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Analysis of Glucocerebrosides of Rye (*Secale cereale* L. cv Puma) Leaf and Plasma Membrane[1]

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ABSTRACT

Glucocerebrosides of whole rye (*Secale cerale* L. cv Puma) leaf and plasma membrane were analyzed using gas chromatography-mass spectrometry and gas chromatography following hydrolysis or as intact molecules purified by reverse-phase high performance liquid chromatography. Fatty acids of acid-hydrolyzed leaf and plasma membrane glucocerebrosides consisted of >98 weight percent saturated and monounsaturated 2-hydroxy fatty acids which contained 16 to 26 carbon atoms. The major fatty acids detected were 2-hydroxynervonic acid (24:1h), 2-hydroxylignoceric acid (24:0h), 2-hydroxyerucic acid (22:1h), and 2-hydroxybehenic acid (22:0h). Long-chain bases of alkaline-hydrolyzed glucocerebrosides consisted primarily of *cis-trans* isomers of the trihydroxy base 4-hydroxysphingenine (t18:1) and the dihydroxy base sphingadienine (d18:2) with lesser amounts of 4-hydroxysphinganine (t18:0) and isomers of sphingenine (d18:1). Intact, underivatized glucocerebroside molecular species of rye leaf and plasma membrane were separated into more than 30 molecular species using reverse-phase HPLC. The molecular species composition of leaf and plasma membrane were quantitatively and qualitatively similar. The major molecular species was 24:1h-t18:1 which constituted nearly 40 weight percent of leaf and plasma membrane extracts. Several other species including 22:1h-t18:1, 24:1h-t18:1 (isomer), 22:0h-t18:1, 24:1h-d18:2, and 24:0h-t18:1 each comprised 4 to 8% of the total. It is anticipated that the high performance liquid chromatography procedure developed in this study to separate intact, underivatized lipid molecular species will be useful in future studies of the physical properties and biosynthesis of plant glucocerebrosides.

Glucocerebrosides are a class of glycosphingolipids that have been reported to constitute less than 1% of the total lipid extract of plant tissues including runner bean leaves (29) and rice bran (8). Recently, however, glucocerebrosides have been identified as a major lipid component of the plasma membrane and tonoplast of cells of several plant species. Plasma membrane isolated from rye (18) and barley (27) leaves, oat root (28), oat coleoptile (28), and mung bean hypocotyl (33) have been reported to contain glucocerebrosides at levels of 16, 23, 10, 26, and 7 mol%, respectively, of the total plasma membrane lipid. In addition, the tonoplast of mung bean hypocotyl cells reportedly contains 17 mol% glucocerebroside (33).

The structure of the glucocerebroside molecule differs from that of the more abundant glycerolipids in having numerous hydroxyl groups and an amide linkage at the interface of the polar region and the hydrophobic acyl chains (6, 13). The generic structure of a glucocerebroside consists of a long-chain base, fatty acid, and glucose residue. The long-chain base is typically a C_{18} amino alcohol which contains two to three hydroxyl groups. The long-chain base is conjugated at its C-1 hydroxyl group to the glucose moiety via a β-1 glycosidic linkage. Fatty acids of plant glucocerebrosides are predominantly 2- (or α-)hydroxy fatty acids and contain 16 to 26 carbon atoms. The fatty acid is bound to the long-chain base through an amide bond between the carbonyl carbon of the fatty acid and the C-2 amine group of the long-chain base.

These structural features instill glucocerebrosides with distinct physical properties. In particular, the hydroxyl groups and amide proton of the glucocerebroside molecule are available to participate in extensive intra- and intermolecular hydrogen bonding (2). This property is believed to be the basis for the rigidity and ion impermeability of animal membranes, such as myelin and the intestinal brush border, which contain high levels of cerebrosides (gluco- or galactocerebrosides) (7, 13). It has been suggested that glucocerebrosides may be of importance with regard to the cryostability of rye plasma membrane (18, 30) and chilling sensitivity of mung bean (34). Despite this, the physical properties of plant glucocerebrosides have received only limited study (19, 34), and the contributions of this lipid to the physical behavior of plasma membrane and tonoplast of plant cells have yet to be determined.

By analogy with other lipid classes such as phospholipids, the physical properties of glucocerebrosides may be dependent upon the molecular species composition. In this regard, we present in this communication the first detailed molecular species analysis of plasma membrane glucocerebrosides. In the course of this study, we have developed a C_{18} reverse-phase HPLC method for the separation and purification of intact, underivatized molecular species of rye glucocerebrosides. The method has several advantages over commonly used procedures of sphingolipid analysis and may be useful in future studies of the physical properties and biosynthesis of these lipids.

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MATERIALS AND METHODS

Plant Material

Seeds of rye (*Secale cereale* L. cv Puma) were germinated in moist vermiculite. Seedlings were grown for 14 to 17 d under growth chamber conditions of 16 h light at 20°C and 8 h dark at 15°C prior to lipid extraction or plasma membrane isolation.

Plasma Membrane Isolation

Plasma membrane was isolated from leaves of rye seedlings using an aqueous two-phase partition system of 5.6 weight%/5.6 weight% polyethylene glycol/dextran as previously described (18, 32).

Glucocerebroside Purification

Lipids were extracted from leaves or plasma membrane of rye seedlings according to the method of Bligh and Dyer (1). Separation of the total lipid extract into neutral lipid (primarily free sterols) and glycolipid fractions was achieved using silica SepPak cartridges (Millipore). The crude lipid extract dissolved in 2 to 3 mL of chloroform:acetic acid (100:1, v/v) was applied to an equilibrated silica cartridge attached to the barrel of a 10 mL glass syringe. Residual lipid was washed into the column with 2 mL of chloroform:acetic acid (100:1, v/v), and neutral lipids were eluted with an additional 15 mL of this solvent. Glycolipids were eluted by the sequential addition of 10 mL of acetone and 8 mL of acetone:acetic acid (100:1, v/v). The glycolipid fraction was taken to dryness under nitrogen or with a rotary evaporator and resuspended in chloroform:methanol (6:1, v/v).

To facilitate glucocerebroside purification, glycolipids were subjected to alkaline hydrolysis resulting in deacylation of glycerolipids and acylated steryl glucoside. Plasma membrane glycolipids dissolved in 2 mL of chloroform and whole leaf glycolipids divided into two aliquots dissolved in 2 mL of chloroform were reacted with 2 mL of 0.6 N methanolic sodium hydroxide. After 1 h of incubation, the hydrolysate consisting of steryl glucoside, glucocerebrosides, and free fatty acids was neutralized with 1.3 mL of HCl and recovered after effecting a phase separation with the addition of 0.5 mL of water.

Glucocerebrosides were purified from the alkaline hydrolysate using silica SepPak cartridges. Glycolipids dissolved in 2 mL of chloroform:acetic acid (100:1, v/v), were transferred to a silica cartridge attached to a glass syringe barrel. Free fatty acids were eluted with the sequential addition of 15 mL of chloroform:acetic acid (100:1, v/v), and 10 mL of chloroform:acetone (80:20, v/v). Steryl glucosides were eluted by the addition of 15 mL of chloroform:acetone (50:50, v/v) and collected in 3 mL fractions. Glucocerebrosides were eluted with 8 mL of acetone followed by 6 mL of acetone:acetic acid (100:1, v/v). The purity of each fraction was checked by TLC on precoated plates (Silica Gel 60, 0.25 mm layer thickness, EM Reagents) using a mobile phase of chloroform:methanol:acetic acid:water (85:15:15:3.5, v/v). If cross-contamination of glucocerebroside with steryl glucoside was observed, all glucocerebroside-containing fractions were combined and rechromatographed using the same procedure. Glucocerebrosides purified from whole rye leaves were subjected to acetone precipitation as described by Kates (15) to remove residual pigments.

GC/MS and GC Analyses of Glucocerebroside Hydrolysates

The fatty acid composition of purified whole leaf and plasma membrane glucocerebrosides was analyzed by GC and GC/MS following acid hydrolysis. Glucocerebrosides (<1 mg) were dissolved in 5 mL of 2 N methanolic HCl and refluxed at 70°C for 18 to 20 h (31). Fatty acid and 2-hydroxy fatty acid methyl esters were extracted with petroleum ether, dried under nitrogen, and silylated with 100 μL of Sylon HTP (Supelco) for 1 h. The resulting fatty acid and *O*-trimethylsilyl (TMS) 2-hydroxy fatty acid methyl esters were identified by GC/MS (4) using a Hewlett-Packard model 5890 GC coupled to a Hewlett-Packard model 5970B mass selective detector (MSD). Fatty acid derivatives were separated using a 15 m × 0.25 mm capillary column wall-coated with SPB-1 (Supelco). Column temperature was programmed from 150°C (1.5 min hold) to 250°C at 10°C/min with a head pressure of 3 psi He. Injector and MSD inlet temperatures were maintained at 270°C. The ionizing potential of the MSD was 70 eV and the mass range was 70 to 600 mass units with a resulting 3.23 scans/s. Mass spectra of fatty acids were identified by comparison with those of Capella *et al.* (4). Alternatively, flame ionization detection was used for quantitation of fatty acid and 2-hydroxy fatty acid methyl ester derivatives. In this case, separation of derivatives was achieved using a 15 m × 0.25 mm SP-2330 wall-coated capillary column (Supelco) with the oven temperature programmed from 185°C (2.5 min hold) to

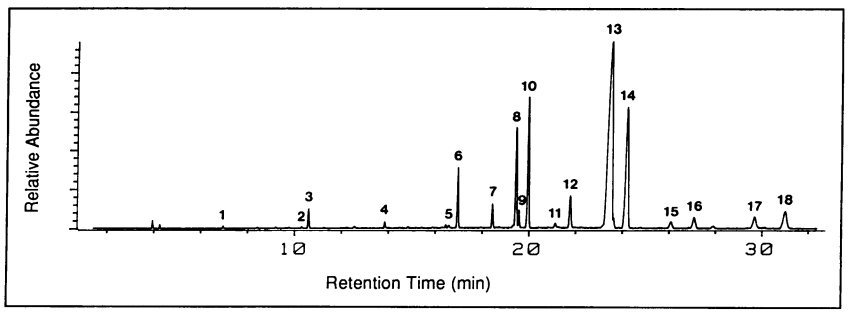

Figure 1. Total ion chromatogram of rye glucocerebroside fatty acids analyzed as *O*-TMS methyl esters (2-hydroxy fatty acids) or as methyl esters (normal chain fatty acids). Peak numbers correspond to the mass spectral identification provided in Table I.

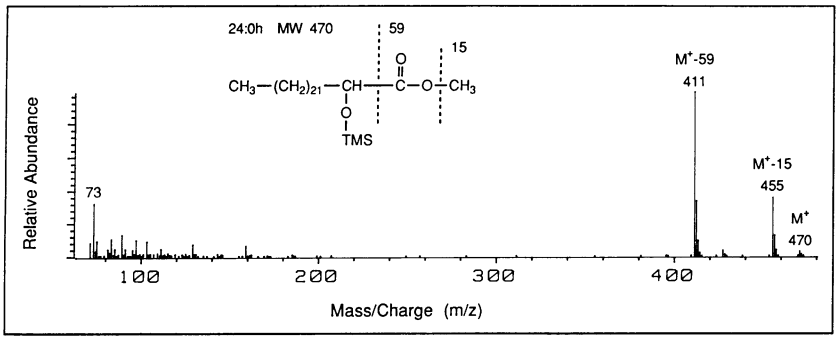

Figure 2. Example of mass spectral fragmentation pattern of a glucocerebroside 2-hydroxy fatty acid *O*-TMS methyl ester derivative. Shown is the mass spectrum of 24:0h (peak 14 of Fig. 1 and Table I).

205°C at 1.5°C/min with a head pressure of 5 psi He. The injector and detector temperatures were maintained at 250°C.

The long-chain base composition of rye glucocerebrosides was determined by GC/MS following strong alkaline hydrolysis as described by Morrison and Hay (22) with slight modification. Glucocerebrosides (<1 mg) were refluxed in 5 mL of 10% barium hydroxide in dioxane (w/v) at 110°C for 24 h. Hydrolysis products were extracted with 15 mL of ethyl ether in 5 mL portions. Acid hydrolysis of glucocerebrosides including the mild hydrolysis of Kadowaki *et al.* (12) were found to produce artifacts similar to those reported by Kawai and Ikeda (16). Long-chain bases were purified from the hydrolysate extract by TLC using the conditions described above. Long-chain bases were converted to *N*-acetyl derivates by reaction with 0.1 mL of methanol:acetic anhydride, 4:1 (v/v), for 16 to 18 h at room temperature (9). Long-chain base derivatives were dried under nitrogen, dissolved in chloroform:methanol:water, 1:1:0.8 (v/v/v), and recovered in the chloroform phase. Following silylation as described previously, *N*-acetyl-*O*-TMS long-chain base derivatives were identified by GC/MS (26). Derivatives were separated using a 15 m × 0.25 mm SPB-1 capillary column with the oven temperature programmed from 185°C (2 min hold) to 235°C at 3.5°C/min with the column head pressure maintained at 3 psi He. Injector and MSD inlet temperatures were both set at 270°C. MSD operating conditions were the same as described above.

IR Analysis of Glucocerebroside Long-Chain Base *Cis-Trans* Isomers

Cis-trans isomerization of dihydroxy and trihydroxy long-chain bases was determined by IR analysis of individual glucocerebroside molecular species purified by HPLC as described below. IR spectra (4000–250 cm^{-1}) of molecular spe-

Table I. *Mass Spectral Identification and Diagnostic Ions of Methyl Ester and O-TMS Methyl Ester Derivatives of Normal Chain and 2-Hydroxy Fatty Acids, Respectively, of Hydrolyzed Rye Glucocerebrosides*

Peak No.[a]	Fatty Acid	M+-59	M+-15	M+
1	16:0			270
2	18:0			298
3	16:0h	299	343	358
4	18:0h	327	371	386
5	20:1h	353	397	412
6	20:0h	355	399	414
7	21:0h	369	413	428
8	22:1h	381	425	440
9	22:1h(i)[b]	381	425	440
10	22:0h	383	427	442
11	23:1h	395	439	454
12	23:0h	397	441	456
13	24:1h	409	453	468
14	24:0h	411	455	470
15	25:1h	423	467	482
16	25:0h	425	469	484
17	26:1h	437	481	496
18	26:0h	439	483	498

[a] Refers to corresponding numbered peaks in Figure 1. [b] (i) = *trans* isomer (tentative identification).

Table II. *Fatty Acid Composition of Rye Leaf and Plasma Membrane Glucocerebrosides*

Plasma membrane samples were purified from 1 to 5 pooled plasma membrane glycolipid extracts. Fatty acids were analyzed as methyl ester (normal chain fatty acids) or *O*-TMS methyl ester (2-hydroxy fatty acids) derivatives. Values are expressed as mean weight percent ± SE (n = 5 for leaf and 3 for plasma membrane).

Fatty Acid	Leaf	Plasma Membrane
16:0	0.7 ± 0.1	1.0 ± 0.1
18:0	0.3 ± 0.1	0.5 ± 0.1
16:0h	2.8 ± 0.2	3.4 ± 0.4
18:0h	0.5 ± 0.1	0.5 ± 0.1
20:0h	3.6 ± 0.2	3.9 ± 0.1
20:1h	0.2 ± 0.1	0.4 ± 0.1
21:0h	1.1 ± 0.1	1.2 ± 0.1
22:0h	8.7 ± 0.2	8.8 ± 0.1
22:1h	7.8 ± 0.2	7.5 ± 0.2
22:1h(i)[a]	1.3 ± 0.1	1.4 ± 0.1
23:0h	1.8 ± 0.1	1.9 ± 0.1
23:1h	0.4 ± 0.1	0.4 ± 0.1
24:0h	12.4 ± 0.4	11.9 ± 0.1
24:1h	52.5 ± 0.6	51.2 ± 0.8
25:0h	1.0 ± 0.1	0.9 ± 0.1
25:1h	0.9 ± 0.1	1.0 ± 0.1
26:0h	2.0 ± 0.1	1.7 ± 0.1
26:1h	1.7 ± 0.1	1.9 ± 0.1

[a] (i) = *trans* isomer (tentative identification).

Table III. *Mass Spectral Identification and Diagnostic Ions of N-Acetyl-O-TMS Derivatives of Long-Chain Bases of Hydrolyzed Rye Glucocerebrosides*

Peak No.[a]	Long-Chain Base	M^+-276	M^+-174	M^+-105	M^+-59	M^+-15
1	d18:2$\Delta^{4trans,8cis}$		309	378	424	468
2	d18:2$\Delta^{4trans,8trans}$		309	378	424	468
3, 4, 5[b]	d18:1$\Delta^{4\ or\ 8,cis\ or\ trans}$		311	380	426	470
6	t18:1Δ^{8cis}	297				558
7	t18:1Δ^{8trans}	297				558
8	t18:0	299				560

[a] Refers to corresponding peaks in Figure 3. [b] Tentative identification.

cies were obtained with a Perkin-Elmer PE783 IR spectrophotometer using KBr pellets consisting of approximately 1:80 (w/w) glucocerebroside:KBr. *Trans* and *cis* isomers were detected by absorption bands at 980 to 960 cm^{-1} and 730 to 665 cm^{-1}, respectively.

HPLC Separation of Intact Glucocerebroside Molecular Species

Glucocerebrosides purified from whole leaf or plasma membrane were separated into their component molecular species by C_{18} reverse-phase HPLC. Separation of intact, underivatized molecular species was achieved using a LiChrospher 100 RP-18e column (E. Merck); (25 cm × 4 mm ID, 20% carbon loading, 5 μm particle size) and a mobile phase of 60% acetonitrile/40% methanol at a flow rate of 1.5 mL/min. Eluted glucocerebrosides were detected by absorbance at 210 nm. The system used was a Perkin-Elmer Series 4 liquid chromatograph and an Isco V[4] variable wavelength detector interfaced with a Hewlett-Packard 3393A integrator. Prior to injection, analytes were dissolved in a minimum volume of chloroform:methanol (1:2, v/v), plus approximately two to three volumes acetonitrile:methanol (6:4, v/v). Slight heating was required to completely dissolve glucocerebrosides in this solvent.

GC/MS Identification of Glucocerebroside Molecular Species

Glucocerebroside molecular species separated by HPLC were collected from the column effluent. Following drying and silylation, intact glucocerebroside molecular species were identified by GC/MS as TMS-ether derivatives (14). Lacking the capability for direct-probe mass spectrometric analysis, derivatized molecular species were vaporized off a fused-silica capillary (15 m × 0.25 mm) column containing no wall coating into the MSD inlet system. The column temperature was programmed from 100°C (1.5 min hold) to 275°C at 10°C/min with a head pressure of 1 to 2 psi He. The ionizing potential of the MSD was 70 eV, and the mass range scanned was 70 to 700 mass units resulting in 2.72 scans/s.

Quantification of HPLC-Separated Glucocerebroside Molecular Species

Due to structural variability of molecular species, particularly in levels of unsaturation, UV detection at 210 nm did not provide a quantitative measurement of HPLC-separated glucocerebrosides. To correct for this, detector response factors were determined for molecular species containing various numbers and positions of double bonds. Known weights of individual HPLC-purified molecular species (24:1h-t18:1, 22:1h-t18:1, 22:0h-t18:1, 24:0h-t18:1, 16:0h-d18:2, and 24:1h-d18:2)[4] were analyzed by HPLC using the procedure described above. Response factors were derived from the UV detector signal at 210 nm for a known weight of injected

[4] Lipid notation: *x*:*y*h, hydroxy fatty acid. Number preceding the colon is the number of carbon atoms in the molecule. The number following the colon is the number of double bonds in the molecule. h, hydroxy. t18:1, 4-hydroxysphingenine (t, trihydroxy long-chain base). t18:0, 4-hydroxysphinganine. d18:2, sphingadienine (d, dihydroxy long-chain base). d18:1, sphingenine.

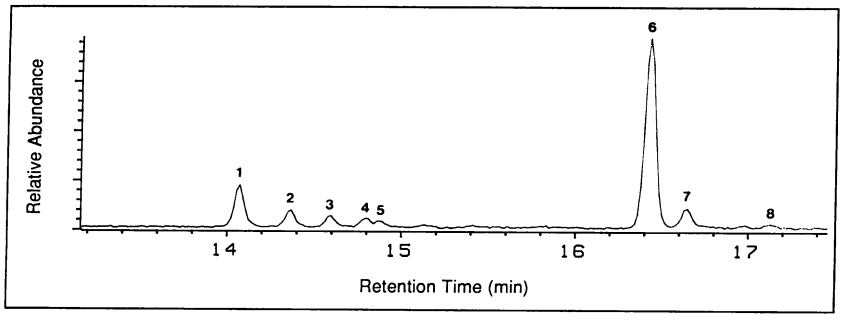

Figure 3. Total ion chromatogram of *N*-acetyl-*O*-TMS long-chain base derivatives obtained from the alkaline hydrolysis of the total rye glucocerebroside extract. Peak numbers correspond to the mass spectral identification provided in Table III.

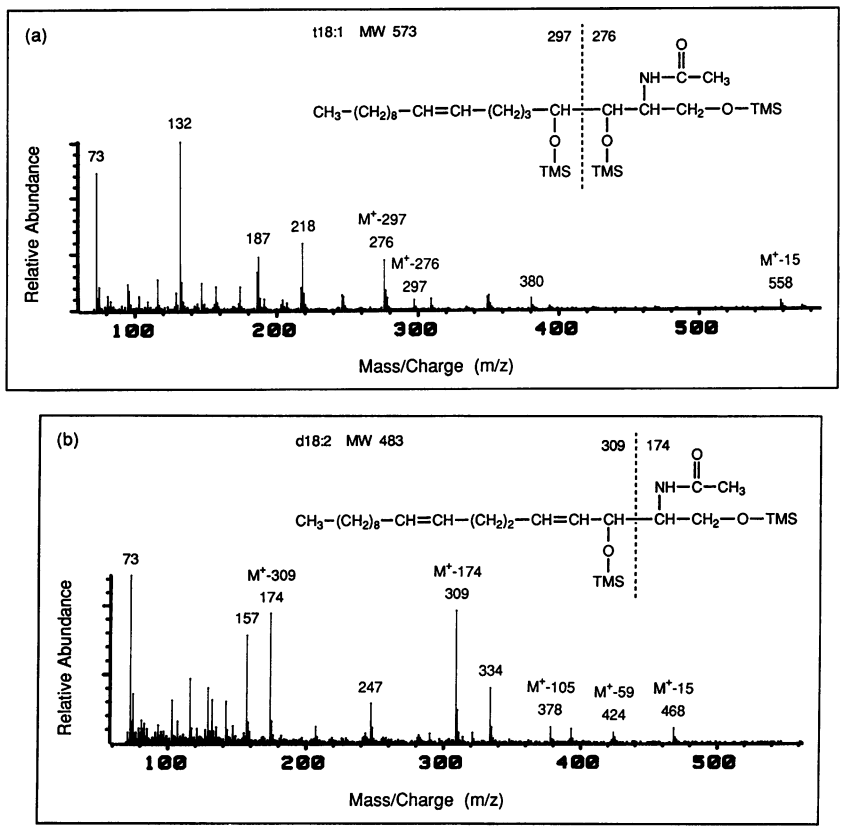

Figure 4. Examples of mass spectral fragmentation patterns of glucocerebroside *N*-acetyl-*O*-TMS long-chain base derivatives. Shown are the mass spectra of (a) the trihydroxy long-chain base 4-hydroxysphingenine and (b) the dihydroxy long-chain base sphingadienine.

molecular species. The relative response factors used to quantify HPLC-separated glucocerebrosides were 1.02 for n:1h-t18:1, 0.83 for n:1h-d18:2, 1.15 for n:0h-t18:1, and 0.87 for n:0h-d18:2. Relative response factors were estimated for the very minor molecular species n:0h-d18:1 (1.11) and n:0h-t18:0 (1.25). Weight percent of molecular species was obtained as the product of the integrated area percent of HPLC peaks and the corresponding response factor. In cases in which HPLC peaks represented more than one molecular species, the response factor of the major peak component was used to quantify the entire peak.

RESULTS

Fatty Acid and Long-Chain Base Composition of Hydrolyzed Rye Glucocerebrosides

The fatty acid and long-chain base compositions of rye glucocerebrosides were analyzed by GC-MS and GC following hydrolysis of the purified leaf or plasma membrane extract. This was done in order to facilitate the identification of HPLC-separated intact molecular species (see below) and to compare glucocerebrosides of rye with those of other species reported in the literature.

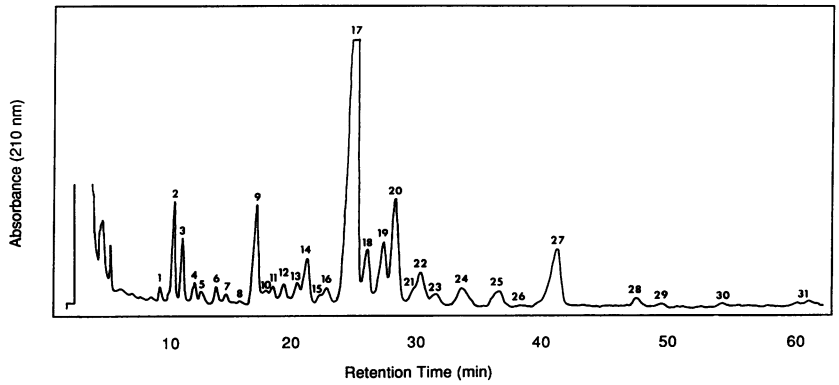

Figure 5. Reverse-phase HPLC separation of rye glucocerebroside molecular species. Peak numbers correspond to the mass spectral identification provided in Table IV.

Using GC-MS, fatty acids of acid-hydrolyzed glucocerebrosides were identified by the mass-to-charge ratio (m/z) of the M^+-59, M^+-15, and M^+ fragments (2-hydroxy fatty acids) or the M^+ fragment (normal chain fatty acids) (Figs. 1, 2; Table I) (4). Rye glucocerebroside fatty acids consisted almost exclusively of 2-hydroxy fatty acids, which ranged in chain length from 16 to 26 carbon atoms. Included were both saturated and monounsaturated 2-hydroxy fatty acids. The major fatty acids detected were 2-hydroxynervonic acid (24:1h), 2-hydroxylignoceric acid (24:0h), 2-hydroxyerucic acid (22:1h), 2-hydroxybehenic acid (22:0h), and 2-hydroxyarachidic acid (20:0h). Appreciable amounts of 2-hydroxy fatty acids containing odd numbers of carbon atoms (C_{21}, C_{23}, and C_{25}) were also identified. The only normal chain fatty acids detected were palmitic acid (16:0) and stearic acid (18:0).

The fatty acid composition of whole leaf and plasma mem-

Table IV. *Mass Spectral Identification and Diagnostic Ions of HPLC-Separated Rye Glucocerebroside Molecular Species Analyzed as O-TMS Ether Derivatives*

Peak No.[a]	Molecular Species	M^+-a-b+73[b]	M^+-a-b+31[b]	d[b]	b[b]	c[b]
1	16:0h-t18:1[c]	458	416	299		297
2	16:0h-d18:2[d]	458	416	299	309	
3	16:0h-d18:2(i)[e]	458	416	299	309	
	16:0-d18:2[f]	370	328		309	
4	16:0h-t18:0	458	416	299		299
	20:1h-t18:1	512	470	353		297
5	18:0h-t18:1	486	444	327		297
	16:0-t18:0[f]					
	18:0-t18:1[f]					
6	16:0h-d18:1	458	416	299	311	
7	18:0h-d18:2	486	444	327	309	
	20:1h-d18:2(i)	512	470	353	309	
	18:0-d18:2[f]					
	16:0-d18:1[f]					
8	18:0h-d18:2(i)	486	444	327	309	
9	22:1h-t18:1	540	498	381		297
10	22:1h-t18:1(i)	540	498	381		297
11	20:0h-t18:1	514	472	355		297
12	22:1h-d18:2	540	498	381	309	
13	23:1h-t18;1	554	512	395		297
	22:1h-d18:2(i)	540	498	381	309	
	18:0h-d18:1	486	444	327	311	
14	20:0h-d18:2	514	472	355	309	
15	22:1h-t18:0	540	498	381		299
16	21:0h-t18:1	528	486	369		297
	20:0h-d18:2(i)	514	472	355	309	
17	24:1h-t18:1	568	526	409		297
18	24:1h-t18:1(i)	568	526	409		297
19	22:0h-t18:1	542	500	383		297
20	24:1h-d18:2	568	526	409	309	
21	25:1h-t18:1	582	540	423		297
22	24:1h-d18:2(i)	568	526	409	309	
	20:0h-d18:1	514	472	355	311	
23	22:0h-d18:2	542	500	383	309	
24	23:0h-t18:1	556	514	397		297
	24:1h-t18:0	568	526	409		299
	22:0h-d18:2(i)	514	472	383	309	
25	26:1h-t18:1	596	554	437		297
26	24:1h-d18:1	568	526	409	311	
	23:0h-d18:2	556	514	397	309	
27	24:0h-t18:1	570	528	411		297
28	24:0h-d18:2	570	528	411	309	
29	25:0h-t18:1	584	542	425		297
30	25:0h-d18:2[g]					
31	26:0h-t18:1[g]					

[a] Refers to corresponding peak in Figure 5. [b] Letters (*e.g.* a, b, c, and d) refer to mass spectral fragmentation pattern illustrated in Figure 6. [c] t18:1 refers to the Δ^{8cis} isomer. [d] d18:2 refers to the $\Delta^{4trans,8cis}$ isomer. [e] (i) = isomer of long-chain base moiety. d18:2(i) = $\Delta^{4trans,8trans}$; t18:1(i) = Δ^{8trans}. [f] Tentative identification. [g] Identified by retention time (Fig. 7).

brane glucocerebrosides was qualitatively and quantitatively similar (Table II). 2-Hydroxy fatty acids accounted for >98 weight% of fatty acids of both extracts. In addition, nearly 65 weight% of the fatty acids of whole leaf and plasma membrane were monounsaturated. The major fatty acid was 24:1h which was present at levels of >50 weight% in whole leaf and plasma membrane samples (Table II).

The long-chain base composition of rye glucocerebrosides was determined following alkaline hydrolysis of purified extracts as described by Morrison and Hay (22). This method was found to produce fewer artifacts than a variety of previously reported acid hydrolyses, including the mild acid hydrolysis described by Kadawaki *et al.* (12). Alkaline hydrolysis, however, did not result in total cleavage of glycosidic bonds between the long-chain base and glucose residue (data not shown). Therefore, this method was used only for the semiquantitative analysis of long-chain bases.

Trihydroxy and dihydroxy bases were identified primarily by the presence and mass-to-charge (m/z) ratios of the M^+-276 or M^+-174 fragments, respectively (Table III; Fig. 3) (26). Examples of the mass spectra of trihydroxy and dihydroxy long-chain bases are shown in Figure 4. The major long-chain bases of plasma membrane and whole leaf extracts were isomers of the trihydroxy base 4-hydroxysphingenine (t18:1) and the dihydroxy base sphingadienine (d18:2) with lesser amounts of 4-hydroxysphinganine (t18:0) and isomers of sphingenine (d18:1).

The positions and *cis-trans* isomerization of the double bonds in these molecules could not be readily determined by mass spectrometry. Based on reports of other species (8, 11, 21, 25) the double bonds of t18:1, d18:2, and d18:1 are likely Δ^8, $\Delta^{4,8}$, and $\Delta^{4 \text{ or } 8}$, respectively. Using HPLC-purified individual molecular species (see below), *cis-trans* orientations of double bonds were determined by infrared spectroscopy (data not shown). The primary isomeric form of t18:1 (Fig. 3, peak 6) in rye glucocerebrosides was identified as 4-hydroxy-8-*cis*-sphingenine and the primary isomeric form of d18:2 (Fig. 3, peak 1) was tentatively identified as sphinga-4-*trans*-8-*cis*-dienine. By default, the minor isomers of t18:1 and d18:2 were tentatively identified as 4-hydroxy-8-*trans*-sphingenine (Fig. 3, peak 7) and sphinga-4-*trans*-8-*trans*-dienine (Fig. 3, peak 2). The three peaks corresponding to d18:1 in the total ion chromatogram (Fig. 3, peaks 3, 4, 5) are likely *cis-trans* isomers of Δ^4 and Δ^8 sphingenine.

Reverse-Phase HPLC Analysis of Rye Glucocerebroside Molecular Species

Glucocerebrosides of leaf and plasma membrane were separated into more than 30 intact, underivatized molecular species by C_{18} reverse-phase HPLC (Fig. 5). GC-MS identification of TMS derivatives of HPLC-purified molecular species is detailed in Table IV. Shown in Figure 6 are examples of partial mass spectra of TMS derivatives of molecular species containing trihydroxy and dihydroxy long-chain bases. The mass spectral identification of HPLC peaks in Figure 5 was confirmed by linearity of retention time plots, which relate the log of HPLC retention times to the number of carbon atoms of fatty acids of molecular species containing a given long-chain base (Fig. 7). Retention time plots were also used

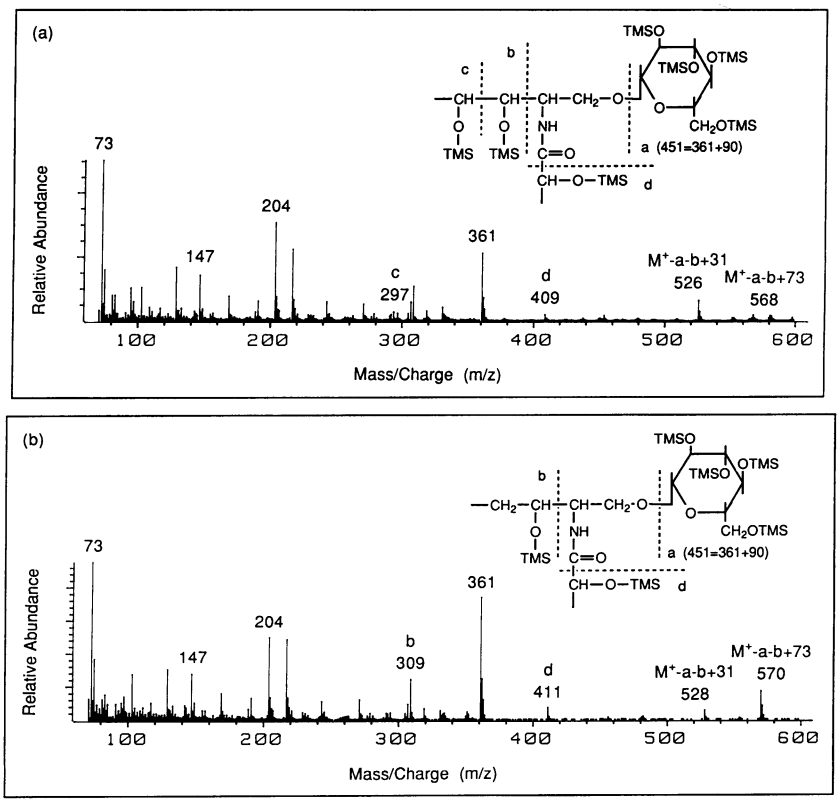

Figure 6. Partial mass spectra of (a) 24:1-t18:1 and (b) 24:0h-d18:2. Also shown are the fragmentation patterns of (a) trihydroxy and (b) dihydroxy long-chain base-containing glucocerebroside molecular species.

to identify several of the late eluting molecular species (Fig. 5, peaks 30, 31) that were not characterized by mass spectrometry. *Cis-trans* configuration of long-chain bases was determined by infrared spectroscopy of purified molecular species as described above.

Using the HPLC procedure developed in this study, a high degree of resolution of molecular species including those containing critical pairs of fatty acids (*e.g.* 22:0h *versus* 24:1h) was achieved. Also resolved were molecular species containing *cis-trans* isomeric forms of the long-chain base, such as 24:1h-t18:1 (Fig. 5, peaks 17, 18). Thus, the procedure described, though developed on an analytical scale, provides a means of isolating essentially pure fractions of the major glucocerebroside molecular species of rye.

Quantification of HPLC-separated molecular species was achieved by obtaining response factors for UV absorbance at 210 nm. The validity of these response factors is illustrated in Figure 8. As shown, general agreement existed between amounts of major leaf glucocerebroside fatty acids determined by GC and amounts of molecular species containing the corresponding fatty acids as determined by HPLC (using response factors). In plasma membrane extracts, levels of 16:0h-containing molecular species determined by HPLC were 2 to 3 weight% higher than levels of this fatty acid in the hydrolyzed extract as determined by GC (Table II). This difference was due most likely to small amounts of steryl glucoside contamination, which were more difficult to detect by TLC (see "Materials and Methods") in the less concentrated plasma membrane extracts. To note, steryl glucoside was observed to coelute with peak 3 of Figure 5. Despite this difference, the detector response factors were found to provide an adequate estimate of the relative amounts of the HPLC-separated molecular species.

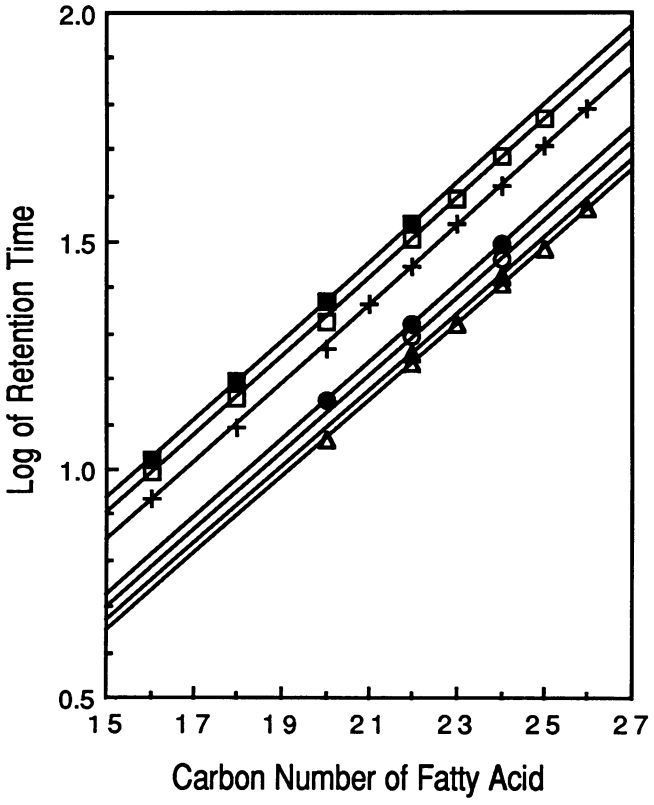

Figure 7. Retention time plots of HPLC-separated glucocerebroside molecular species: (□), n:0h-d18:2; (■), n:0h-d18:2(i); (+), n:0h-t18:1; (○), n:1h-d18:2; (●), n:1h-d18:2(i); (△), n:1h-t18:1; (▲), n:1h-t18:1(i).

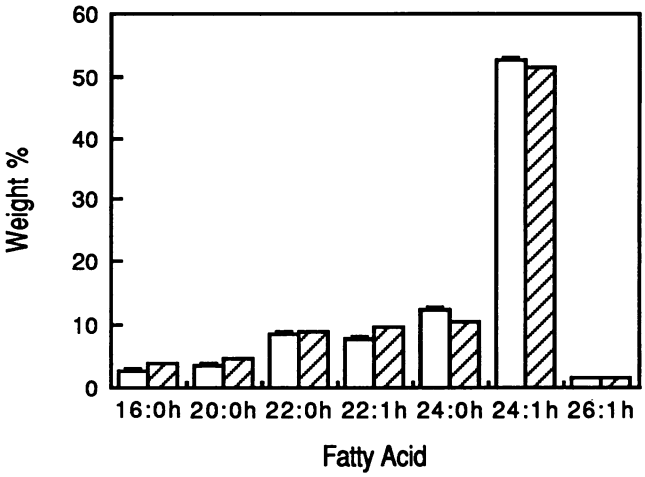

Figure 8. Comparison of weight% of major glucocerebroside fatty acids of the total hydrolyzed extract determined by GC (open bars) and the weight% of molecular species containing the corresponding fatty acids as determined by HPLC using retention time response factors (hatched bars).

The glucocerebroside molecular species composition of leaf and plasma membrane samples were strikingly similar and consisted primarily (>65 weight%) of monounsaturated and saturated C_{22} and C_{24} hydroxy fatty acids paired with t18:1 Δ^{8cis} (Table V). The ratio of molecular species containing trihydroxy to those containing dihydroxy long chain bases was about 80:20. Molecular species containg the *cis* isomer of t18:1Δ^8 accounted for nearly 70 weight% of the total leaf and plasma membrane glucocerebrosides. As expected from analyses of fatty acids and long-chain bases of hydrolyzed glucocerebrosides (see above), the primary molecular species was 24:1h-t18:1Δ^{8cis}, which accounted for about 37 weight% of plasma membrane glucocerebrosides. In an accompanying study (3), glucocerebrosides were reported to constitute 11.4 mol% of the total plasma membrane lipid. Thus, 24:1h-t18:1Δ^{8cis} represents roughly 4 mol% of the total plasma membrane lipids.

DISCUSSION

Prior to very recent analyses of the lipid composition of plasma membrane and tonoplast of plant cells (3, 18, 27, 28, 33), glucocerebrosides were identified as a minor component of plant extracts (8, 29). As such, the physical properties of plant glucocerebrosides have received only limited study, and the contributions of this lipid to the observed properties of the plasma membrane and tonoplast are not known. Also, the biosynthesis of plant glucocerebrosides has yet to be characterized. In contrast, cerebrosides (galacto- or glucocer-

Table V. *Weight Percent of HPLC-Separated Glucocerebroside Molecular Species of Rye Leaf and Plasma Membrane*

Plasma membrane glucocerebroside samples were purified from pooled glycolipid extracts of 1 to 5 plasma membrane isolations. Values were obtained using detector response factors and are expressed as weight% ± SE (n = 5 for leaf extract, n = 3 for plasma membrane extract).

Peak No.[a]	Molecular Species	Leaf	Plasma Membrane
1	16:0h-t18:1	0.4 ± 0.1	0.6 ± 0.1
2	16:0h-d18:2	1.6 ± 0.1	2.9 ± 0.2
3	16:0h-d18:2(i);[b] 16:0-d18:2	0.8 ± 0.1	2.7 ± 0.2
4	16:0h-t18:0;[b] 20:1h-t18:1[c]	0.9 ± 0.2	0.8 ± 0.1
5	18:0h-t18:1;[b] 16:0-t18:0;[c] 18:0-t18:1[c]	0.7 ± 0.1	0.5 ± 0.1
6	16:0h-d18:1	0.9 ± 0.1	0.9 ± 0.1
7	18:0h-d18:2;[b] 20:1h-d18:2(i);[c] 18:0-d18:2;[c] 16:0-d18:1[c]	0.4 ± 0.1	0.5 ± 0.1
8	18:0h-d18:2(i)	tr[d]	tr
9	22:1h-t18:1	6.6 ± 0.3	6.2 ± 0.1
10	22:1h-t18:1(i)	1.0 ± 0.1	1.1 ± 0.1
11	20:0h-t18:1	0.9 ± 0.1	1.2 ± 0.1
12	22:1h-d18:2	0.8 ± 0.1	0.6 ± 0.1
13	22:1h-d18:2(i);[b] 23:1h-t18:1; 18:0h-d18:1[c]	1.6 ± 0.1	1.6 ± 0.1
14	20:0h-d18:2	2.0 ± 0.1	2.3 ± 0.3
15 & 16	21:0h-t18:1;[b] 20:0h-d18:2(i); 22:1h-t18:0	2.4 ± 0.1	1.9 ± 0.1
17	24:1h-t18:1	39.5 ± 0.5	37.3 ± 0.1
18	24:1h-t18:1(i)	5.0 ± 0.2	5.2 ± 0.2
19	22:0h-t18:1	6.9 ± 0.4	6.8 ± 0.4
20	24:1h-d18:2	5.2 ± 0.1	4.6 ± 0.1
21 & 22	24:1h-d18:2(i);[b] 25:1h-t18:1; 20:0h-d18:1[c]	2.7 ± 0.1	2.4 ± 0.2
23	22:0h-d18:2	0.9 ± 0.1	1.0 ± 0.1
24	23:0h-t18:1;[b] 24:1h-t18:0; 22:0h-d18:2(i)	3.2 ± 0.2	3.6 ± 0.4
25	26:1h-t18:1	1.6 ± 0.1	1.3 ± 0.2
26	24:1h-d18:1; 23:0h-d18:2	tr	tr
27	24:0h-t18:1	9.4 ± 0.4	10.6 ± 0.5
28	24:0h-d18:2	1.0 ± 0.1	0.9 ± 0.1
	25:0h-t18:1,d18:2[e]	1.0	0.9
	26:0h-t18:1,d18:2[e]	2.0	1.7

[a] Refers to corresponding peak in Figure 5. [b] Major component of peak. [c] Trace component. [d] Trace. [e] Values derived from GC determination of fatty acid composition of total extract (see Table II).

ebrosides) have long been known to be major components of a number of animal membranes, particularly the myelin sheath of mammalian nervous tissues (7, 13). Animal cerebrosides are believed to be localized primarily in the outer leaflet of plasma membrane and are characterized by extensive hydrogen bonding ability and high gel-to-liquid crystalline phase transition temperatures (Tm) (2, 6). This lipid class is therefore generally considered to physically stabilize the plasma membrane and reduce ion permeability of various animal cells (7, 13). It is not unlikely that glucocerebrosides may play a similar role in plant plasma membrane, given that this membrane is the primary interface between the cell and its environment. In a previous study (18), glucocerebrosides were reported to constitute 16 mol% of the plasma membrane lipid of cells of noncold-acclimated rye leaves. It was also suggested that this lipid may be of importance in altering cryostability of rye plasma membrane (18, 30). Thus, as part of an effort to fully characterize the lipid composition and cryobehavior of rye plasma membrane and to gain insight into the function of this lipid in plant membranes, it was our desire to provide a detailed analysis of glucocerebroside molecular species.

Prior studies of plant glucocerebrosides have relied primarily upon analysis of fatty acids and long-chain bases following acid hydrolysis of whole tissue extracts (5, 8, 11, 29). Using such methods, rye glucocerebrosides were found to consist of a preponderance of monounsaturated 2-hydroxy fatty acids (>65 weight% of glucocerebroside fatty acids) as well as the *cis* isomer of the long-chain base t18:1Δ^8 (>70 weight% of glucocerebroside long-chain bases). In both regards, glucocerebrosides of rye leaves are distinct from those of most other plant tissues. Fatty acids of glucocerebrosides of tissues including leaves of runner bean (29), *Phaseolus vulgaris* (5), and spinach (25) as well as seeds of Azuki bean (23), pea (11), and soybean (24) consist almost entirely of saturated 2-hydroxy fatty acids. Similarly, in the few cases where *cis-trans*

isomerization of long-chain bases has been reported, the *trans* isomer of t18:1 is typically more abundant than the *cis* isomer (*e.g.* 8, 23–25). In addition, the composition of intact glucocerebroside molecular species detected in the leaf and plasma membrane of rye were similar. Assuming the tonoplast is the only other cellular location of glucocerebrosides, this result would suggest that the molecular species composition of the plasma membrane is nearly identical to that of the tonoplast.

Whether differences in glucocerebroside composition are reflected in different properties of the plasma membrane and tonoplast of plant cells is not known. It has been observed that trihydroxy rather than dihydroxy long-chain bases are more prevalent in cerebrosides of animal membranes that are exposed to harsh or fluctuating environments (13). These additional hydroxyl groups theoretically increase lateral hydrogen bonding and thus add to the stability of the membrane (2, 13). Examples of this phenomenon include the brush border of the small intestine, which is exposed to high levels of bile salts; kidney tubules, which are exposed to high concentrations of toxins; and epidermal cells of frog skin, which are exposed to moisture extremes (7, 13). Glucocerebrosides of leaves tend to contain high levels of trihydroxy long-chain bases (5, 24, 29) while those of seeds contain primarily dihydroxy long-chain bases (11, 23, 24). Comparative biochemical studies of different plant tissues from a range of species, however, are required before any generalizations or inferences can be made. Nevertheless, it is possible that the composition of glucocerebrosides may be related to the environmental status of the plant cell.

Unlike any prior analyses of plant glucocerebrosides, C_{18} reverse-phase HPLC was used in this study to separate and identify intact, underivatized molecular species of whole tissue and specific membrane extracts. Reverse-phase HPLC has been successfully employed in analyses of phospholipid (21) and galactolipid (20) molecular species. In contrast to these lipids, glucocerebrosides are characterized by a much greater structural complexity. In rye leaf and plasma membrane, 18 different fatty acids may be paired with at least 8 different long-chain bases to form the total glucocerebroside complement. The resolution of such a complex mixture of molecules thus requires a very powerful chromatographic method. In animal systems, perbenzoylated derivatives of bovine brain cerebrosides have been separated into simple mixtures by reverse-phase HPLC (17). In this case, however, the long-chain base composition was nearly homogenous, and molecular species were separated mainly on the basis of differences in fatty acid composition. In addition, Hirabayashi *et al.* (10) have reported a high degree of resolution of underivatized Japanese quail intestine cerebrosides by reverse-phase HPLC using an isocratic solvent system of methanol. This procedure, however, was found to be ineffective in separating rye glucocerebroside molecular species (data not shown), due most likely to differences in long-chain base composition. Using the HPLC method developed in this study, we were able to not only separate underivatized glucocerebrosides containing critical fatty acid pairs but also molecular species containing *cis-trans* isomeric forms of the two major long-chain bases, t18:1 and d18:2.

In addition, the HPLC method described here, though developed on an analytical scale, provided a rapid means of analyzing and purifying the major glucocerebroside molecular species of rye. Such capabilities may facilitate future studies of the physical properties and metabolism of plant glucocerebrosides. With regard to the physical properties of this lipid, it has been reported that the Tm of rye glucocerebrosides is 56°C (19) and that of mung bean hypocotyl plasma membrane and tonoplast glucocerebrosides are 38°C and 35°C, respectively (34). In the former study, limited information was provided on the composition of the glucocerebrosides analyzed, and, in the latter, no information was provided. The physical properties of lipids, especially phospholipids, are dependent upon the molecular species present. Therefore, the use of reverse-phase HPLC to characterize the exact pairings of fatty acids and long-chain bases of tissue and membrane glucocerebroside extracts would provide some structural basis for observed thermotropic behavior. Also, the use of reverse-phase HPLC as a purification step would allow one to determine the contributions of individual molecular species to the physical properties of the entire glucocerebroside extract.

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