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Wheat flour lipids: II. isolation and characterization of glycolipids of wheat flour and other plant sources*

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SUMMARY

Methods involving solvent fractionation and silicic-acid column chromatography have been developed for isolating monogalactosyl- and digalactosylglycerol lipids and cerebrosides from the benzene-extracted lipids of wheat flour. A comparative study has been made of the composition of the lipid mixtures obtained from bleached and unbleached flours. The lipids from bleached flour were found to contain covalently bound chlorine, apparently resulting from reaction with chlorine, the bleaching agent. A preliminary study of the occurrence of galactosylglycerol lipids in other plant materials is reported.

In a previous paper (1) we reported the preparation from wheat flour lipids of a crude mixture of glycolipids which, on alkaline hydrolysis, yielded among other substances, monogalactosyl- and digalactosylglycerol. The structures of the two glycerol derivatives were established as β-D-galactopyranosyl-1-glycerol and α-D-galactopyranosyl-(1 → 6)-β-D-galactopyranosyl-1-glycerol. A partial separation of the intact galactosylglycerol lipids was achieved by virtue of the greater solubility in organic solvent of the monogalactosylglycerol lipid as compared to the digalactosylglycerol lipid.

During the past three years an extensive study has been made of the composition of wheat flour lipids. A simple procedure has been devised for preparing a glycolipid fraction relatively free of triglyceride and sterol ester, and chromatographic procedures have been developed for separation of the glycolipids into relatively pure monogalactosyl- and digalactosylglycerol lipid fractions and a crude cerebroside fraction.

These studies are described in the present paper, together with data on the comparative composition of lipids from bleached and unbleached wheat flour.

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EXPERIMENTAL AND RESULTS

Analytical Methods. Elementary analysis for C, H, Cl, and N (Dumas) was obtained. Phosphorus was determined by the method of Harris and Popat (2), total nitrogen by the micro-Kjeldhal method, and the long-chain base nitrogen by the method of McKibbin and Taylor (3). Sugars were determined by the method of Radin *et al.* (4).

The infrared spectra were obtained from smeared material on a Perkin-Elmer 21 double beam spectrophotometer, using sodium chloride prism.

The conditions for gas chromatographic analysis are described in the experimental part.

Unbleached Wheat Flour. Isolation of Monogalactosylglycerol Lipid, Digalactosylglycerol Lipid, and Cerebrosides. One hundred pounds of unbleached wheat

flour[1] was extracted with benzene at room temperature, courtesy of the Procter and Gamble Company. The benzene extract was concentrated *in vacuo*, and the residual heavy syrup was stored in the cold room at 4°.

In a typical experiment, 1 liter of benzene extract (containing about 280 g of lipid), was treated with 10 liters of acetone. After standing overnight at 4°, the precipitate was filtered, redissolved in 100 ml of benzene, and reprecipitated with 1 liter of acetone. The insoluble material (BAI)[2] was filtered (12.3 g), and the filtrate was combined with the previous one.

The combined benzene-acetone solutions, after evaporation of solvent *in vacuo* at room temperature, gave 241.6 g of benzene-acetone soluble (BAS) yellow oil. This material was dissolved in 10 volumes of *n*-heptane (pre-equilibrated with 95% methanol) and extracted twice with equal volume of 95% methanol (pre-equilibrated with *n*-heptane). The methanol-soluble material (BAS-MS) was obtained by evaporation of the solvent, giving 30.8 g of brown oil. The heptane-soluble material (BAS-HS) was obtained in a similar manner, yielding 192.5 g of brown oil.

The analyses summarized in Table 1 show a clean separation of carbohydrate-containing material in the methanol phase. The heptane phase contained, mainly, triglyceride plus sterol and sterol ester.

For silicic-acid chromatography, 30.7 g of BAS-MS was dissolved in 100 ml of chloroform and applied to a 1-kg silicic-acid column (35 × 7 cm). Silicic acid (Mallinckrodt, 20% maximum water content, 100 mesh) was activated by heating in an oven for 12 hours at 110°. This activated silicic acid was washed thoroughly with methanol until the supernatant became clear. The slurry was then poured into a glass column and washed with 3 volumes of chloroform, after which the sample solution was applied. The elution was started with chloroform and continued with increasing concentrations of methanol in chloroform (0% to 30%); 500-ml fractions were collected. Table 2 contains data on the resultant combined fractions. The characterization of each fraction was carried out by paper chromatography. After mild alkaline hydrolysis (1 N sodium hydroxide at 37° for 24 hours), followed by neutralization with Dowex-50 (HCO_3^-), the water-soluble material was applied to Whatman No. 1 filter paper and chromatographed in an *n*-butanol:pyridine:water (6/4/3, v/v) solvent system, using the ascending technique. Standards of glycerol, monogalactosylglycerol, and digalactosylglycerol were applied under the same conditions. The combined fractions, listed in Table 2, were analyzed in a similar manner and also by infrared spectroscopy.

[1] General Mills Soft-as-Silk unbleached cake (wheat) flour.

[2] Abbreviations used: BAI: benzene-acetone insoluble material; BAS: benzene-acetone soluble material; BAS-MS: benzene-acetone soluble-methanol soluble material; BAS-HS: benzene-acetone soluble-heptane soluble material.

TABLE 1. ANALYSES OF HEPTANE- AND METHANOL-SOLUBLE MATERIAL FROM BENZENE-ACETONE SOLUBLE EXTRACT OF UNBLEACHED WHEAT FLOUR

	Heptane Soluble (BAS-HS)	Methanol Soluble (BAS-MS)
	per cent	*per cent*
Sugar (anthrone)	negative	13.3
Nitrogen (Kjeldhal)	0.03	0.11
Long-chain base nitrogen (McKibbin-Taylor)	0.02	0.17
Phosphorus	0.01	0.12

On the basis of these analyses, fractions 1 to 29 contained neutral fats; fractions 34 to 40 were rich in the monogalactosylglycerol lipid; digalactosylglycerol lipid was concentrated in fractions 44 to 72, and cerebrosides in fractions 61 to 65. The recovery was 82.3% of the starting material.

Purification of Monogalactosylglycerol Lipid. A pure sample of monogalactosylglycerol lipid was obtained by rechromatography of combined fractions 34 to 40. This material (5.69 g) was dissolved in a minimum amount of chloroform and applied to a 200-g column of silicic acid (3.5 × 50 cm). Two hundred and ninety fractions (each 12.5 ml) were eluted using chloroform (fractions 1 to 140) and chloroform-methanol mixtures (fractions 141 to 290), as solvents. Monogalactosylglycerol lipid, free from the neutral glycerides and

TABLE 2. SILICIC-ACID CHROMATOGRAPHY OF METHANOL-SOLUBLE MATERIAL (30.7 G) FROM BENZENE-ACETONE SOLUBLE EXTRACT OF UNBLEACHED WHEAT FLOUR

Combined Fractions	Solvent (Chloroform:-Methanol)	Weight	Paper Chromatographic Analysis*		
			Glycerol	Mono-	Di-galactosylglycerol
		g			
1–29	100:0	10.76	+++	+	−
30–33	98:2	0.11	++	+	−
34–40	98:2	5.69	+−	+++	−
41–43	98:2	0.56	+−	+++	−
44–60	96:4	0.91	+−	+	+++
61–65	94:6	0.60	+−	−	++
66–72	90:10	5.11	−	−	+++
73–80	80:20	1.30	−	−	+−
81–91	70:30	0.24	−	−	−

* Abbreviations: +++ strongly positive; ++ positive; + slightly positive; − negative. Detected with permanganate-periodate spray.

digalactosylglycerol lipid, was accumulated in fractions 181 to 289 (eluted with chloroform:methanol 98/2, v/v). These combined fractions, after evaporation of solvent, gave 1.88 g of white amorphous powder containing 23.9% of sugar. The infrared spectrum showed strong absorption due to hydroxyl and ester bonds. Paper chromatographic analysis of the alkaline hydrolysis products, as described above, showed only the presence of monogalactosylglycerol (detected with permanganate-periodate spray).

Purification of Digalactosylglycerol Lipid. The combined fractions 66 to 72 (Table 2) showed only digalactosylglycerol in paper chromatography, but in the infrared spectrum, absorptions due to free fatty acids (1705 cm^{-1}) and cerebrosides (1525, 1650 cm^{-1}) were also present. A solution of 5.10 g of the combined fractions 66 to 72 in 50 ml of 95% methanol was passed three times through an Amberlite®-MB-3 column (30 × 4.5 cm). The column was washed with 500 ml of the same solvent. The combined methanolic solutions were evaporated to dryness *in vacuo* at room temperature, giving 3.34 g of white amorphous, rather hygroscopic material. The analysis showed 33.5% of carbohydrate, and in the infrared spectrum the absorption due to free acids was completely absent. However, the absorption due to amide bonds (1525, 1650 cm^{-1}) was still present, indicating (on the basis of ratio between ester and amide bonds) 5% to 10% of cerebrosides as impurity. The *complete* separation of digalactosylglycerol lipid from cerebrosides was achieved only on partially methylated material, as is described in the next article in this periodical (5).

Saponification Equivalents of Mono- and Digalactosylglycerol Lipids. Saponification equivalents were determined on 100 to 150 mg samples of purified monogalactosylglycerol lipid, and on 200 to 400 mg samples of digalactosylglycerol lipid. A solution of 139.8 mg of monogalactosylglycerol lipid in 10 ml of 0.1 N methanolic sodium hydroxide and 1 ml of water was refluxed for 2 hours. The excess of sodium hydroxide was determined by titration with 0.1 N hydrochloric acid, using a pH meter for estimation of the end point. The consumption was 3.70 ml 0.1 N sodium hydroxide. For digalactosylglycerol lipid, a solution of 378.8 mg of sample in 10 ml of 0.1 N methanolic sodium hydroxide and 1 ml of water was refluxed for 2 hours, consuming 8.14 ml of base. The saponification equivalent for monogalactosylglycerol lipid obtained in this way was 378, and for digalactosylglycerol lipid 466, giving molecular weights, respectively, of 378 and 466 (one fatty acid per molecule) or 756 and 932 (two fatty acids per molecule). (Calculated saponification equivalents for monostearoyl-monogalactosylglycerol, 520.7; distearoyl-monogalactosylglycerol, 393.5; monostearoyl-digalactosylglycerol, 682.8; distearoyl-digalactosylglycerol, 474.6.)

For determination of neutralization equivalents, fatty acids were extracted from the acidified aqueous-methanolic saponification mixture with ether. The ether solution was washed with water, dried, and evaporated; the resulting mixture of fatty acids was dried *in vacuo* and titrated with 0.1 N sodium hydroxide. For monogalactosylglycerol lipid a value of 258, and for digalactosylglycerol lipid, a value of 303 was obtained.

Composition of Fatty Acids of Mono- and Digalactosylglycerol Lipid. The fatty acids, obtained as described above, were converted to methyl esters with diazomethane. The gas-liquid chromatographic analyses of methyl esters were performed in the Research and Development Department of the Procter and Gamble Company, Cincinnati, Ohio. Conditions for the analysis: The adipic acid-ethylene glycol polyester (12% on 80 to 100 mesh Chromosorb W) column (200 cm); helium flow rate, 91 ml/minute; column temperature, 208°; sample volume, 0.4 ml of 50% solution in methanol. The results of the analyses are given in Table 3.

TABLE 3. ANALYSES OF METHYL ESTERS OF FATTY ACIDS FROM GALACTOSYLGLYCEROL LIPIDS FROM UNBLEACHED WHEAT FLOUR

Corresponding Acid	Corrected Retention Time	Composition*	
		Mono-galactosylglycerol lipid	Di- lipid
	minutes	*per cent*	*per cent*
Myristic	4.39	0.5	
Palmitic	8.76	13.9	41.6
Palmitoleic	9.55	3.4	
Stearic	16.94	1.3	4.4
Oleic	18.76	17.2	12.1
Linoleic	22.73	57.0	29.3
Linolenic	28.49	2.0	
All others		5.1	12.6

* Values listed can be in error as much as ±15% relative.

Isolation of Crude Cerebroside Fraction. Combined fractions 61 to 65 (600 mg), obtained by silicic-acid chromatography of benzene-methanol soluble material, as described above, were dissolved in 50 ml of 1 N sodium hydroxide in aqueous methanol and left at 37° for 36 hours. After dilution with the same volume of water and neutralization with 1 N hydrochloric acid to pH 7 (in ice), the suspension was extracted three times with chloroform. The extract was washed with water, dried, and evaporated to dryness. The residue was redissolved in about 100 ml of 95% methanol and the solution was passed through a Dowex-2 (OH^-) column to remove fatty acids. After evaporation of

solvent, 297 mg of colorless powder (m.p. 159°–162°) was obtained (analyses: C 66.72, H 11.09, N 2.00%).

The infrared spectrum showed strong absorption due to hydroxyl, amide-, and *trans*-double bonds, and complete absence of ester bonds.

Isolation of Sitosteryl-β-D-Glucoside. Fractions eluted with chloroform:methanol 98/2 (v/v) from silicic acid in the course of chromatography of methanol-soluble material from benzene extracts of unbleached wheat flour were contaminated with Liebermann-Burchard positive material. This material was separated in the course of mild alkaline hydrolysis of fractions, described above, as methanol and acetone-insoluble material. For purification, in one experiment, 500 mg of this material was suspended in chloroform and applied to a silicic-acid column (16 × 2.5 cm). In fraction 3, eluted with chloroform:methanol 97/3 (v/v), 159.8 mg of material was recovered. The elementary analysis of this material indicated the presence of sitosteryl-β-D-glucoside (6).

$C_{35}H_{60}O_6$ (576.83)

Calculated:	C 72.88, H 10.50%
Found:	C 72.02, H 10.39%

Acidic hydrolysis of material released glucose, as detected by paper chromatography.

The polyacetyl derivative of the same material, prepared in the usual way, after crystallization from *n*-hexane, gave a crystalline compound (m.p. 166°–168°). The reported melting point for tetraacetyl-sitosteryl-β-D-glucoside is 166°–168°(7). The elementary analyses were in fair agreement with calculated values:

$C_{43}H_{68}O_{10}$ (744.97)

Calculated:	C 69.32, H 9.20%
Found:	C 68.78, H 9.12%

The infrared spectrum of this compound was superimposable with that of authentic sample.

Degradation of Sitosteryl-Glucoside to Sitosterol. To a solution of 52 mg of sitosteryl-glucoside, isolated as described above, in 4 ml of chloroform and 4 ml of methanol, 120 mg of periodic acid in 5 ml of water was added. After standing overnight at room temperature, the reaction mixture was passed through Dowex-2-(OH^-) column. To the clear filtrate a mixture of 50 mg of hydroxylamine hydrochloride and 50 mg of sodium acetate was added, and the solution was left overnight at room temperature. After deionization and evaporation to dryness, 52 mg of crude oxime was obtained, which was dissolved in 0.3 N hydrochloric acid in methanol-chloroform 1/1 (v/v) and left at 30° for 24 hours. The reaction mixture was extracted with ether. The ether solution was evaporated to dryness and the residue (36 mg), after two recrystallizations from ethanol, gave 20 mg of crystalline compound melting at 142° (β-sitosterol, m.p. 137°–137.5° [8]).[3] The elementary analyses were in good agreement with the calculated values for sitosterol with 1 mole of water:

$C_{29}H_{52}O_2$ (432.91)

Calculated:	C 80.31, H 12.08%
Found:	C 81.01, H 11.70%

Bleached Wheat Flour.[4] *Isolation and Characterization of Lipids.* The benzene extract of bleached wheat flour, obtained in the same way as described for unbleached wheat flour, was precipitated with acetone. To the solution of about 600 g of lipid in 900 ml of benzene, 9 liters of acetone was added, and after standing at 4° overnight, the precipitate was filtered, redissolved, and reprecipitated in the same way. The insoluble material (130 g) was filtered off, and the benzene-acetone soluble material was obtained by evaporation of solvent. This brown oil (430 g) was distributed between 95% methanol and *n*-heptane, as described above, giving, after evaporation of solvents, 192 g of BAS-MS and 213 g of BAS-HS. The comparative yields of these fractionations are listed in Table 4.

TABLE 4. COMPARATIVE YIELDS OF VARIOUS FRACTIONS AFTER SOLVENT FRACTIONATION OF BENZENE EXTRACT OF UNBLEACHED AND BLEACHED WHEAT FLOUR LIPIDS

Material	BAS	BAS-HS	BAS-MS	BAI
	per cent	*per cent*	*per cent*	*per cent*
Unbleached wheat flour	73–93 (78)*	43–81 (43)*	12–30 (15)*	21–23
Bleached wheat flour	71–86 (86)*	42–58 (50)*	27–35 (34)*	14–23 (17)*

* Figures in parentheses are values most frequently obtained.

The silicic-acid chromatography of 50.0 g of BAS-MS material on 1 kg of silicic acid (35 × 7 cm), under the previously described conditions, gave fractions which were characterized as listed in Table 5. On the basis of these analyses (combined with results obtained by infrared spectroscopy), fractions 1 to 5 were shown to be rich in neutral fats, fractions 13 to 24 in monogalactosylglycerol lipid, and fractions 33 to 71 in digalactosylglycerol lipid. The main cerebroside fraction appeared in fractions 66 to 71, although absorptions (at 1650 to 1660 and 1530 to 1550 cm^{-1}) due to amide bonds in the infrared spectrum were weaker and less

[3] The observed higher melting point probably indicates the presence of α-sitosterol as an impurity (8).

[4] General Mills Soft-as-Silk cake flour milled from soft red winter wheat and bleached with chlorine.

TABLE 5. SILICIC-ACID CHROMATOGRAPHY OF METHANOL-SOLUBLE MATERIAL (50.0 G) FROM BENZENE-ACETONE SOLUBLE EXTRACT OF BLEACHED WHEAT FLOUR

Combined Fractions	Solvent (Chloroform:-Methanol)	Weight	Paper Chromatographic Analysis*		
			Glycerol	Monogalactosylglycerol	Digalactosylglycerol
1–5	100:0	19.83	+++	−	−
11–12	99:1	1.67	+	+++	−
13–18	98:2	4.34	+	+++	−
19–24	97:3	2.73	+	++	−
25–30	96:4	2.50	++	++	++
31–36	95:5	4.59	−	+	++
37–42	94:6	2.64	−	+−	+++
43–48	93:7	0.79	−	+−	+++
49–54	92:8	0.59	−	+−	+++
55–60	90:10	1.15	++	−	+++
61–65	80:20	0.97	+	−	+++
66–68	80:20	1.09	+	−	+++
69–71	50:50	0.64	−	−	+++

* Abbreviations: +++ very positive; ++ positive; + slightly positive; − negative. Detected with permanganate-periodate spray.

sharp than in the case of the cerebroside fraction isolated from unbleached wheat flour. Recovery was 89% of starting material.

Comparative analytical data of the various fractions from silicic-acid chromatography of bleached and unbleached material are listed in Table 6.

Benzene-Acetone Insoluble Fractions of Unbleached and Bleached Wheat Flour Lipids. Benzene-acetone insoluble materials (BAI) of unbleached and bleached wheat flour lipids were distributed between 95% methanol and *n*-heptane by dissolving the material (16.83 g of material from unbleached, and 67.0 g of material from bleached wheat flour) in 95% methanol, and extracting the solution several times with *n*-heptane. Results of these distributions are given in Table 7, showing a low yield of heptane-soluble material from unbleached wheat flour in comparison with the high yield from bleached wheat flour. The methanol-soluble material of unbleached wheat flour is far less rich in sugar than the corresponding fraction from bleached flour.

The formation of interfacial material, not soluble in methanol or heptane, was observed. The amount of interfacial material from unbleached wheat flour lipid was 53%, and from bleached wheat flour lipid 49.3% (variation in range of 17% to 50%). This material, with content of phosphorus in range of 0.60% to 1.00% and nitrogen 7% to 11%, is lipoprotein or lipopeptide-like material, a detailed study of which fraction will be the subject of a subsequent communication.

TABLE 6. COMPARATIVE ANALYTICAL DATA ON FRACTIONS ELUTED FROM SILICIC-ACID CHROMATOGRAPHY OF METHANOL-SOLUBLE MATERIAL FROM BENZENE-ACETONE SOLUBLE EXTRACT OF UNBLEACHED AND BLEACHED WHEAT FLOUR LIPIDS

Fraction (Chloroform:-Methanol)	Analyses						
	Sugar		Nitrogen		Phosphorus		Chlorine
	Unbleached	Bleached	Unbleached	Bleached	Unbleached	Bleached	Bleached Only
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
100:0	1.23	0.31	0.07	0.01	0.07	0.02	7.75
99:1	20.00	11.91	0.07	0.21	0.30	0.08	*
98:2	19.33	11.63	0.17	0.30	0.16	0.38	5.15
96:4	32.93	15.90	0.36	0.28	0.13	0.48	*
95:5	35.36	26.39	0.10	0.33	0.06	0.18	4.01
90:10	*	37.65	*	0.20	*	0.12	*
80:20	*	37.21	*	0.60	*	0.29	5.74
60:40	4.37	26.04	1.43	2.00	2.09	1.96	*

* Not determined on this fraction.

TABLE 7. SOLVENT DISTRIBUTION OF BENZENE-ACETONE INSOLUBLE MATERIAL OF UNBLEACHED (16.83 G) AND BLEACHED (67.0 G) WHEAT FLOUR LIPIDS*

Phase	Transfer Number	Yield		N		P		Sugar		Liebermann-Burchard Test	
		U	B	U	B	U	B	U	B	U	B
		g	*g*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
Heptane	1	0.09	8.75							+++	+++
Heptane	2	0.04	0.33							++	+++
Heptane	3	0.03	0.13							+	+++
Heptane	4	0.02	0.08							+	++
Methanol	5	0.09	0.19	1.77	1.80	1.96	1.11	3.21	9.24	−	−
Methanol	6	0.76	0.51	2.94	2.63	2.52	1.21	4.80	13.60	−	−
Methanol	7	6.37	20.04	3.71	4.48	2.25	1.17	†	15.82	−	−
Methanol	8	9.01	11.13	7.34	4.17	1.62	1.03	†	8.70	−	++
Methanol	9		3.41		5.17		1.20		8.65	−	+
Methanol	10		18.49		7.50		0.76		19.88	−	−

* Abbreviations: U = unbleached; B = bleached; +++ very positive; ++ positive; + slightly positive; − negative.
† Not determined on this fraction.

Isolation of Cerebroside-like Compounds from Unbleached Material. Methanol-soluble material from BAI of unbleached wheat flour (combined fractions 6 and 7, Table 7) was fractionated on silicic acid. The solution of 6.32 g of this material in chloroform: methanol 9/1 (v/v) was applied to a 200-g silicic-acid column. The fractions obtained (77% recovery), using solvent mixtures of chloroform-methanol (9/1 to 0/10, v/v), were analyzed after acidic hydrolysis, giving the sugars listed in Table 8. The infrared spectrum showed still very strong ester absorption. In order to obtain purified cerebrosides, fractions 1 and 2 from Table 8 were combined and hydrolyzed in 0.1 N aqueous sodium hydroxide at 37° for 33 hours. After cooling, the alkaline solution was neutralized with Amberlite® IR-120 (H^+) and repeatedly extracted with chloroform and methanol, giving 992 mg of waxy material. After trituration with acetone, 840 mg of acetone-insoluble substance was obtained, which still showed an ester absorption band in the infrared (1725 cm^{-1}). This material was again hydrolyzed at 37° for 14 hours, but in 1 N potassium hydroxide in methanolic solution. The solution was treated as above, giving 387 mg of ester-free material. This crude fraction was a mixture of manno-, gluco-, and possibly galactocerebrosides, but preliminary attempts to separate these cerebrosides were unsuccessful. Further studies on this fraction are under way.

TABLE 8. SILICIC-ACID CHROMATOGRAPHY OF METHANOL-SOLUBLE MATERIAL (6.32 G) FROM BENZENE-ACETONE INSOLUBLE EXTRACT OF UNBLEACHED WHEAT FLOUR LIPIDS

Fraction	Solvent	Weight	Analysis			Paper Chromatographic Analysis		
	Chloroform:-Methanol		P	N	Sugar	Mannose	Glucose	Galactose
		g	*per cent*	*per cent*	*per cent*			
1	9:1	1.931	2.21	0.91	7.28	−	+	+
2	8:2	0.594	2.23	1.62	14.92	+	−	+
3	7:3	0.555	3.04	2.80	6.51	−	−	+
4	6:4	0.387	2.87	4.03	*	−	−	+
5	1:1	0.493	2.63	3.67	22.20	−	+	+
6	0:10	0.587	2.87	4.28	*	−	−	+
7	0:10	0.220	2.59	4.68	*	Not determined		

* Atypical color with anthrone test

Isolation of the Cerebroside-like Compounds from Bleached Material. The methanol-soluble material from BAI of bleached wheat flour (combined fractions 6 and 7, Table 7) was chromatographed over silicic acid. Twenty grams of this material was applied to a 600-g silicic-acid column. The fraction eluted with chloroform:methanol 7/3 (v/v) gave 9 g of material with 22.1% of sugar, 1.03% of phosphorus, 0.99% of nitrogen, and 0.17% of long-chain base nitrogen. From two similar preparations 13.6 g of such material was obtained with strong ester absorption in the infrared spectrum. These combined materials were hydrolyzed with mild alkali (aqueous methanolic 0.1 N potassium hydroxide, 37°, 22 hours), and the solution was repeatedly extracted with chloroform. After evaporation of solvent, 9.6 g of a crude mixture of cerebrosides was obtained from bleached wheat flour. By fractionation over a 400-g silicic-acid column the chloroform-methanol 9/1 (v/v) fraction contained 1.05 g of glucocerebrosides; the chloroform-methanol 8/2 (v/v) fraction gave 1.26 g of mannocerebrosides. Both cerebrosides were characterized by infrared spectra and by paper chromatography of water-soluble acid hydrolysis products.

The crude mannocerebroside fraction (1.2 g) was triturated with acetone and centrifuged. The insoluble cerebrosides (580 mg) were dissolved in a minimum amount of cold methanol, and the insoluble material was filtered. The clear filtrate was diluted with acetone and cooled at 4°, giving 240 mg of white powder melting at 160°–200°. (Analyses: C 61.11, H 9.39, N 1.13, Cl 2.37, and sugar 45.77 and 47.11%.) These data are in reasonable agreement with a lignocerylsphingosine trimannoside structure (47.5% sugar; dimannoside, 37% sugar). This material, after acidic hydrolysis, gave a sharp and intensive spot for mannose, but glucose in very low concentration was also detected.

Distribution of chlorine in this material was determined by hydrolysis with 10% methanolic sulfuric acid for 4.5 hours in a sealed tube. After cooling and evaporation of methanol, the reaction mixture was extracted with *n*-hexane. The hexane solution was concentrated to dryness, yielding 27 mg of fatty acids, with 1.96% of chlorine.

The aqueous solution was made alkaline to pH 13 and repeatedly extracted with ether. The ether solution was washed with water, dried, and evaporated to dryness, giving 34 mg of long-chain base(s) with 2.99% of chlorine.

The remaining aqueous solution, after extraction with ether and isolation of long-chain base(s), was deionized with Amberlite®-IR-120 (H^+) and Dowex-2 (CO_3^-) resin. For complete hydrolysis of glucosidic linkage, this solution was again hydrolyzed with sulfuric acid (0.5 N, 12 hours) in a sealed tube, neutralized with Dowex-2 ($CO_3^=$), and evaporated to small volume. Four drops of phenylhydrazine were added and the reaction mixture was shaken vigorously for a few minutes and left standing at room temperature for a few hours. The crystalline material (4 mg) was

filtered and, after recrystallization from methanol, melted at 190°–191° (decomp.). The reported melting point for D-mannosephenylhydrazone is 195°–200° (decomp.) (9). The analysis was:

$C_{12}H_{18}N_2O_5$ (270.28)

Calculated: N 10.36%
Found: N 11.34%

Detection of Mono- and Digalactosylglycerol in Various Plants Material. Whatman No. 1 filter paper was used for ascending chromatography in *n*-butanol:pyridine:water 6/4/3 (v/v) solvent system. Permanganate-periodate spray was used for the detection of spots.

Corn phosphatides[5] (11.6 g) were extracted three times with dry acetone, yielding 6.13 g of acetone-soluble material and 5.19 g of acetone-insoluble fraction. Portions of these materials (1.85 g of acetone-soluble and 1.88 g of acetone-insoluble material) were refluxed with 30 ml of 0.1 N sodium hydroxide for several hours, then acidified to pH 1 with 1 N hydrochloric acid, and extracted several times with ether. The water solution was deionized by passing through Amberlite®-MB-3 column. After evaporation of the solvent, 62 mg of material from the acetone-soluble lipids and 75 mg of material from the acetone-insoluble lipids were obtained. These materials were redissolved in a small amount of water and chromatographed as described above. In the acetone-insoluble fraction, digalactosylglycerol was detected; in the acetone-soluble fraction, only monogalactosylglycerol was present.

Corn gluten[6] (75 g) was extracted with benzene for 48 hours in a Soxhlet apparatus. After concentration of benzene to dryness, 3.70 g of brown oil was obtained. A portion of this material (0.83 g) was hydrolyzed with alkali and treated as described above. The water-soluble material (21 mg), on paper chromatography, gave spots corresponding to the monogalactosylglycerol and digalactosylglycerol.

Wheat germ oil[7] (2.93 g) was hydrolyzed in the same manner as described above, giving 69 mg of water-soluble material. This material, on paper chromatography, gave digalactosylglycerol.

Euglena gracilis, strain Z, was grown on a sucrose-containing medium (10) in the light and the lipids were extracted with chloroform:methanol 2/1 (v/v). After evaporation of solvent, the crude lipids were treated with acetone. The acetone-insoluble lipids were hydrolyzed with alkali and chromatographically examined. In the water-soluble material, monogalactosylglycerol was detected.

After similar treatment of organism grown on the same medium in the dark, no galactosylglycerol could be detected.

Green oat groat oil[8] (1.01 g) was heated on a steam cone for 3 hours with 30 ml of 0.1 N sodium hydroxide, cooled, acidified with 6 N hydrochloric acid, and extracted with ether. The aqueous solution was then deionized by passing over an Amberlite®-MB-3 column and the effluent lyophilized, yielding 0.041 g of material. This material was only partially soluble in water. The paper chromatography revealed the presence of a large amount of digalactosylglycerol, a trace of monogalactosylglycerol, glycerol, and an unidentified material with lower R_f than digalactosylglycerol.

DISCUSSION

In the previous paper (1) preliminary evidence was presented that benzene-extracted wheat flour lipid contains glycerides, sterol and sterol derivatives, lipids containing galactosylglycerol, and a lipoprotein (peptide?) fraction. In undertaking a study of the glycolipid fraction, our first efforts were directed to the development of simple procedures for preparing a glycolipid fraction relatively free of other constituents.

The main part of the peptide-containing material could be removed from the benzene-extracted lipids by precipitation with acetone. The precipitate contained the majority of the amino acid nitrogen together with a considerable amount of lipid, some of which could be readily separated by repeated precipitation, giving a lipopeptide fraction with lipid more firmly bound.

Distribution of the benzene-acetone soluble material between *n*-heptane and methanol gave a sharp separation of glycolipids in the methanol phase, with triglycerides and sterol esters concentrated in the heptane phase (Table 1). The methanol-soluble material was rich in sugar-containing substances (Table 1), and was used as the starting material in most of the further fractionation studies. A number of samples of wheat flour lipid obtained from both bleached and unbleached hard and soft flour have been investigated. The flours used were commercial preparations, and in only one case did the bleached and unbleached flour come from the same batch of flour. It is not surprising, therefore, that yields of the various fractions were quite variable, precluding the drawing of any conclusions as to variation of lipid content with type or treatment of the flour. The range of values obtained, together with some typical figures, are presented in Table 4.

In preliminary studies on bleached flour lipids, it was found that the crude glycolipid mixture could be

[5] Supplied by Corn Products Corp., Pekin, Ill.
[6] See footnote 5.
[7] A gift from the Viobin Corporation, Monticello, Ill.
[8] A gift from The Quaker Oats Co., Chicago, Ill.

fractionated on a silicic-acid column, using as the eluant chloroform, with gradually increasing concentrations of methanol. The results of a typical column are shown in Table 5. A monogalactosylglycerol lipid fraction was eluted with chloroform-methanol 98/2 (v/v), followed by a digalactosylglycerol lipid fraction with increasing methanol concentrations. Obviously there is some overlapping of these main fractions, and a glycerol-containing substance is present in many of the fractions. As shown in Table 6, these fractions contained substantial amounts of chlorine, which probably accounts for some of the heterogeneity observed, since the separation obtained with a typical unbleached glycolipid sample (Table 2) was much cleaner. In both cases, however, the digalactosyl fraction showed amide absorption in the infrared. Rechromatography of the monogalactosylglycerol lipid fraction from unbleached flour gave a relatively homogeneous material free of glycerides and almost devoid of nitrogen and phosphorus. The saponification equivalent of this material (378) is in close agreement with that required for two long-chain acyl groups per molecule. Digalactosylglycerol lipid further purified by rechromatography over silicic acid gave a saponification equivalent of 466, also in close agreement with the calculated values for a diacyl derivative. The structure of these lipids is the subject of the following paper.

The infrared amide absorption of the digalactosylglycerol lipid fraction was very similar to that of a cerebroside. It was decided, therefore, to attempt the isolation of this (minor) constituent. Alkaline hydrolysis (to destroy esters) gave a crude chloroform-extractable material which, after passage over Dowex-2 (OH^-) to remove fatty acids, gave analytical data and physical properties in agreement with those for a cerebroside. The carbohydrate component proved to be glucose and the long-chain base constituent to be a mixture of three, perhaps four, components. A preliminary communication (11) has been made regarding these bases, and evidence for the structure of the cerebrosides will be presented in a subsequent paper.

In view of the relatively low solubility of cerebrosides in acetone, it seems possible that some cerebroside might have precipitated in the benzene-acetone insoluble fraction. This material, therefore, was subjected to the heptane-methanol distribution with substantial further separation of components, as shown in Table 7.

Much of the peptide nitrogen remained as insoluble interfacial material. The heptane phase contained sterol and sterol ester with little or no nitrogen or carbohydrate. The methanol phase contained (in addition to a significant amount of peptide nitrogen) the galactosylglycerol lipids and at least two different types of cerebroside, one containing glucose, the other containing mannose. Data on the separation of these two materials on silicic acid are given in Table 8. Fraction 1 (chloroform:methanol 9/1, v/v) on alkaline hydrolysis yielded the usual glucocerebroside. Fraction 2 (chloroform:methanol 8/2, v/v) gave, after alkaline hydrolysis, mannocerebroside, whose mannose content corresponded to that of a trimannoside derivative. The chloroform:methanol 6/4 (v/v) fraction from this column appeared to contain a lipopeptide.

It thus appears that wheat flour contains two galactosylglycerol lipids and a complex mixture of gluco- and mannocerebrosides, for whose isolation preparative methods are presented in this paper.

In extending these studies of the galactosylglycerol lipids to other plant sources, we have found similar materials in corn gluten, wheat germ oil, green oat groat oil, and lipids of *Euglena gracilis*, strain Z. The galactosylglycerols have been found also after hydrolysis of *Chlorella* lipids (12), and in lipids of forage grasses and clovers (13), although the lipids from these sources have not, as far as we are aware, been separated and characterized.

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