Ceramide Di- and Trihexosides of Wheat Flour†

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ABSTRACT: Ceramide di- and trihexosides were isolated from wheat flour; they amounted to 0.2 and 0.3%, respectively, of the total lipids. Their properties were compatible with structures of *O*-β-D-mannopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→1)-ceramide and *O*-β-D-mannopyranosyl-(1→4)-*O*-β-D-mannopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→1)-ceramide. The ceramide units of the molecules consisted of D-2-hydroxy fatty acids and of derivatives of sphinganine and 4-sphingenine. The structural analysis involved (1) gas–liquid chromatography of the component sugars, fatty acids, and long-chain bases; (2) thin-layer chromatography of the ceramides to establish the configuration of the fatty acids at C-2; (3) partial acetolysis to establish the sequence of the hexoses and their pyranose structures; (4) permethylation studies coupled with gas–liquid chromatography and mass spectrometry to locate the bonds between the hexose units; and (5) polarimetry and CrO_3 oxidation of noncrystalline, fully acetylated glycolipids to establish the stereochemistry of the hexose units and the anomeric configuration of the glycosidic links.

Wheat flour ceramide hexosides have been studied more thoroughly than other plant sphingolipids. Carter *et al.* (1961a) showed that glucosyl ceramide, previously considered a typically animal lipid, is present. They found that 2-hydroxy fatty acids and sphinganines, D-4-hydroxysphinganines, and related molecules are the building blocks of the ceramide portion of this lipid (Carter *et al.*, 1961a). Their data on the optical rotation of the intact lipid suggested that the sample may have been a D-glucopyranosyl ceramide. Subsequent work by Carter's group (1961b), by MacMurray and Morrison (1970), and by ourselves (Laine and Renkonen, 1973) showed that even molecules resembling animal ceramide oligosaccharides may be present in wheat flour. In the present report we describe in detail the structures of ceramide di- and trisaccharides. As suggested by Carter *et al.* (1961b) these molecules proved to be examples of mannose containing ceramide hexosides previously found only in shellfish (Hori *et al.*, 1968) and in the glycolipids of cultured insect cells (Luukkonen *et al.*, 1973).

Materials and Methods

Two samples of wheat flour (Manitoba II, 1969, and a mixture of soft wheat varieties, 1972) were obtained from the State Granary (Helsinki). They had not been subjected to any previous chemical treatment.

The samples were extracted overnight at 20°, once with 200 volumes with chloroform–methanol (2:1) and once with chloroform–methanol (1:2). The alkali-stable lipids were isolated as described previously (Laine and Renkonen, 1973).

Isolation of Ceramide Mono-, Di-, and Trihexosides of Wheat Flour. Three major glycolipid zones were present in the alkali-stable fraction. These were separated on silica gel G plates with chloroform–methanol–7 N ammonia (65:25:4 v/v). Care was taken to collect fairly wide zones in order to include all molecular species. The lipids eluted from the adsorbent required further purification: the ceramide monosaccharide was contaminated with a sterol glucoside, which was removed, after acetylation, on thin-layer chromatography (Morrison, 1973); the ceramide di- and trisaccharides were purified from contaminating phospholipids by the procedure of Saito and Hakomori (1971).

Analysis of Fatty Acids, Long-Chain Bases, and Carbohydrates. Quantitation of long-chain bases (Lauter and Trams, 1962) and of hexoses (Scott and Melvin, 1953) was carried out by standard colorimetric methods. Hexoses were also measured by quantitative gas–liquid chromatography after cleavage according to Yang and Hakomori (1971) and subsequent conversion of the liberated hexoses to alditol acetates (Sawardeker *et al.*, 1965). Inositol was used as an internal standard. Fatty acids of the sphingoglycolipids were converted into methyl esters as described elsewhere (Renkonen, 1965) and subsequently estimated colorimetrically (Renkonen, 1961).

Compositional analysis of the long chain bases was carried out by gas–liquid chromatography of *N*-acetyl-*O*-trimethylsilyl ether derivatives of the bases liberated after hydrolysis with 1 N HCl in 10 M H_2O in methanol (Gaver and Sweeley, 1965). The major bases were rather poorly separated on SE-30 (Laine and Renkonen, 1973).

For compositional analysis the fatty acids were converted into methyl esters (Renkonen, 1965) and these were separated with preparative thin-layer chromatography on silica gel G with hexane–ether (85:15 v/v) as solvent. After recovery from the adsorbent the methyl esters of unsubstituted fatty acids were directly analyzed by gas–liquid chromatography (Renkonen, 1965), whereas the methyl esters of 2-hydroxy fatty acids were first silylated (Carter and Gaver, 1967) and then subjected to gas–liquid chromatography on a 4-m column of 3% SE-30 on Gas-Chrom Q at 230°. Internal standards, methyl heneicosanoate (21:0) and methyl 2-hydroxybehenate, were added to the lipids before hydrolysis. The configuration of the 2-hydroxy fatty acids at C-2 was established by thin-layer chromatography (Karlsson and Pascher, 1971).

The component hexoses of the lipids were liberated by the method of Yang and Hakomori (1971) and analyzed by the method of Sawardeker *et al.* (1965).

Partial Acetolysis of the Lipids. The ceramide oligosaccharides (0.2 mg) were treated with 0.30 ml of 0.5 N H_2SO_4 in 90% aqueous acetic acid for 1 hr at 80°. Chloroform (1 ml) and water (1 ml) were added. The two layers were separated and the lipids were deacetylated by mild alkaline methanolysis

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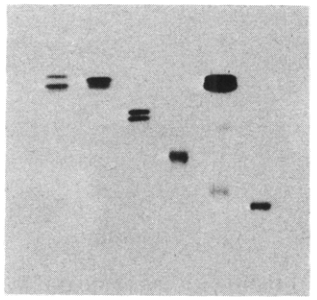

FIGURE 1: Thin-layer chromatography of alkali-stable glycolipids from wheat flour: (I) ceramide galactoside from ox brain; (II) alkali-stable glycolipids from wheat flour (50 μg); (III) ceramide dihexoside from human red blood cells; (IV) ceramide trihexoside from human red blood cells; (V) alkali-stable glycolipids from wheat flour (300 μg); (VI) globoside from human red blood cells. The precoated silica gel plate [Merck] was developed with chloroform–methanol–7 N aqueous NH_4OH (65:25:4) and stained with aniline–diphenylamine (Bailey and Bourne, 1960).

(Renkonen, 1963). The deacetylated lipids were fractionated by thin-layer chromatography and the ceramide mono-, di-, and trihexosides were isolated. The individual lipids, and also the water soluble fraction, were then subjected to complete hydrolysis and the component hexoses were analyzed using gas–liquid chromatography.

Methylation Analysis of the Glycolipids. The glycolipids were permethylated in dimethyl sulfoxide by methyl sulfinyl carbanion base and methyl iodide (Hakomori, 1964; Hellerqvist *et al.*, 1968). The methylated lipids were isolated by partitioning the reaction mixture between chloroform and water. The subsequent acetolysis and hydrolysis were carried out according to Yang and Hakomori (1971). The liberated carbohydrates were reduced with $NaBH_4$ and acetylated. In some experiments the reduction was carried out with $NaBD_4$ (Merck) in D_2O (Norsk hydroelektrisk kvaelstof aktieselskab). The partially methylated alditol acetates were analyzed with gas–liquid chromatography on 3% ECNSS-M or on 1% OV-225 on Gas-Chrom Q.

Gas–liquid chromatography–mass spectrometry was carried out with a Varian 1700 gas chromatograph coupled to a Varian MAT CH-7 mass spectrometer and to spectrosystem 100 MS computer. Ionization potential was 70 eV, and ionization current was 300 μA.

CrO_3 Oxidation. Chromium trioxide oxidation of acetylated glycolipids with myoinositol hexaacetate as internal standard was performed as described by Hoffman *et al.* (1972). The sonication time was 15 min in an ultrasonic cleaning bath at 40°.

Measurement of Optical Rotation. Specific rotations were determined at 20° in chloroform using fully acetylated derivatives of the lipids. A Perkin-Elmer polarimeter, Type 141, with a thermostated cuvet of 10-cm optical path and a volume of 0.9 ml was used. The molecular rotations were calculated by using 691 as the molecular weight of the acetylated ceramides. This value is based on the fatty acid composition of the ceramides reported in this paper and on the long-chain base composition reported previously (Laine and Renkonen, 1973). An increment of 288 was added for each additional acetylated hexose unit of the glycolipids.

The ceramides were prepared from the ceramide hexosides by Smith degradation as described previously (Renkonen, 1969).

Infrared spectra were recorded with a Perkin-Elmer Infracord Type 457; the samples were in the form of a film on potassium bromide.

Reference Lipids. Ceramide di- and trihexosides as well as globoside were isolated from human red blood cells according to the procedure of Saito and Hakomori (1971). The fatty acids of the red blood cell ceramide dihexoside were unsubstituted C_{16}–C_{24} acids and those of the red blood cell ceramide trihexoside were unsubstituted molecules of 22–24 carbon atoms. Our findings concerning the fatty acids of the red blood cell lipids agree with those of Tschöpe (1973). *O*-β-D-Glucosyl-(1→1)-ceramide was isolated from a sample of spleen tissue of a patient suffering from Gaucher's disease (Rosenberg and Chargaff, 1958). *O*-β-D-Galactosyl-(1→1)-ceramide was isolated from ox brain; it was further separated with thin-layer chromatography into two fractions, the fast migrating component with unsubstituted fatty acids and the slow component with D-2-hydroxy fatty acids.

The D,L-2-hydroxystearoylsphingenine was obtained from Dr. K. A. Karlsson, Göteborg, Sweden. Ceramides containing unsubstituted fatty acids were obtained by hydrolysis of serum sphingomyelin with phospholipase C (Renkonen, 1965).

3-*O*-[-α-D-Galactopyranosyl-(1′ → 2′)-*O*-α-D-glucopyranosyl]-*sn*-1,2-diacylglycerol and 3-*O*-[α-D-glucopyranosyl-(1′→2′)-*O*-α-D-glucopyranosyl]-*sn*-1,2-diacylglycerol from bacteria were obtained from Dr. N. Shaw, Newcastle upon Tyne, England.

Reference Carbohydrates. The following carbohydrates were purchased from commercial sources and used as reference materials in the methylation analysis: glucose, galactose, mannose, lactose, and gentiobiose. 4-*O*-β-D-Mannopyranosyl-D-mannose was obtained from Dr. E. Sjöström, Helsinki, Finland. Partially methylated alditol acetates as obtained from these compounds were used as reference standards.

Results

Isolation and Preliminary Characterization of Wheat Flour Ceramide Hexosides. The alkali stable fraction of wheat flour lipids amounted to 0.043 ± 0.015% of the flour weight (mean ± half of the range; $n = 3$).[1] From this fraction three ceramide glycosides were isolated by thin-layer chromatography after some phospholipids and a sterol glucoside had been removed by special procedures. Pure ceramide monohexosides amounted to about 55%, ceramide dihexosides to about 5%, and ceramide trihexosides to about 7% of the total alkali-stable fraction ($n = 1$). These three lipids were first identified and characterized in a preliminary manner by thin-layer chromatography, infrared spectra, and chemical analysis of the molar ratios of the constituent fatty acids, long-chain bases, and hexose units.

Thin-layer chromatography showed that the ceramide monohexoside of wheat (Figure 1, samples II and V, the major spot) has a migration rate similar to that of *O*-β-D-galactosyl ceramide and *O*-β-D-glucopyranosyl ceramide. The wheat flour ceramide dihexoside (Figure 1, sample V, the second fastest, faint spot) had a slightly lower mobility than that of the ceramide dihexoside of human red blood cells. The ceramide trihexoside of wheat (Figure 1, sample V, the third fastest, faint spot) had a slower migration rate than ceramide trihexoside of red blood cells, but it migrated faster than the globoside of the blood cells. The fourth spot of sample V which is faintly visible

[1] Abbreviations used are: *n*, number of samples analyzed; CMH, ceramide monohexoside; CDH, ceramide dihexoside; CTH, ceramide trihexoside. The nomenclature used for the carbohydrates is that proposed by the IUPAC–IUB Commission (1971).

TABLE I: Molar Ratios of Component Hexoses of Wheat Lipids.

Lipid	Glucose	Mannose[a]
Ceramide monohexoside	1.00	0.00 (n = 3)
Ceramide dihexoside	1.00	0.91 ± 0.13[b] (n = 6)
Ceramide trihexoside	1.00	1.75 ± 0.25[b] (n = 6)

[a] Mean ± SD. [b] Traces of galactose were occasionally seen also.

in Figure 1 was probably a ceramide tetrasaccharide.

The infrared spectra of acetylated ceramide hexosides of wheat resembled those of reference samples of acetylated ceramide glucoside and of acetylated ceramide di- and trihexosides (not shown). None of the wheat lipids showed an absorption peak at 844 cm^{-1} which suggests that they did not contain α-glycosidic linkages (Barker *et al.*, 1954). The ceramide trihexoside of human red blood cells revealed a small absorption peak at 845 cm^{-1} as expected (Hakomori *et al.*, 1971), but so did the ceramide dihexoside of red blood cells though it should not contain any α-glycosidic linkages. These findings suggest that our samples were too small and too impure to give reliable spectra in the range 840–920 cm^{-1}. Hakomori *et al.* (1971) have also reported inconsistencies in this area of infrared spectra of ceramide glycosides.

Assay of the constituent fatty acids, long-chain bases, and hexose units revealed that these building blocks were present in the ceramide dihexoside of wheat in a molar ratio of 1.0:0.91:2.0 and in the ceramide trihexoside in a ratio of 1.0:1.0:2.7.

The carbohydrate of the monohexoside was identified by gas–liquid chromatography as glucose (Table I). The ceramide disaccharide revealed equimolar amounts of glucose and mannose, and the ceramide trisaccharide contained glucose and mannose in a ratio of 1:2. The lipid tentatively identified as ceramide tetrasaccharide also contained glucose and mannose.

The long-chain bases have been previously analyzed from the mixed ceramide hexosides of wheat; they are a mixture of sphinganine and 4-sphingenine derivatives (Laine and Renkonen, 1973). Gas–liquid chromatography suggested that the individual ceramide hexosides contained similar mixtures of long-chain bases (data not shown).

All the ceramide hexosides were similar in their fatty acid composition, too; they contained predominantly 2-hydroxy fatty acids (Table II). The major individual components were

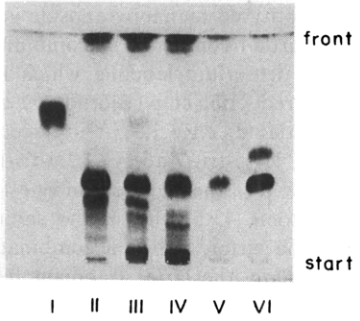

FIGURE 2: Thin-layer chromatography of ceramides: (I) ceramides containing unsubstituted fatty acids; (II) Smith degradation product of wheat flour CMH; (III) Smith degradation product of wheat flour CDH; (IV) Smith degradation product of wheat flour CTH; (V) Smith degradation product of brain CMH containing D-2-hydroxy fatty acids; (VI) synthetic D,L-2-hydroxystearoylsphingenine. The plate (silica gel G) was developed with chloroform–methanol (95:5) and stained with 20% ammonium hydrogen sulfate (Ziminski and Borowski, 1966).

TABLE II: Fatty Acid Composition (%) of Ceramide Hexosides of Wheat.

Fatty Acid[a]	Ceramide Monohexoside	Ceramide Dihexoside	Ceramide Trihexoside
16:0	0.5	0.7	0.2
16:1		0.2	0.1
18:0	0.2	0.8	0.1
18:1	0.3	3.1	0.5
18:2	0.4	1.0	1.0
20:0	0.2	0.2	0.1
22:0	0.1		
23:0	0.1		
24:0	0.2		
h16:0	62	62	60
h18:0	7.6	6.6	7.0
h19:0	2.1	1.3	0.7
h20:0	26	24	30

[a] The first numeral indicates chain length, the second indicates the number of double bonds, h = 2-hydroxy.

2-hydroxypalmitic and 2-hydroxyarachidic acid. The small amounts of unsubstituted fatty acids observed (Table II) were probably true components of the ceramide glycosides since Smith degradation of the lipids gave trace amounts of ceramides which contained unsubstituted fatty acids (Figure 2). The glycerolipids of wheat flour contained mainly linoleic acid and palmitic acid (R. Laine, unpublished observations).

Thin-layer chromatography of the constituent ceramides showed also that the wheat lipids contained D-2-hydroxy fatty acids (Figure 2). Ceramides containing L-2-hydroxy fatty acids move faster than those containing D-2-hydroxy fatty acids (Karlsson and Pascher, 1971).

Sequence of the Hexose Units. A preliminary experiment showed that 1-hr acetolysis of the wheat flour lipids produced a complete series of partially deglycosylated derivatives, whereas longer reaction times resulted in a too extensive hydrolysis (Figure 3). The reaction mixtures obtained after partial cleavage were fractionated so that each of the lipid components present, and also the lipid-free carbohydrates, were isolated. The monosaccharide composition of all these fractions was then determined. Both the ceramide dihexoside and the ceramide trihexoside gave a lipid-free carbohydrate fraction which contained mainly mannose (Table III). Both gave ceramide monohexoside fractions which contained only glucose. The ceramide trihexoside yielded in addition a ceramide dihexoside

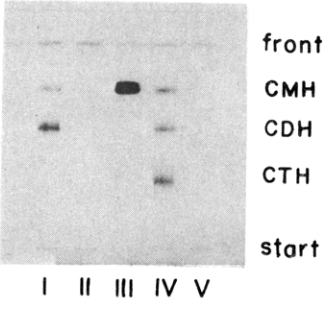

FIGURE 3: Thin-layer chromatography of partial acetolysates of wheat flour ceramide saccharides: (I) 1-hr acetolysis of wheat flour CDH; (II) 4-hr acetolysis of wheat flour CDH; (III) wheat flour CMH; (IV) 1-hr acetolysis of wheat flour CTH; (V) 4-hr acetolysis of wheat flour CTH. The precoated silica gel G plate was developed with chloroform–methanol–7 N NH_4OH and stained with aniline–diphenylamine (Bailey and Bourne, 1960).

TABLE III: Hexose Composition (%) of Different Fractions Isolated from Partial Acetolysates of Ceramide Hexosides of Wheat.

Substrate Acetolyzed	Component Hexose	Fractions Obtained from the Acetolysates: Lipid-Free Carbohydrate	Ceramide Monohexoside	Ceramide Dihexoside	Ceramide Trihexoside
Ceramide dihexoside	Glucose	36	100	46	
	Mannose	64	0	54	
Ceramide trihexoside	Glucose	0	100	54	40
	Mannose	100	0	46	60

which contained equimolar amounts of glucose and mannose. These observations show that the ceramide dihexoside was a mannosylglucosyl ceramide, and the trihexoside a mannosylmannosylglucosyl ceramide.

Ring Size of the Hexose Units. Naturally occurring furanosic glucose and mannose units appear to be so rare (Green, 1966) that their pyranosic structure is almost self-evident even in the wheat ceramide hexosides.

Methylation analysis (see below) showed that the distal hexoses in all three ceramide hexosides of wheat indeed had pyranose rings. This was evidenced by the formation of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitols; the 1,4-di-*O*-acetyl-2,3,5,6-tetra-*O*-methylhexitols were not formed.

The fact that all glycosidic linkages of the wheat lipids appeared to be cleaved at fairly similar rates suggests that also the inner hexose units are pyranosic; furanosic units should be cleaved faster than the corresponding pyranose structures both by hydrolysis and by acetolysis (Green, 1966; Janson and Lindberg, 1960). The similarity in the rate of cleavage of all glycosidic linkages of the wheat lipids during acetolysis is shown by the formation of approximately equal amounts of all possible products of partial deglycosylation (Figure 3). The small glucose content of the lipid-free carbohydrate fraction isolated from the acetolysates confirms this also (Table III): the bond between glucose and ceramide obviously was not broken faster than the other glycosidic links of the two lipids.

Positions of the Glycosidic Linkages in the Carbohydrate Chains. Upon methylation and subsequent hydrolysis, reduction, acetylation, and gas–liquid chromatography the ceramide trihexoside gave three peaks of partially methylated alditol acetates (peaks A, B, and C in Figure 4) which were of approximately equal sizes. The relative areas of the peaks were 1.0:0.8 (±0.1):1.0 (±0.2) (mean ± half of the range; n = 2). The peaks running faster than A remained unidentified.

The methylation analysis of the ceramide dihexoside gave peaks A and C in relative areas 1.0:0.6 (±0.02) (mean ± half of the range; n = 2), and that of the ceramide monohexoside gave only peak A. Subsequent analysis with a combination of gas–liquid chromatography and mass spectrometry showed that peak A was derived from the distal hexose unit of the lipids, which was either glucose (in ceramide monohexoside) or mannose (in ceramide di- and trihexoside). Peak B was formed from the internal mannose unit of the ceramide trihexoside, and peak C was formed from the internal glucose of the ceramide di- and trihexoside. Peak B of the ceramide trihexoside and peak C of the ceramide dihexoside were slightly smaller than expected. This suggests small contamination: galactose was occasionally observed among the hydrolysis products of these lipids (see Table I), and the presence of, *e.g.*, a mannose-galactose-glucose ceramide lipid would give rise to a peak B which is too small. Another possibility is that the analysis was contaminated with glucose which would explain the large size of peak A.

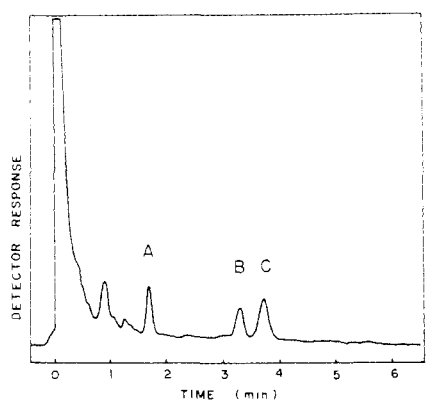

FIGURE 4: Gas–liquid chromatography of partially methylated alditol acetates from wheat flour ceramide trihexoside. Conditions for Glc: 2-m 1% OV-225 on Gas-Chrom Q, temperature 190°.

Mass spectrometry confirmed that peak A derived from ceramide mono-, di-, and trihexosides was in all cases a 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol. The major fragments (Figure 5) were in all cases those reported to be characteristic to these molecules (Björndal *et al.*, 1967a). The fragmentation patterns of 1,4-di-*O*-acetyl-2,3,5,6-tetra-*O*-methylhexitols are reportedly quite different (Björndal *et al.*, 1967a). These observations confirm that the distal hexose units were of pyranose structure in all three ceramide hexosides of wheat. The chromatographic properties of peak A were the same as those of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol and of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol (Table IV).

The mass spectrum of peak B (Figure 5) was identical with that obtained from a reference sample of a 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol (mannitol). The spectrum was also different from those reported by Björndal *et al.* (1967a) for the other isomers of triacetyltrimethylhexitols which can be derived from monosubstituted mannopyranoside units. The identity of peak B was further confirmed by combining the methylation analysis with deuterium labeling which is obtained by using $NaBD_4$ in the reduction step (Björndal *et al.*, 1970). This experiment differentiated, *e.g.*, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylhexitols from 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitols: the former gives, *e.g.*, the fragment of *m/e* 161 which contains the carbon atoms 4, 5, and 6; the same fragment is formed also from the latter, but as a combination of carbon atoms 1, 2, and 3. Only the latter fragment becomes tagged during $NaBD_4$ treatment. As expected the deuteride reduction transformed the *m/e* 161 fragment of peak B into one of *m/e* 162 (Figure 5). Finally, peak B had also the same chromatographic retention time as the reference standard of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylmannitol (Table IV). Comparison with reported retention times (Björndal *et al.*, 1967b) suggests that it is separated from 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylmannitol, whereas 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylmanni-

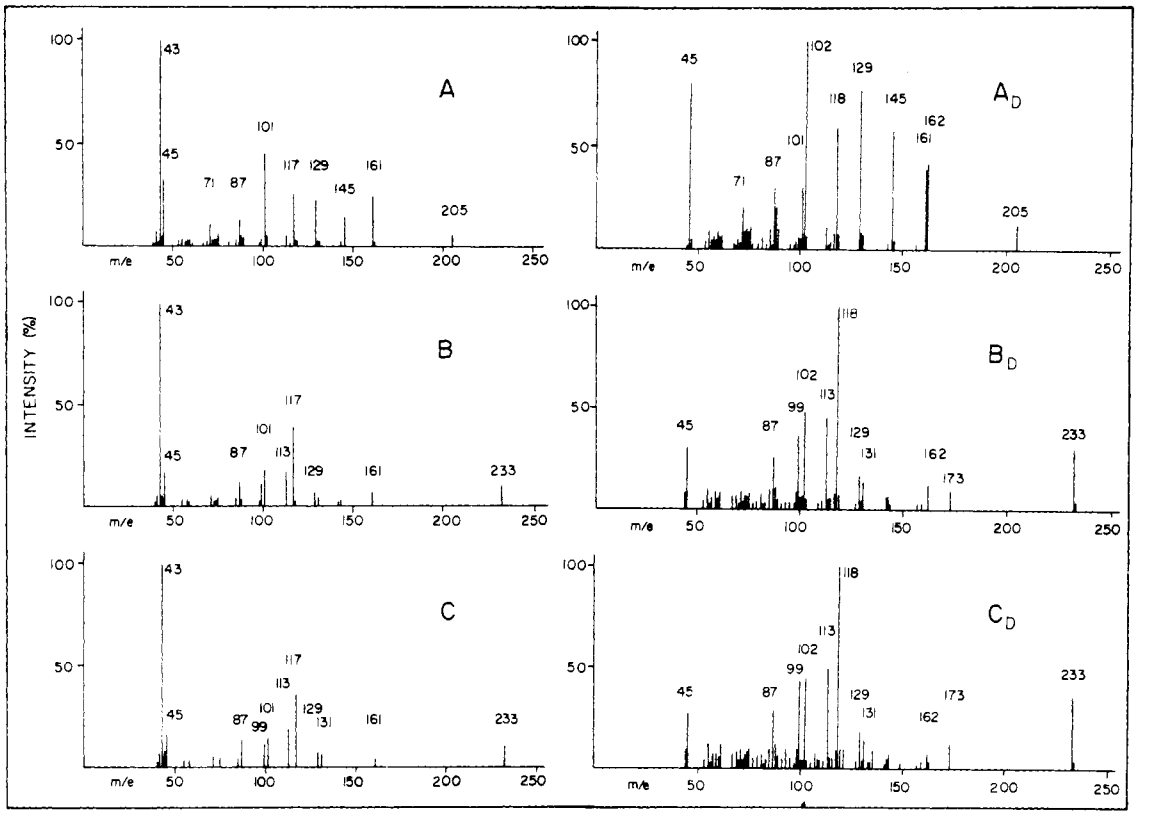

FIGURE 5: Mass spectra of partially methylated alditol acetate peaks derived from ceramide trihexoside of wheat. Peaks A, B, and C were reduced with $NaBH_4$ and peaks A_D, B_D, and C_D with $NaBD_4$.

tol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylmannitol may have similar retention times under our chromatographic conditions. These observations suggest that the central unit of the trihexoside chain of the wheat lipid was a 1,4-substituted mannopyranose residue.

Peak C from both the ceramide di- and trihexoside gave mass spectra (Figure 5) identical with that of a reference sample of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol (mannitol). Their spectra, like that of peak B, were also different from those reported for the other isomers of tri-*O*-acetyl-tri-*O*-methylhexitols which can be derived from monosubstituted glucopyranoside units (Björndal *et al.*, 1967a). The identity of peak C of ceramide trihexoside was confirmed further by deuterium labeling after which the material gave a mass spectrum very similar to that of peak B. Peak C was also chromatographically identical with 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol (Table IV). All other tri-*O*-acetyltri-*O*-methylglucitols which can be derived from monosubstituted glucopyranoside units reportedly have different retention times (Lönngren and Pilotti, 1971). However, in our experiments like in those of Lönngren and Pilotti, the separation between 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol was not very good (Table IV). All these observations suggest that peak C was derived from a 1,4-substituted glucopyranose unit in both two lipids.

Anomeric Nature of the Glycosidic Linkages and the Configuration of the Hexoses at C_5. All glycosidic linkages in the ceramide mono-, di-, and trihexosides were shown to have β structures. This was demonstrated by applying CrO_3 oxidation of the acetylated lipids. The β-linked hexose units are rapidly oxidized to give 5-hexulosonates, whereas the α-linked units are much more resistant to oxidation (Angyal and James, 1970). The 5-hexulosonates are then destroyed during acid hydrolysis of the oxidized carbohydrate chain and only the α-glycosidically linked carbohydrates are found intact in the hydrolysate. All the carbohydrate units of ceramide mono-, di-, and trihexosides of wheat were almost completely oxidized under conditions where α-glycosidically bound galactose and glucose of reference lipids were almost completely stable (Table V).

The optical rotations of the (noncrystalline) acetylated samples of ceramide mono-, di-, and trisaccharides and also that of the acetylated ceramides derived from the ceramide monosaccharides were determined and the molecular rotations were calculated by using the average molecular weights obtained from gas–liquid chromatography data on the component hydrocarbon chains. Table VI shows that the "carbohydrate contribution" in molecular rotation of glucosyl ceramide was $-79 \pm 28°$. This value is similar to the "carbohydrate contribution"

TABLE IV: Relative Retention Times of Partially Methylated Alditol Acetates in Gas–Liquid Chromatography.

	R_t[a]
Peak A of ceramide monohexoside	1.00
Peak A of ceramide dihexoside	0.99
Peak C of ceramide dihexoside	2.44
Peak A of ceramide trihexoside	0.99
Peak B of ceramide trihexoside	2.11
Peak C of ceramide trihexoside	2.44
Reference compounds	
1,5-Di-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol	1.00
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol	1.00
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol	2.44
1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol	2.41
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-mannitol	2.11

[a] R_t = retention time on 3% ECNSS-M relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

TABLE V: Chromium Trioxide Oxidation of Ceramide Hexosides of Wheat.

Glycoside	Ratio of Oxidation-Resistant Carbohydrate to Original Carbohydrate (%)		
	Glucose	Mannose	Galactose
Wheat lipids			
Ceramide monohexoside[a]	2		
Ceramide dihexoside[a]	7	3	
Ceramide trihexoside	11	10	
Reference lipids			
3-*O*-[α-D-galactopyranosyl-(1′→2′)-*O*-α-D-glucopyranosyl]-*sn*-1,2-diacylglycerol	85		94
3-*O*-[α-D-glucopyranosyl-(1′→2′)-*O*-α-D-glucopyranosyl]-*sn*-1,2-diacylglycerol	115		
O-α-D-galactopyranosyl-(1→4)-*O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→1)-ceramide	10		33

[a] Oxidation time, exceptionally, 60 min.

in methyl β-D-glucopyranoside (Hough and Richardson, 1967), but it is quite different from that in the other three methyl glucopyranosides (Table VI). These data suggest that the glucose was of D series, and they confirm the presence of a β-glycosidic linkage in the ceramide monohexosides.

The "carbohydrate contribution" of the distal mannose unit in the $[M]_D$ of the mannosylglucosyl-(1→1)-ceramide of wheat flour is $-120 \pm 40°$ (Table VI). If it is assumed that the β-glucose unit belongs even here to the D series, this finding rules out β-L-mannopyranosidic and α-D-mannopyranosidic structures because they should have strong dextrarotary contributions (Table VI). The β-D-mannopyranosidic and α-L-mannopyranosidic structures are equally likely on the basis of rotational data, but only the β-D structure of the distal manose is compatible with the CrO_3 oxidation data.

The molecular rotation of the ceramide trihexoside shows, by analogous reasoning, that the distal mannose unit of this lipid too has a β-D-pyranosidic structure. The assumption has been made in this reasoning that the β-mannose unit in the middle of the carbohydrate chain is of D series like the mannose in the isolated ceramide dihexoside.

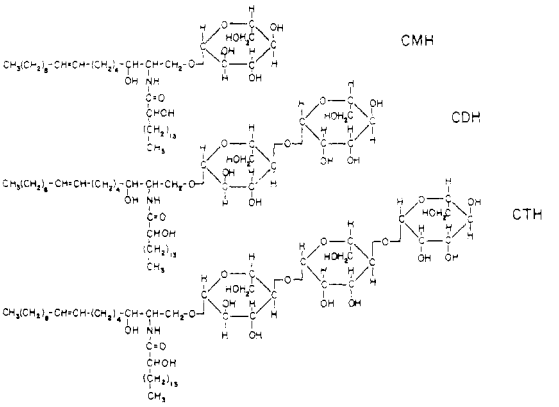

FIGURE 6: Suggested structures for wheat flour ceramide hexosides.

Discussion

The present work shows that mannose-containing ceramide hexosides are present in plants. Similar lipids are present also in animals (Hori *et al.*, 1968; Luukkonen *et al.*, 1973). Mannose-containing glycoproteins, too, are widespread in animals and in plants (Buddecke, 1972).

The present findings allow an assignment of complete structures of wheat flour ceramide mono-, di-, and trisaccharides. The monosaccharide is *O*-β-D-glucopyranosyl-(1→1)-ceramide, the disaccharide is *O*-β-D-mannopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→1)-ceramide, and the trisaccharide is *O*-β-D-mannopyranosyl-(1→4)-*O*-β-D-mannopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→1)-ceramide (Figure 6). It is noteworthy that mannose units appear to be α-glycosidically linked to each other in the soybean flour hemagglutinin (Lis *et al.*, 1969), which is one of the most thoroughly studied examples of plant glycoproteins.

The fatty acids of the ceramide hexosides of wheat flour were almost completely saturated and of longer chains than in the glycerolipids; in addition they were hydroxylated. Also in animal cells the ceramide hexosides are invariably more saturated and contain longer chains than the other membrane lipids. The similarity of the plant and animal ceramide hexosides extends to the hydroxyl groups in the fatty acids and to the similarity of the sphingosine bases (Wiegandt, 1971; Laine and Renkonen, 1973). The ceramide hexosides amount to about 2% of the polar lipids of wheat flour (MacMurray and Morrison, 1970). Comparable values have been reported for animal sphingoglycolipids, which amount to 3–4% of the phospholipids

TABLE VI: Optical Rotation of Fully Acetylated Ceramide Hexosides of Wheat in Chloroform.

	$[\alpha]^{20}D$, deg	$[M]_D$, deg	"Carbohydrate Contribution," deg
Glycolipids of wheat			
Ceramide (*c*, 0.74)	+14.6	+101	
Ceramide monohexoside (*c*, 0.99)	+2.3	+22	−79 ± 28
Ceramide dihexoside (*c*, 0.76)	−11.3	−142	−120 ± 40
Ceramide trihexoside (*c*, 0.91)	−24.0	−373	−231 ± 98
Reported values for reference glycosides (Hough and Richardson, 1967)			
Methyl α-D-glucopyranoside tetraacetate			+474
Methyl β-D-glucopyranoside tetraacetate			−69
Methyl α-D-mannopyranoside tetraacetate			+177
Methyl β-D-mannopyranoside tetraacetate			−181

in cultured BHK cells (Renkonen *et al.*, 1972) and to 0.6% of the phospholipids in cultured mosquito cells (Luukkonen *et al.*, 1973). These similarities in structure and quantity suggest, perhaps, similar locations and similar functions of the sphingoglycolipids in plant and animal cell membranes.

It appears that the application of the chromium trioxide oxidation (Angyal and James, 1970) is a useful tool in the elucidation of anomeric structures of glycolipids. It has been already applied to the analysis of *Salmonella* lipopolysaccharides (Hoffman *et al.*, 1972) and the present data suggest that it is applicable to ceramide and diglyceride oligosaccharides as well.

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