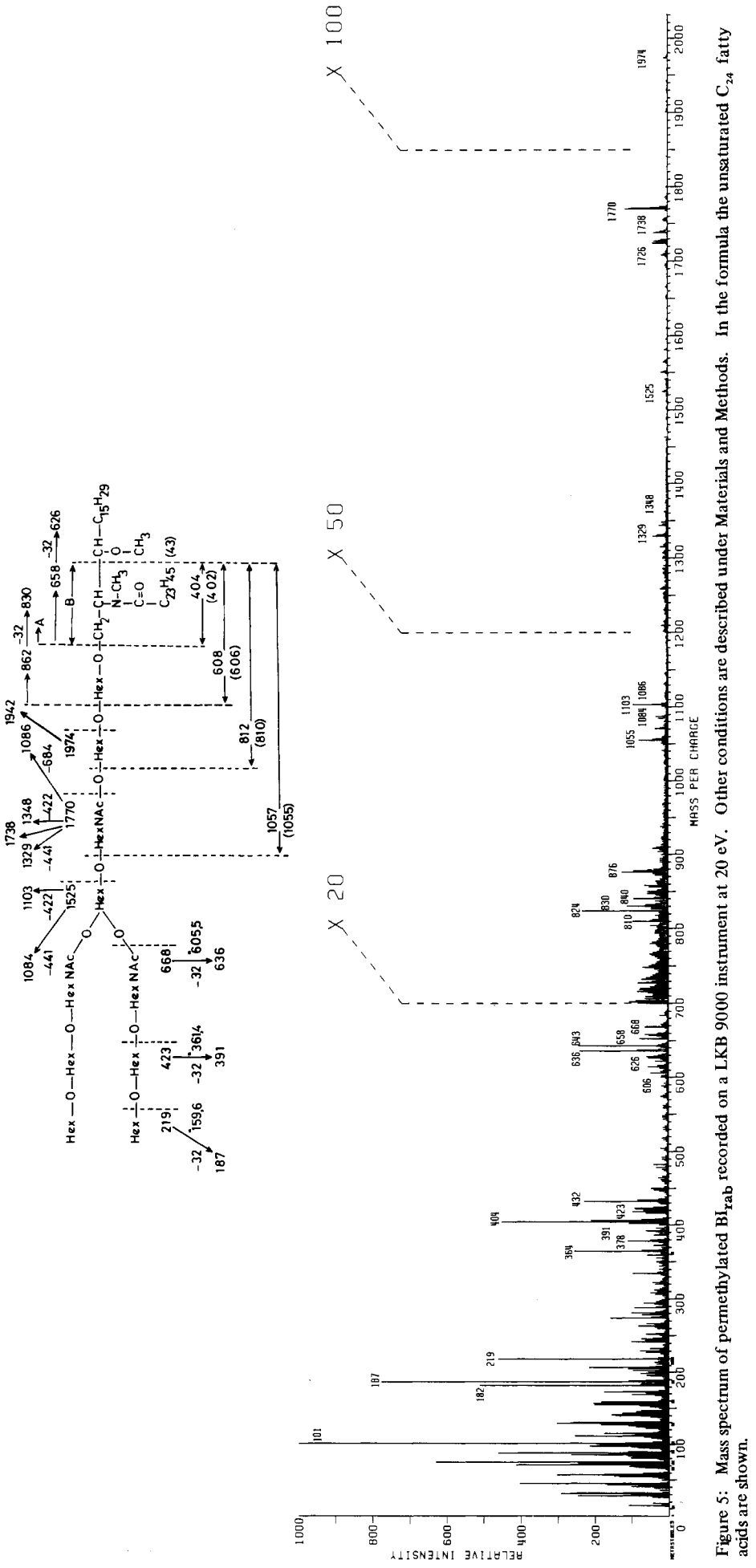


Figure 5: Mass spectrum of permethylated BI_{rab} recorded on a LKB 9000 instrument at 20 eV. Other conditions are described under Materials and Methods. In the formula the unsaturated C_{24} fatty acids are shown.

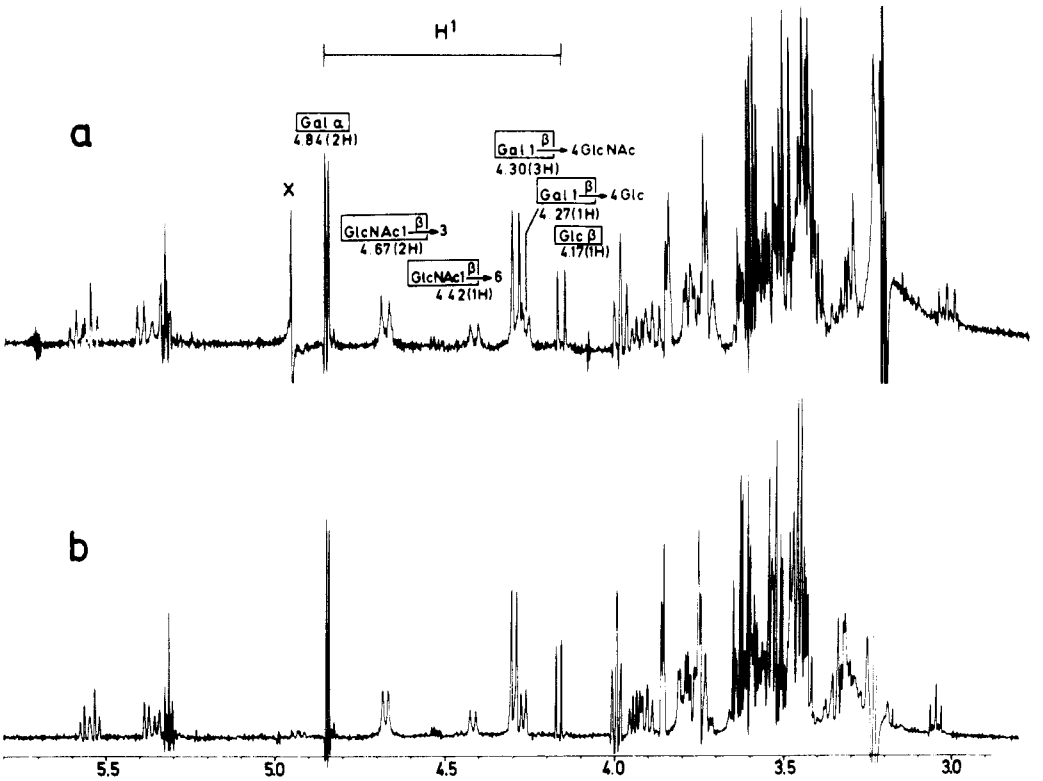


FIGURE 6: Sugar proton region of the (a) 360- and (b) 500-MHz ^{1}H NMR spectrum of BI_{rab} in Me_2SO-d_6. Lorentzian-to-Gaussian transformation was applied. (×) Spike. For chemical shifts and coupling constants, see Table III. The integrals of the anomeric proton signals are given in parentheses.

Table III: Sugar Proton Chemical Shifts (ppm from Me_4Si) and $^3J_{1,2}$ Coupling Constants (Hz) of the Ceramide Decasaccharide (BI_{rab}) and the Ceramide Pentasaccharide (B_{rab})[a] in Me_2SO-d_6

	7	6	5		4	3	2	1
BI_{rab}	Galα1→3Galβ1→4GlcNAcβ1 ⟶ 3				Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer			
	Galα1→3Galβ1 ⟶ 4GlcNAcβ1→ 6							
	7′	6′		5′				
δ(H-1)	4.84	4.30	4.67	4.42	4.30[b]	4.67	4.27	4.17
J	3.6	7.3	8.5	8.1	7.3	8.5	7.2	7.7
δ(H-2)	3.59[c]	3.44	3.48	3.45	3.44	3.44	3.43	3.05
δ(H-3)	3.64	3.48[c,d]	3.58[e]	3.58[e]	3.46[c,d]	3.58[e]	3.46[c,d]	3.32
δ(H-4)	3.75	3.86[d]			3.80[b]		3.86	3.31[f]
δ(H-5)	3.99	3.33[e]	3.31[e]	3.32[e]	3.61[e]	3.31[e]	3.33[e]	
B_{rab}	Galα1→3Galβ1 ⟶					4GlcNAcβ1→3Galβ1→4Glcβ1 ⟶ 1Cer		
δ(H-1)	4.85	4.29				4.70	4.27	4.17
J	(3.8)	(7.4)				(8.4)	(8.0)	(7.8)
δ(H-2)	3.59	3.42				3.44	3.42	3.04
δ(H-3)	3.64	3.48					3.48	3.32
δ(H-4)	3.75	3.85					3.85	
δ(H-5)	3.99							

[a] Data from Dabrowski et al. (1980b). [b] Confirmed by NOE (H-5 of the same sugar ring irradiated). [c] Obtained by both SDDS and NOE (H-1 of the same ring irradiated). [d] Obtained by SDDS and observed by NOE (H-1 of the subsequent sugar residue irradiated). [e] Obtained by NOE (H-1 of the same ring irradiated). [f] Obtained by NOE (H-2 of the same ring irradiated).

units can be excluded by the perfect coincidence of all their H-1, H-2, H-3, H-4, and H-5 signals, both between themselves and with those of the terminal α-galactose unit of B_{rab}. Indeed, glycosidic substitution by another sugar unit was shown (loc. cit.) to deshield the ring protons, especially those at the site of substitution or vicinal to it. In particular, although the H-1 resonance of the terminal α-galactose of a ceramide trisaccharide was an exception to this rule in that it was not shifted by elongation of the oligosaccharide chain, all other signals of that galactose were strongly affected. Thus, the identification of *two* terminal units in BI_{rab} furnishes an evidence of its branched structure.

The glycosidic linkage of those terminal α-galactose residues to β-galactoses was directly established by NOE. The NOE

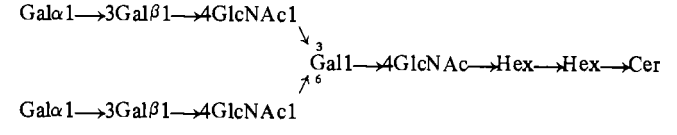
of the signal of an aglyconic proton can be obtained upon preirradiation of the anomeric proton of the subsequent sugar unit. While this scheme was recently used for calculation of the twist angles between sugar rings in synthetic tri- and tetrasaccharides of established sequence (Lemieux et al., 1980), it can also be applied to the determination of unknown sequences. An additional benefit is the identification of the signals of protons of the same sugar ring which are close in space to this anomeric proton. With BI_{rab}, the preirradiation of H-1 of Gal-7 and -7′ produced a NOE difference signal at the resonance frequency of H-2 of the same sugar ring (3.59 ppm) and two more at 3.48 and 3.86 ppm where H-3 and H-4 signals of β-galactose residues have been found by SDDS. Although the sequence Galα→Galβ is thus unambiguously determined, the question of the glycosidic linkage to site 3 or 4 remains open. However, substitution at site 3 of Gal-6 and -6′ unequivocally follows from their δ 3.44 (H-2) found by SDDS, because glycosylation at site 4 would require δ 3.31–3.34 (Dabrowski et al., 1980b). Once this is established, the NOE signal of H-4 at 3.86 ppm can be used for estimating the secondary structure of this fragment of the oligosaccharide chain. Although no quantitative approach will be attempted at this stage, it is obvious that the Ψ and Φ angles assume values leading to the localization of α-Gal-7 and -7′ H-1 between H-3 and H-4 of the penultimate β-galactose unit (Gal-6 and -6′). The H-1 signal at 4.30 ppm assigned to Gal-6 and -6′ has a three-proton intensity, and we propose a location at the branching point of BI_{rab} for the third β-galactose residue, as shown in Table III. Since bifurcation at galactose-2 of the lactosyl head group is a priori equally possible, it is important to substantiate the choice made here: of the two sets of chemical shifts to be decided upon, one is almost identical with that found for the lactosyl galactose unit of B_{rab} and is accordingly assigned to Gal-2; in the other set, the H-5 resonance is strongly shifted to lower field which can only be explained by the additional glycosidic substitution at the neighbouring C-6. Like with Gal-6 and -6′, the glycosidic substitution at site 3 of Gal-4 is independently confirmed by its H-2 chemical shift of 3.44 ppm.

The two-proton signal at 4.67 ppm ($J_{1,2}$ = 8.5 Hz) corresponds to H-1 of *N*-acetylglucosamine units. Its preirradiation yields a differential NOE signal of the H-3 of β-galactose residues thus indicating the sequence shown in Table III. Here again, the glycosylation at site 3 of the galactose-2 is confirmed by its δ 3.43 (H-2). Although the two H-2 resonances of *N*-acetylglucosamines at 3.48 and 3.44 ppm were found directly by a SDDS experiment, their relative assignment is tentative.

The arguments discussed below allow one to assign the remaining one-proton H-1 signal at 4.42 ppm ($J_{1,2}$ = 8.1 Hz) to another *N*-acetylglucosamine unit glycosidically linked to the 6-methylene group of a galactose. First, the integral of the three signals at 1.82, 1.83, and 1.84 ppm corresponds to three *N*-acetylamino groups. Second, the upfield shift by 0.25 ppm of that H-1 signal as compared to 4.67 ppm of the two other *N*-acetylglucosamines is readily explained by the effect of their aglycon substituents. Indeed, as already mentioned, the glycosidic linkage to a methylene group (of a ceramide unit) instead of a methine group of a sugar resulted in an upfield shift of 0.17 ppm. Finally, analogous upfield shifts have been reported for 6- vs. 3-linked mannose (Dorland et al., 1978) and glucose derivatives (De Bruyn, 1975; De Bruyn et al., 1975).

Discussion

As demonstrated by the GC–MS methylation analysis, the I-active and B blood group active ceramide decasaccharide (BI_{rab}) obviously consists of two terminal galactoses, three 1→3-linked galactoses, three 1→4-linked *N*-acetylglucosamines, one 1→4-linked glucose, and one 3,6-di-*O*-substituted hexose (Figure 3). Since the molar ratio of glucose/galactose/*N*-acetylglucosamine is 1:6:3, the latter hexose conclusively is a galactose, indicating the branching point in the oligosaccharide chain. Degradation by α-galactosidase produces a glycolipid, which in methylation analysis furnishes again two terminal galactoses but one 1→3-linked galactose; the number of 1→4-linked *N*-acetylglucosamines (3) and of 1→4-linked glucose (1), however, is unchanged (Figure 3). Reduction of 3 mol of 1→3-linked galactose to 1 mol by α-galactosidase degradation allows the conclusion that two terminal galactoses are linked 1→3 glycosidically to the penultimate sugar units. These penultimate sugars obviously both are galactoses, since after α-galactosidase degradation, methylation analysis again yields two terminal galactose residues. At least one of these galactoses (if not both) is linked β-glycosidically to the next sugar units, since incubation with β-galactosidase enables further degradation. The terminal sequence of hexose–hexose of both branches is confirmed by direct inlet mass spectrometry (Figure 5), producing the characteristic strong fragments of *m/e* 423 and *m/e* 391. Trisaccharide ions at *m/e* 668 and *m/e* 636 further exclusively establish the linkage of *N*-acetylglucosamine to each of the two terminal digalactosyl units. 1→4 linkage of *N*-acetylglucosamine is evaluated first by GC–MS analysis, which clearly excludes any other linkage. Furthermore, the neglegible intensity of *m/e* 228 during direct inlet mass spectrometry and the presence of an intense ion at *m/e* 182 strongly support the sole occurrence of the 1→4 substitution of all amino sugars. The lack of ions caused by tetra-, penta-, or hexasaccharide units and the appearance of hepta-, octa-, and nonasaccharide ions at *m/e* 1525, *m/e* 1770, and *m/e* 1974 strongly suggest that the above-mentioned trisaccharides both are linked to a hexose–hexosamine–hexose unit forming the corresponding hepta-, octa-, or nonasaccharide fragments. The double-substituted hexose is formed by a galactose, to which the above-mentioned trisaccharides are linked 1→3 or 1→6 glycosidically as previously shown by GC–MS. Ions at *m/e* 630 and *m/e* 830 suggest that two hexoses are linked to the ceramide part. All these major fragments of the spectrum and the results of the FD measurements giving the molecular weight are in good agreement with the following partial structure of a ceramide decasaccharide:



The data obtained by NMR analysis are in complete accordance with this structure and, in addition, identify the two-hexose head fragment as a lactose unit.

Finally, it should be stressed that such a detailed NMR analysis is only possible with the help of the knowledge accumulated on studying series of highly purified and structurally related compounds. In particular, the application of SDDS at high field opens new sources of structural information enabling one to elucidate the key features of the molecular structure of a glycosphingolipid by nondestructive experiments on ~1 mg of a native compound. The NOE is an additional powerful method of identifying the signals and determining the sequence, the site of glycosidic linkage, and the secondary structure. Although NMR is usually combined with other

FIGURE 7: Structure of the oligosaccharide part of the I-active and blood group B active ceramide decasaccharide from rabbit erythrocyte membranes (BI_{rab}). R = ceramide residue mainly consisting of nervonic acid and C_{18}-sphingosine.

analytical methods, it is interesting to see to what extent it can be independent of them. In the particular case of BI_{rab}, the sequence in the lactosyl head group could only be established by NMR, and the elucidation of the structure of the remaining fragments was possible without resorting to the data obtained by MS or enzymatic degradation.

In summary, the combination of all these analytical data listed above strongly support the structure of BI_{rab}, the oligosaccharide part of which is demonstrated in Figure 7. The close relationship to the ceramide pentasaccharide of rabbit erythrocytes (Eto et al., 1968; Stellner et al., 1973) and of bovine erythrocytes (Chien et al., 1978b; Uemura et al., 1978) ($IV^3Gal\alpha$-nLcOse$_4$Cer) is evident. Both glycosphingolipids have identical terminal trisaccharide units. Their neolactotetraosylceramide part also is identical. Fatty acid moieties and sphingosine base compositions of the rabbit red cell ceramide penta- and decasaccharide are strikingly similar. With regard to the branching region, analogous glycosphingolipids have previously been identified from human erythrocytes as H-(H_3) or A-(A_c) blood group active substances (Watanabe et al., 1975; Hakomori et al., 1972). It can be anticipated that an analogous ceramide dodecasaccharide (B-III) also exists in human B erythrocytes. This still hypothetical substance merely differs from BI_{rab} by two α1→2fucosyl units, each additionally linked to the two subterminal galactoses.

In accordance with the close structural relationship of *N*-acetylglucosamine-containing glycolipids of human, bovine, and rabbit erythrocyte origin (Hakomori & Kobata, 1974; Chien et al., 1978a,b; Chien et al., 1979; Hanfland et al., 1978b; Koscielak et al., 1973), also gangliosides sharing a lacto-*N*-isooctaosylceramide as core structure recently have been identified in human (Watanabe et al., 1978, 1979b) and in bovine (Watanabe et al., 1979a) erythrocytes; the latter merely differ from BI_{rab} by replacement of the terminal Galα1→3 unit of the 1→3 linked oligosaccharide branch by an α2→3 glycosidically linked *N*-acetyl- or *N*-glycolylneuraminic acid.

BI_{rab} is of immunological interest for the following reasons: first, when tested against some I antisera, the substance displays a strong I activity. Moreover, as mentioned above, the oligosaccharide backbone is identical with those of I-active ceramide deca or dodecasaccharides from bovine as well as from human erythrocyte origin. Therefore, it can be predicted that after α-galactosidase treatment, BI_{rab} will react with the majority of I antisera in a manner similar to that of the correspondingly degraded above-mentioned complex ganglioside from bovine erythrocytes (Watanabe et al., 1979a; Feizi et al., 1979). Due to the various degradation possibilities, beginning with either α-galactosidase or sialidase, the latter substance was more advantageous for the structural elucidation of different antigenic specificities of several I/i antisera (Watanabe et al., 1979a). However, the yield of BI_{rab} seems to be much higher. Furthermore, because there is a lack of gangliosides in rabbit erythrocytes and the glycolipids are less heterogeneous, the isolation procedure from rabbit erythrocytes is much easier and more rapid. In addition, the immediate acetylation of the Folch upper phase and the purification of the acetylated material by silicic acid column and silica gel high-performance thin-layer chromatography further simplify the isolation procedure. It can be expected that the use of Iatrobeads will further facilitate the isolation.

After purification, conventional deacetylation of the acetylated BI_{rab} by using 0.1% sodium methoxide in chloroform–methanol (2:1) was not complete. Up to today no evidence has been presented for the completeness of this reaction when applied to glycosphingolipids having more than six sugars. A series of experiments, using a variety of solvents and sodium—as well as barium—methoxide in various concentrations for different reaction times, finally lead to the very efficient deacetylation of the complex glycolipid. This method can also be used successfully for glycolipids having shorter carbohydrate chains, fucosyl residues included. Alteration of the original structures could not be observed.

Due to the high complexity, heterogeneity, and variety of the I/i antigen–antibody systems, a number of questions concerning structural, metabolical, and immunological aspects remain still unresolved. For example, there is still no assignment of serological subspecificities like I^F, I^T, I^S, and I^D as classified by Dzierzkowa-Borodej et al. (1975) to structurally well-defined homogeneous glycoconjugates. Secondly, a higher standardization of the radioimmunoassay measuring I activities of substances from different sources (Wood et al., 1979) may be achieved when, for example, α-galactosidase-degraded BI_{rab} coupled to a water-soluble matrix may be taken as labeled material in analogy to the determination of Forssman activity by radioimmunoassay (Young et al., 1979). Thus, it seems of particular importance to obtain a reference substance or a starting material for further I/i system studies which can easily and rapidly be obtained in a homogeneous form. BI_{rab} in degraded as well as in undegraded form offers this chance. Studies further simplifying the isolation and enlarging the yield of BI_{rab} now are being performed in our laboratories.

Acknowledgments

We thank Marie-Luise Corzelius and Monika Schmidt for their skillful technical assistance and Dr. T. Feizi, Medical Research Center, Harrow, England, for valuable discussion of the manuscript. The authors are indebted to Dr. W. E. Hull (Bruker Analytische Messtechnik, Karlsruhe-Forchheim, Germany) for recording the 500-MHz spectrum and for a few NOE and decoupling experiments confirming the findings obtained on the 360-MHz spectrometer and to Dr. A. Dell (Imperial College, London) for the field desorption measurements.

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