Enzymatic Determination of the Anomeric Structures of Two Blood-Group B Active Glycosphingolipids Recombined with Apolipoproteins

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Anomeric configuration of oligosaccharides usually is established by specific glycosidases. For this purpose detergents achieving water solubility of primarily insoluble glycosphingolipids as substrates have been replaced by delipidated human serum high density lipoproteins. The new method, tested by several well characterized glycosphingolipids and glycosidases, finally was applied to the evaluation of anomeric structures of two blood-group B active glycosphingolipids [ceramide hexasaccharide (B-I) and ceramide octasaccharide (B-II)] from human erythrocyte membranes. In both B-I and B-II, α-glycosidic linkage was demonstrated for the terminal galactose and fucose residues. β-glycosidic linkage has been evaluated for backbone saccharides. Together with the results previously obtained by composition analysis, linkage analysis and sequence analysis the following complete structure can be established:

B-I: $Gal\alpha1 \rightarrow 3Gal(2 \leftarrow 1\alpha Fuc)\beta1 \rightarrow 4GlcNAc\beta1 \rightarrow 3Gal\beta1 \rightarrow 4Glc\beta1 \rightarrow 1Cer$;

B-II: $Gal\alpha1 \rightarrow 3Gal(2 \leftarrow 1\alpha Fuc)\beta1 \rightarrow 4GlcNAc\beta1 \rightarrow 3Gal\beta1 \rightarrow 4GlcNAc\beta1 \rightarrow 3Gal\beta1 \rightarrow 4Glc\beta1 \rightarrow 1Cer$.

Introduction

The biological importance of glycosphingolipids concerning blood-group activities, cellular recognition, regulation of cell growth and malignant transformation [1, 2] has promoted the development of microscale characterization methods: combined gas-chromatography-mass spectrometry of partially methylated alditol acetates [3–5], direct inlet mass spectrometry of permethylated glycolipids [6–8] and enzymatic degradation by specific glycosidases [9, 10] allow to evaluate sugar linkage and sequence as well as anomeric configuration, respectively. Because of the water-insolubility of glycosphingolipids, detergents (taurocholate, cholate, Triton X-100) have been previously applied in enzymatic analysis [11–20]. In the present investigation, these detergents have been replaced by the use of delipidated human serum high density lipoproteins (apoHDL). Glycosphingolipid-apoprotein complexes proved to be suitable substrates for several glycosidases. Glycosphingolipids used at first for these purposes

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Abbreviations (in accordance with the IUPAC-IUB recommendations, Hoppe-Seyler's Z. Physiol. Chem. **358**, 617–631 [1977]): glycosphingolipids from human erythrocyte membranes: $LcOse_2Cer_{hu}$, ceramide disaccharide; $GbOse_3Cer_{hu}$, ceramide trisaccharide; $nLcOse_4Cer_{hu}$, lacto-N-neotetraosylceramide; $GbOse_4Cer_{hu}$, globoside; B-I, blood-group B active ceramide hexasaccharide; B-II, blood-group B active ceramide octasaccharide; H_b-I, ceramide pentasaccharide from B-I after α-galactosidase treatment; H_b-II, ceramide heptasaccharide from B-II after α-galactosidase treatment; $Tetra_b$-I, ceramide tetrasaccharide from H_b-I and $Hexa_b$-II ceramide, hexasaccharide from H_b-II after partial hydrolysis with 0.1 N trichloracetic acid or α-fucosidase treatment; $C5S_b$, ceramide pentasaccharide from B-I after α-fucosidase treatment; $C7S_b$, ceramide heptasaccharide from B-II after α-fucosidase treatment; glycosphingolipids from rabbit erythrocyte membranes: $GbOse_3Cer_{rab}$, ceramide trisaccharide; $C5S_{rab}$, ceramide pentasaccharide; $nLcOse_4Cer_{rab}$, lacto-N-neotetraosylceramide from $C5S_{rab}$ after α-galactosidase treatment; HDL, high density lipoprotein: apo HDL, delipidated high density lipoprotein.

all have been extensively characterized including their anomeric structures. Additionally, the new assay was applied to the evaluation of the anomeric configurations of two blood-group B active glycosphingolipids from human erythrocyte membranes. They represent a ceramide hexasaccharide (B-I), which already has been characterized by Koscielak *et al.*[21] and a ceramide octasaccharide (B-II). Composition, sugar linkage and sequence of both glycosphingolipids previously have been studied in our laboratories[22–24]. In consequence, the complete structure of B-II now can be presented for the first time.

Materials and Methods

Isolation of glycosphingolipids

$LcOse_2Cer_{hu}$, $GbOse_3Cer_{hu}$, $nLcOse_4Cer_{hu}$, $GbOse_4Cer_{hu}$, B-I and B-II were isolated from human erythrocyte membranes[22]. H_b-I and H_b-II were prepared from B-I and B-II by α-galactosidase treatment[23]. Removal of the terminal fucosyl residue by 0.1 N trichloracetic acid at 100 °C for 2 hours[25] from H_b-I and H_b-II yielded $Tetra_b$-I and $Hexa_b$-II, respectively[23]. $GbOse_3Cer_{rab}$ and $C5S_{rab}$ were isolated according to Eto *et al.*[26]. $nLcOse_4Cer_{rab}$ was obtained from ceramide pentasaccharide by α-galactosidase treatment (see below). Glucocerebroside was obtained after partial hydrolysis of a crude human ganglioside fraction (slightly contaminated by sulfatides) with 0.05 N HCl for 45 min at 100 °C. This cerebroside fraction was further purified by silicic acid column chromatography.

Enzyme sources

α-Galactosidase (E.C. 3.2.1.22) from coffee beans (10 U/mg), N-acetyl-β-D-hexosaminidase (E.C. 3.2.1.30) from bovine kidney (0.6 U/mg with *p*-nitrophenyl-N-acetyl-β-D-galactosaminide as substrate) and α-L-fucosidase (E.C. 3.2.1.51) from bovine kidney (2 U/mg) were purchased from Boehringer, Mannheim. β-Galactosidase from jack bean (0.25 U/mg) and N-acetyl-β-D-hexosaminidase (0.5 U/mg) from jack bean (isolated according to Li and Li[9]) were a generous gift from Prof. Lagrou and Dr. van Dessel, Laboratory of Biochemistry, University of Antwerpen. β-Galactosidase from Charonia lampas (1.1 U/mg) was purchased from Miles, Frankfurt. α-L-fucosidase from Charonia lampas (4.5 U/mg) was provided by Dr. P. Okuyama of Seikagaku Chemical Co., Tokyo.

Preparation of apo HDL

HDL used in these studies was obtained from the plasma of three male volunteers. The lipoproteins were isolated by preparative ultracentrifugal flotation between KBr densities 1.063 and 1.210 g/ml, and delipidated with chloroform-methanol, 2 : 1[27]. Apo HDL was solubilized in 0.05 M NH_4HCO_3 buffer, pH 8.8 (5 mg/ml).

Reassembly procedure

Individual glycosphingolipids (250 μg/ml) were sonicated in water and yielded clear dispersions on sonication for 5 – 10 min. Apo HDL was added to the sonicated glycosphingolipids (2 : 1 w/w) and then subjected, in an ice bath and under nitrogen, to short sonication (3 × 10 sec) with a microtip probe of a Branson sonifier at a probe setting of 5. The sonicated protein-lipid solutions were dialysed against 1.000 volumes of incubation buffers (0.05 M) at 4 °C and used within 4 hours for enzymatic degradation.

Enzymatic degradation

After dialysis of glycosphingolipid-apoprotein complexes enzyme protein was added and the resulting solutions were sterilized by millipore ultrafiltration (0.40 μm) at 4 °C. Incubation was performed at 37 °C for 18 or 36 hours in sterile glass tubes containing a magnetic stirring bar with screw caps teflon lined on the inside (see also Table I). Incubations were stopped by freezing. After lyophilization, glycosphingolipid reaction products were extracted with chloroform-methanol 2 : 1 v/v, 1 : 2 v/v and/or chloroform-methanol-water 1 : 1 : 0.1. Glycosphingolipids were analyzed by chromatography with authentic compounds. For glucocerebroside identification, borate plates were used with chloroform-methanol-water-15 M NH_4OH (280 : 70 : 6 : 1) as solvent system[28]. The spots were visualized by spraying with 5% sulfuric acid in water and heating at 125 °C or with anthrone reagent[29]. Substrate-product ratios were estimated by the degree of disappearance of the original glycosphingolipid and by the appearance of the new glycolipid as a degradation product.

Results and Discussion

The conversion rates of glycosphingolipids after incubation in the presence of apolipoproteins or sodium taurocholate are listed in Table I. In most experiments, degradation of glycosphingolipids was observed, when apolipoprotein-glycolipid mixtures were employed as substrates for individual enzymes. Fig. 1 demonstrates the degradation of $nLcOse_4Cer_{rab}$ to ceramide disaccharide as major and ceramide trisaccharide as minor reaction products in the

Table I. Enzyme-glycolipid incubation mixtures, reaction products and incubation conditions. See also "Materials and Methods". α-gal'ase c.b.: α-galactosidase from coffee bean, β-gal'ase j.b.: β-galactosidase from jack bean, β-h'ase j.b.: β-N-acetyl-hexosaminidase from jack bean, β-gal'ase c.l.: β-galactosidase from Charonia lampas, β-h'ase b.k.: β-N-acetyl-hexosaminidase from bovine kidney, α-fuc'ase b.k.: α-fucosidase from bovine kidney, α-fuc'ase c.l.: α-fucosidase from Charonia lampas.

No.	Substrate [100 μg]	Enzyme	Amount of enzyme [units]	Product	Conversion rate [%]	Buffer	pH	Incubation time [hr]
1	$GbOse_3Cer_{hu}$	α-gal'ase c.b.	2	$LcOse_2Cer$	50	Na phosphate	6.5	18
2	$GbOse_3Cer_{rab}$	α-gal'ase c.b.	2	$LcOse_2Cer$	50	Na phosphate	6.5	18
3	$nLcOse_4Cer_{hu}$	β-gal'ase j.b.	0.1	$LcOse_3Cer$	80	Na citrate	4.5	18
4	$nLcOse_4Cer_{hu}$	β-gal'ase c.l.	0.25	$LcOse_3Cer$	80	Na citrate	4.0	18
5	$nLcOse_4Cer_{hu}$	β-gal'ase +β-h'ase j.b.	0.4+0.4	$LcOse_2Cer$ (Cerebroside)	80 (10)	Na citrate	4.5	36
6	$nLcOse_4Cer_{hu}$	β-gal'ase j.b.	0.1	$LcOse_3Cer$	80	Na citrate	4.5	18
7	$nLcOse_4Cer_{hu}$	β-gal'ase c.l.	0.25	$LcOse_3Cer$	80	Na citrate	4.0	18
8	$nLcOse_4Cer_{rab}$	β-gal'ase +β-h'ase j.b.	0.1+0.1	$LcOse_2Cer$	80	Na citrate	4.5	18
9	$GbOse_4Cer_{hu}$	β-h'ase b.k.	1.5	$GbOse_3Cer$	50	Na citrate	4.5	18
10	$C5S_{rab}$	α-gal'ase c.b.	1	$nLcOse_4Cer$	100	Na phosphate	6.5	18
11	B-I	α-gal'ase c.b.	1	H_b-I	100	Na phosphate	6.5	18
12	B-II	α-gal'ase c.b.	1	H_b-II	100	Na phosphate	6.5	18
13	B-I	α-fuc'ase b.k.	2	$C5S_b$	traces	Na citrate-phosphate	5.0	36
14	B-II	α-fuc'ase b.k.	2	$C7S_b$	traces	Na citrate-phosphate	5.0	36
15	B-I	α-fuc'ase c.l.	0.25	∅	0	Na citrate	4.0	36
16	B-II	α-fuc'ase c.l.	0.25	∅	0	Na citrate	4.0	36
17	$Tetra_b$-I	β-gal'ase +β-h'ase j.b.	0.4+0.4	$LcOse_2Cer$ (Cerebroside)	80 (10)	Na citrate	4.5	36
18	$Hexa_b$-II	β-gal'ase +β-h'ase j.b.	0.4+0.4	$LcOse_2Cer$ (Cerebroside)	80 (10)	Na citrate	4.5	36
19	H_b-I	α-fuc'ase c.l.	0.25	$Tetra_b$-I	60	Na citrate	4.0	18 *
20	H_b-II	α-fuc'ase c.l.	0.25	$Hexa_b$-II	80	Na citrate	4.0	18 *

* Incubation without apo HDL in presence of Na taurocholate [taurocholate-glycolipid ratio 1 : 1 (w/w)].

presence of jack bean β-galactosidase and β-hexosaminidase. Within 18 hours of incubation, only negligible amounts of ceramide monosaccharide were formed. Control experiments proved, that the degradation of glycosphingolipids was largely dependent on the presence of apolipoprotein-glycolipid complexes (Fig. 1, lane b), whereas in the incubation mixture only trace amounts of reaction products were formed in the absence of apoproteins (Fig. 1, lane a).

B-I and B-II, reassembled with apo HDL and incubated with coffee bean α-galactosidase, quantitatively yielded H blood-group active ceramide pentasaccharide H_b-I and ceramide heptasaccharide H_b-II, respectively. Appreciable degradation of H_b-I and H_b-II by α-fucosidases from different sources to the corresponding ceramide tetrasaccharide $Tetra_b$-I or ceramide hexasaccharide $Hexa_b$-II, however, was only observed in the presence of sodium taurocholate as detergent. Alternatively, $Tetra_b$-I and $Hexa_b$-II were also obtained from H_b-I and H_b-II by partial hydrolysis with 0.1 N trichloracetic acid. Further degradation studies of B-I and B-II were exclusively performed with glycosphingolipid-apolipoprotein complexes. Fig. 2 demonstrates degradation of $Tetra_b$-I (lane b) and $Hexa_b$-II (lane c) down to glucocerebroside with ceramide dihexoside being the most abundant reaction product. The formation of glucocerebroside was found to be dependent on prolonged incubation periods (36 hours). Galactocerebroside could not be detected as reaction product. The incubation was performed with a mixture of β-galactosidase and β-N-acetyl-hexosaminidase from jack bean, thus permitting the consequence, that all saccharide linkages of $Tetra_b$-I and $Hexa_b$-II are of β-glycosidic nature.

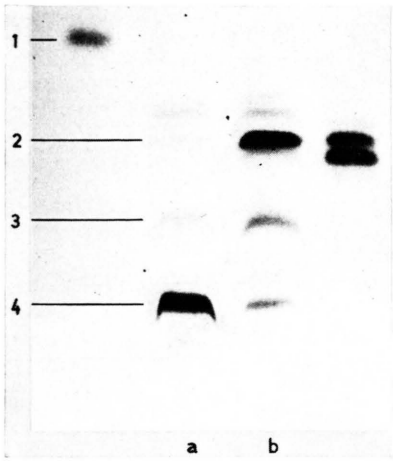

Fig. 1. Enzymatic degradation of $nLcOse_4Cer_{rab}$ with a mixture of jack bean β-galactosidase and β-N-acetyl-hexosaminidase (assay no. 8, Table I). Lane a: incubation mixture in the absence of apolipoproteins; lane b: incubation mixture in the presence of apolipoproteins. 1: cerebroside; 2: ceramide disaccharide; 3: ceramide trisaccharide; 4: ceramide tetrasaccharide. The remaining bands arise from anthrone negative contaminants of the enzyme source.

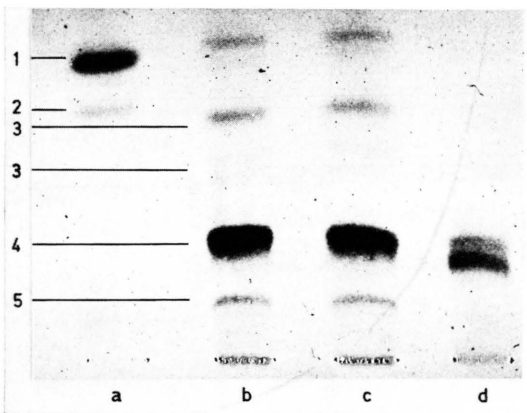

Fig. 2. Borate-silica gel-thin layer chromatography of $Tetra_b$-I and $Hexa_b$-II after 36 hours incubation with a mixture of jack bean β-galactosidase and β-N-acetyl-hexosaminidase (assay no. 17 and 18, Table I). Lane a: cerebroside mixture; lane b: $Tetra_b$-I after incubation; lane c: $Hexa_b$-II after incubation; lane d: ceramide disaccharide from human erythrocytes. 1: glucosyl ceramide; 2: galactosyl ceramide; 3: anthrone negative contaminant of enzyme source; 4: ceramide disaccharide; 5: ceramide trisaccharide.

The results obtained from composition analysis [22, 23], linkage analysis by combined gas chromatography-mass spectrometry of partially methylated alditol acetates [23], sequence analysis of permethylated glycolipids [24] and from the enzymatic evaluation of the anomeric configuration, presented in this paper, allow to deduce the following structures, for B-I:

α-D-galactopyranosyl-(1 → 3)-[α-L-fucopyranosyl-(1 → 2)]-β-D-galactopyranosyl-(1 → 4)-N-acetyl-β-D-glucosaminosyl-(1 → 3)-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 1)-ceramide

and for B-II:

α-D-galactopyranosyl-(1 → 3)-[α-L-fucopyranosyl-(1 → 2)]-β-D-galactopyranosyl-(1 → 4)-N-acetyl-β-D-glucosaminosyl-(1 → 3)-β-D-galactopyranosyl-(1 → 4)-N-acetyl-β-D-glucosaminosyl-(1 → 3)-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 1)-ceramide.

Sphingoside and C_{24}-fatty acid are the main components of the ceramide residues of both glycolipids [23, 24]. The complete structures of these erythrocyte membrane antigens are demonstrated in Fig. 3. The results presented confirm the structure of B-I previously evaluated by Koscielak *et al.* [21]. The complete structure of B-II is presented for the first time.

A further purpose of this paper is to illustrate the usefulness of glycosphingolipid-lipoprotein complexes in enzymatic analysis of the anomeric structure of the oligosaccharide moiety. Previously, in enzymatic analysis of glycosphingolipids, taurocholate, cholate and Triton X-100 have been used to solubilize the respective compounds [15–17]. However, tedious search for the adequate detergent mixture is often inconvenient [17, 20, 23, 30–32] and inhibition of enzymes may occur. It is suggested that hydrophobic interaction between the ceramide moiety and lipophilic surface sides of apolipoprotein [33, 34] facilitates the solubility of glycosphingolipids without perturbation of enzymatic activity.

The usefulness of reconstituted glycosphingolipid-apolipoprotein complexes is further emphasized by the ease, rapid isolation and high yield of reaction products without detergents as contaminants. Complete analysis of complex (branched) glycosphingolipids requires stepwise degradation of reaction products, which, in general, are available in only minor amounts. Presence of detergents, however, diminishes purity and yield of the reaction products and impairs subsequent analytical procedures especially methylation studies.

Another important aspect is the application of recombined glycosphingolipid-apoproteins to the diagnosis of glycosphingolipid storage diseases.

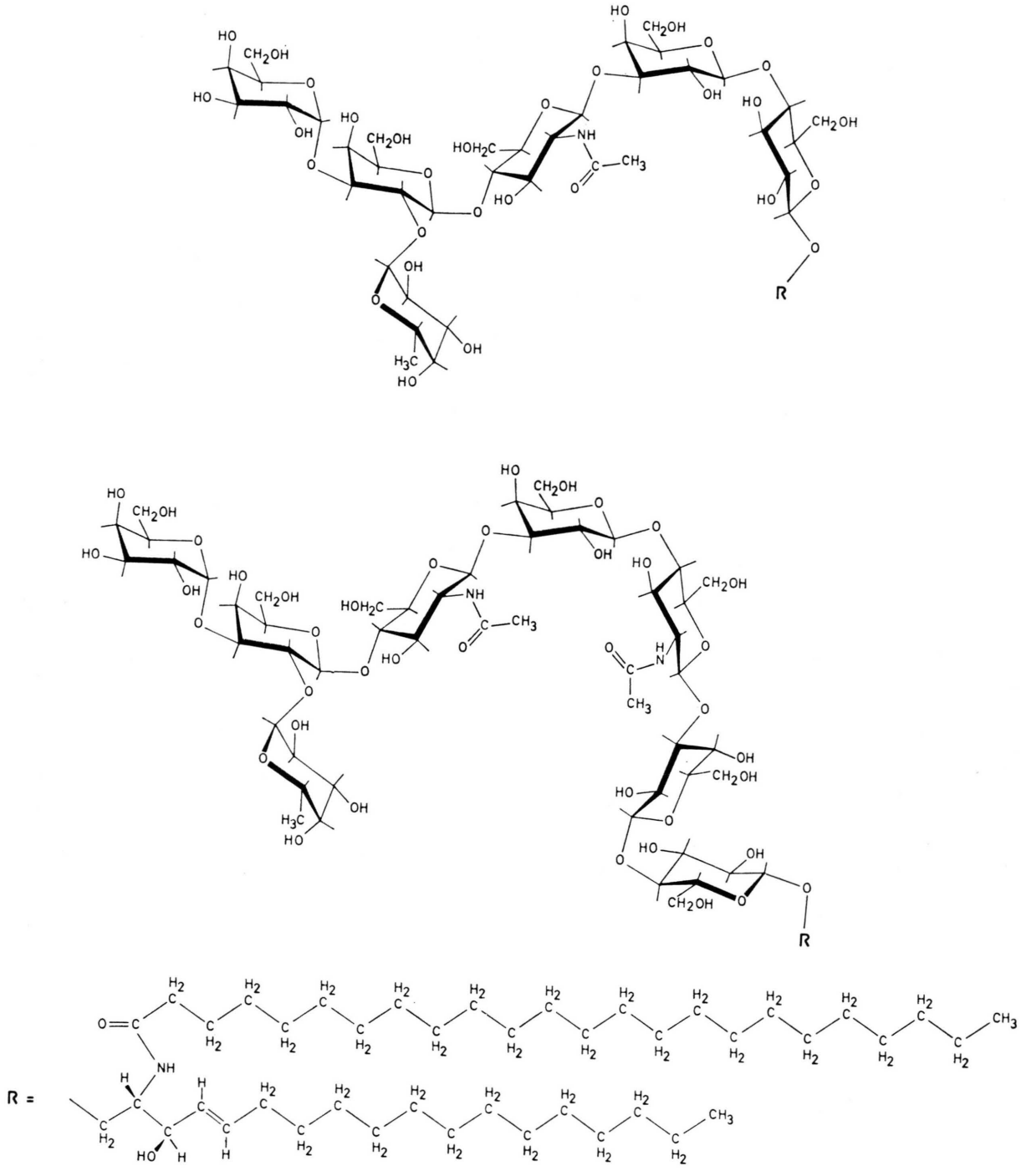

Fig. 3. Structures of blood-group B active ceramide hexa- (B-I) and octa-saccharide (B-II) of human erythrocyte membranes. They are based on composition analysis [22, 23], linkage analysis determined by combined gaschromatography-mass spectrometry [23] and sequence analysis by direct inlet mass spectrometry [24]; evaluation of anomeric configuration is presented in this paper.

Studies employing α-galactosidase (Fabry disease), β-galactosidase (GM_1-gangliosidosis) and β-N-acetyl-hexosaminidase (Tay-Sachs disease) in the degradation of apolipoprotein-glycosphingolipid substrates now are intended to perform in our laboratories.

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