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Wheat flour lipids: III. structure of the mono- and digalactosylglycerol lipids*

H. E. Carter, R. A. Hendry,† and N. Z. Stanacev‡

Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana, Illinois

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SUMMARY

Determination of the structures of the mono- and digalactosylglycerol lipids of wheat flour is reported. Methylation of these lipids, followed by alkaline saponification, yielded partially methylated derivatives. Periodate oxidation of these substances in each case gave 1 mole uptake with the formation of formaldehyde. These data establish the presence of two vicinal hydroxyls in the glycerol residue, and strongly indicate that in the original lipid the two acyl groups are attached to these hydroxyl groups. This assignment was confirmed by acidic hydrolysis studies. The monogalactosylglycerol derivative gave 2,3,4,6-tetra-O-methyl-D-galactose plus glycerol; the digalactosylglycerol derivative yielded, in addition, 2,3,4-tri-O-methyl-D-galactose. In a control experiment, free digalactosylglycerol was methylated and hydrolyzed, giving only on papergrams 2,3,4-tri-O-methyl- and 2,3,4,6-tetra-O-methyl-D-galactose. These results clearly define the structure of the galactosylglycerol lipids as a 2,3-diglyceride with a carbohydrate moiety attached in the 1-position. Infrared data suggest the D-configuration for the glycerol residue.

In the preceding paper (1) we described methods for the preparation from benzene extracts of wheat flour of a glycolipid fraction and its separation into mono- and digalactosylglycerol lipids on silicic-acid columns. Each of the lipids was shown to contain two fatty acid groups per molecule. The present paper reports methylation studies on these lipids leading to a complete elucidation of the structures.

EXPERIMENTAL AND RESULTS [1]

Isolation and Purification of Mono- and Digalactosylglycerol Lipids. The mono- and digalactosylglycerol lipids employed were those reported in the preceding paper (1) (the fractions 181 to 289, obtained by rechromatography of fractions 34 to 40 and 66 to 72, respectively, as shown in Table 2 of the preceding article).

Methylation of Monogalactosylglycerol Lipid. To a solution of 1.5 g of purified monogalactosylglycerol lipid in 30 ml of absolute methanol, 5.0 g of freshly prepared silver oxide and 5.0 ml of redistilled methyl iodide were added. The suspension was stirred at room temperature for 24 hours, after which time fresh portions of 5.0 g of silver oxide and 5.0 ml of methyl iodide were added. This operation was repeated twice more at intervals of 12 hours, using the same quantities of silver oxide and methyl iodide. The reaction mixture was stirred at room temperature for 48 hours after the final addition of the reagents, and then was filtered from insoluble material. The insoluble material was washed twice with methanol, and the combined methanolic solutions were evaporated to dryness *in vacuo*, giving 2.09 g of a brown viscous oil. This material was dissolved in 20 ml of absolute ether and filtered from insoluble material. The clear solution applied to 60 g of silicic acid ("Bio-Rad"; column 2.2 × 30 cm). The first fraction, eluted with ether, after evaporation of solvent, gave 1.53 g of yellow oil. The infrared spectrum of this material still showed absorption at 3600 cm^{-1}, indicating the presence of unmethylated hydroxyl groups. However, the cerebroside amide bonds at 1550 and 1650 cm^{-1}, which were present in the starting material, had com-

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† Postdoctoral Research Associate in Chemistry. Present address: Department of Chemistry, Westminster College, New Wilmington, Pa.

‡ Postdoctoral Research Associate in Chemistry.

[1] Infrared spectra were recorded with a Perkin-Elmer double beam spectrometer Model 21 with a sodium chloride prism.

pletely disappeared. Attempts to separate completely methylated from partially methylated lipid, using neutral aluminum oxide grade II, failed.

Remethylation of Monogalactosylglycerol Lipid. The partially methylated lipid was remethylated as follows, using dioxane as solvent: A suspension of 356 mg of partially methylated lipid in 30 ml of purified dioxane and 20 ml of methyl iodide was refluxed with stirring for 72 hours in the presence of 10 g of silver oxide. Fresh portions of 10 ml of methyl iodide and 10 g of silver oxide were added and the reaction mixture refluxed a further 24 hours. The hot suspension was then centrifuged, the supernatant filtered, and the yellow filtrate evaporated to dryness *in vacuo* at 45°, giving 505 mg of yellow, very viscous oil. The infrared spectrum showed complete absence of hydroxyl bonds. The paper chromatographic examination of this material did not give any permanganate-periodate or aniline-phthalate positive spots.

Alkaline Hydrolysis of Methylated Monogalactosylglycerol Lipid. A solution of 420 mg of fully methylated monogalactosylglycerol lipid in 10.0 ml of methanolic-aqueous 0.1 N sodium hydroxide was refluxed for 4 hours. After cooling at room temperature, 1.0 N hydrochloric acid was added to bring the solution to pH 1. A sufficient amount of methanol was then added to give a clear solution, which was passed through Dowex-2 (OH^-) and Amberlite®-MB-3 columns to remove fatty acids. This operation was repeated three times to achieve complete removal of the acidic material. After evaporation of solvent *in vacuo*, 193 mg of yellow oil was obtained. This oil was purified by chromatography on Whatman No. 3 filter paper in a *n*-butanol : pyridine : water 6/4/3 (v/v) solvent system. After 20 hours at room temperature (using the ascending technique), two permanganate-periodate positive substances were detected (main fraction, R_f 0.70–0.75; minor spot, R_f 0.90–0.95).

The material with R_f 0.90–0.95, in the above solvent system, gave a rather elongated spot and was eluted from the filter paper with methanol at 37° for 24 hours. Evaporation of solvent gave 38.4 mg of material, which showed a poor infrared spectrum and could not be completely characterized. The acidic acid hydrolyzate of this substance gave a spot (detected by aniline-phthalate) with an R_f similar to that of tetra-O-methyl-D-galactose. No permanganate-periodate spots were present.

The main fraction (R_f 0.70–0.75 detected by permanganate-periodate spray) was obtained by elution with methanol at 37° for 24 hours. Evaporation of the solvent gave 120.4 mg of a colorless oil. The infrared spectrum of this material showed strong absorption at 3550 cm^{-1} (hydroxyl) and the complete absence of absorption in the region of 1700 to 1750 cm^{-1}, thus establishing complete removal of ester groups. Quantitative periodate oxidation of this material will be described later.

Acidic Hydrolysis of Methylated Monogalactosylglycerol Lipid. For acidic hydrolysis, 20 to 30 mg of the R_f 0.70–0.75 material described above was used. The sample was heated with 0.5 N aqueous sulfuric acid in a sealed tube at 100° for 16 hours. The reaction mixture was neutralized with Dowex-2 (OH^-) resin, filtered free from resin, and concentrated to small volume *in vacuo*, but never to dryness. Paper chromatography, using ascending and descending techniques and Whatman No. 1 and No. 3 filter paper in two solvent systems—(*a*) *n*-butanol:pyridine:water 6/4/3 (v/v), for ascending chromatography or (*b*) ethyl acetate:acetic acid:water 3/1/3 (v/v), for descending chromatography—on the basis of at least five experiments, always showed (*a*) one intensive spot, detected with permanganate-periodate spray, with identical R_f (0.57 in ascending technique) as that of a glycerol standard; (*b*) one sharp spot, detected with aniline-phthalate spray, with same R_f (0.87 in ascending technique) as that of a 2,3,4,6-tetra-O-methyl-D-galactose standard. Co-chromatography of the unknown sample with the above standards gave similar results.

Methylation of Digalactosylglycerol Lipid. The digalactosylglycerol lipid was methylated in a similar manner to that described for the remethylation of monogalactosylglycerol lipid. A solution of 1.834 g of the lipid in 50 ml of purified dioxane was refluxed with 50 ml of methyl iodide and 25 g of silver oxide for 24 hours, after which time a new portion of 15 g silver oxide and 15 ml of methyl iodide was added and the refluxing continued for a further 24 hours. The same quantity of silver oxide and methyl iodide was again added to the reaction mixture and the refluxing continued, with stirring, for another 24 hours. After this time (72 hours of refluxing in all) the reaction mixture was worked up as described earlier, and the resultant yellow oil was purified on silicic acid. This purification is essential to remove methylation products of the contaminating cerebrosides. The partially methylated lipid was dissolved in 20 ml of ether and applied to 100 g of silicic acid ("Bio-Rad"; 2.7 × 20 cm). The first fraction, eluted with 300 ml of ether, gave a yellow oil, which still had strong absorption due to unmethylated hydroxyls, but did not show amide absorption. After two further methylations under the same conditions as described above, 1.339 g of yellow oil was obtained. This material

showed only a faint trace of infrared absorption due to hydroxyl, and on chromatographic examination was periodate-permanganate and aniline-phthalate negative.

Alkaline Hydrolysis of Methylated Digalactosylglycerol Lipid. The methylated digalactosylglycerol lipid (1.241 g) was refluxed 4 hours with 20 ml of 0.5 N methanolic-aqueous sodium hydroxide. After cooling, acidification with 1 N hydrochloric acid, and the removal of fatty acids as described—by using Dowex-2 (OH^-) and Amberlite-MB-3 column—381.5 mg of colorless oil was obtained. The infrared spectrum of this material showed strong hydroxyl absorption, but no absorption in the region of 1700 to 1750 cm^{-1}. This oil was further purified either by using preparative paper chromatography, as described for the methylated monogalactosylglycerol, or, more efficiently, by using a carbon:celite column.

For purification on a carbon:celite (1:1) column, 100 mg of the alkaline hydrolysis product was dissolved in 5 ml of 80% ethanol and applied to the column (1.8 × 7 cm), prepared by the procedure described earlier (2). Fraction 1, eluted with 50 ml of 80% ethanol, gave, after evaporation of solvent, 74 mg of colorless oil. This material, on paper chromatography, was permanganate-periodate positive and aniline-phthalate negative, and showed strong absorption in the infrared at 3500 cm^{-1} (hydroxyl) with no absorption in the region of 1700 to 1750 cm^{-1}. The absorption due to amide bonds (cerebrosides) also disappeared. Quantitative periodate study of this substance will be described later.

Acidic Hydrolysis of Methylated Digalactosylglycerol Lipid. The acidic hydrolysis of deacylated methylated derivative was performed in the same manner as described above, using 0.5 N aqueous sulfuric acid in a sealed tube for 16 hours at 100°. Paper chromatography, using the same techniques and solvent systems as described for methylated monogalactosylglycerol, showed (*a*) one intensive spot, detected with permanganate-periodate spray, with the same R_f (0.57 in ascending technique) as glycerol standard; (*b*) two spots, detected with aniline-phthalate spray, with the same R_f as standards of 2,3,4-tri-O-methyl-D-galactose (0.78 in ascending technique), and 2,3,4,6-tetra-O-methyl-D-galactose (0.87 in ascending technique). Co-chromatography of the unknown sample with above standards gave similar results, indicating the identity of the unknown mixture with the applied standards of glycerol, 2,3,4-tri-O-methyl- and 2,3,4,6-tetra-O-methyl-D-galactose.

Methylation of Digalactosylglycerol. Digalactosylglycerol, obtained by alkaline hydrolysis of lipid, as described above, was methylated by the procedure of Kuhn *et al.* (3). To a solution of 350 mg of carbohydrate in 10 ml of redistilled dimethylformamide, 6.8 g of methyl iodide and 3.2 g of silver oxide were added. The reaction mixture was shaken 16 hours in a stoppered glass cylinder. The contents of the cylinder were then transferred to a centrifuge tube and centrifuged. The supernatant, combined with the washings (dimethylformamide and chloroform), was washed with a solution of 0.4 g of potassium cyanide in 60 ml of water. The dimethylformamide-chloroform layer was separated, and the aqueous solution extracted five times with 10 ml of chloroform. The combined extracts, after washing four times with 25 ml of water, were dried over sodium sulfate and evaporated to dryness. The residual syrup was purified by distillation at 280° to 290° *in vacuo* (1 to 2 mm of Hg). A yield of 158 mg of colorless oil (or 36.5% of theory) of the nona-O-methyl-digalactosylglycerol was obtained. The analysis was in agreement with calculated values.

$C_{24}H_{46}O_{13}$ (542.61)

Calculated:	C 53.11;	H 8.55
Found:	C 53.22;	H 8.61

This oil, after several weeks, crystallized to a white solid with m.p. 52°–54°. Infrared analysis showed only a faint trace of absorption due to hydroxyl group.

The methylated digalactosylglycerol (10 mg) was hydrolyzed with acid in the usual way. The hydrolyzate, after neutralization with Dowex-2 (HCO^-_3), was analyzed by the described paper chromatographic techniques. The solvent system was the upper layer of *n*-butanol:ethanol:water:ammonia, 40/10/49/1 (v/v). Only two spots were detected with 5% ammoniacal silver nitrate spray: one corresponded to 2,3,4,6-tetra-O-methyl-D-galactose (R_f 0.80) and the other to 2,3,4-tri-O-methyl-D-galactose (R_f 0.60). No glycerol was found in the hydrolyzate, although a strong spot was obtained with the standard (R_f 0.41). This indicated that the glycerol moiety had been methylated and was released on acid hydrolysis as 2,3-di-O-methylglycerol, which would not be expected to react with the spray employed

Periodate Oxidation Studies. Oxidation of Deacylated Methylated Monogalactosylglycerol Lipid (Tetra-O-methylgalactosylglycerol). The method used for these studies was described by Reeves (4). To a solution of 16.2 mg of deacylated methylated monogalactosylglycerol lipid, in 2 ml of absolute methanol, 1 ml of a 0.2 M aqueous solution of sodium metaperiodate was added. The reaction mixture was left at room temperature for 12

hours in the dark, after which time an aliquot was taken for a determination of periodate consumption. The estimated consumption of sodium periodate was 1.03 mole per mole of methylated monogalactosylglycerol.

The main part of the reaction mixture was reduced with 4 ml of 0.2 M disodium arsenite solution at room temperature for 3 hours, and the reaction mixture then buffered with 2 ml of 1 N sodium acetate:1 N hydrochloric acid 1/1 (v/v) solution. One milliliter of 5% ethanolic solution of dimedon was added, the reaction mixture was heated to reflux for 2 to 3 minutes, and then cooled at 4° for 6 hours. A dimedon derivative of formaldehyde (6.2 mg) with m.p. 189.5°–190.5° was obtained in yield of 43% of theory.

Oxidation of Deacylated Methylated Digalactosylglycerol Lipid (Hepta-O-methyl-digalactosylglycerol). To the solution of 32.9 mg of deacylated methylated digalactosylglycerol lipid in 10 ml of absolute methanol, 5 ml of 0.2 M aqueous sodium periodate was added and the reaction mixture left for 12 hours at room temperature. An aliquot was removed for determination of periodate consumption. The estimated consumption of sodium periodate was 1.34 mole per mole of methylated digalactosylglycerol. The remaining reaction mixture was reduced with 25 ml of 0.2 M sodium arsenite for 4 hours at room temperaure, buffered with 20 ml of 1 N sodium acetate:1 N hydrochloric acid 1/1 (v/v), and to this solution 10 ml of 5% dimedon in absolute ethanol was added. The clear reaction mixture was heated to boiling for 2 to 3 minutes, cooled at 4° for 6 hours, and filtered, giving 10.4 mg of white crystals with m.p. 183°–185°. After one crystallization from aqueous methanol, this material gave 9.0 mg (55% of theory) of the dimedon derivative of formaldehyde with m.p. 190.5°–191.5°.

Determination of the Configuration of the Glycerol Residue in Monogalactosylglycerol Lipid. The alkaline hydrolysis of monogalactosylglycerol lipid gave an aqueous solution which was deionized by passing through Amberlite®-MB-3 resin and then lyophilized. The residue gave crystalline monogalactosylglycerol (m.p. 139.5°–142°, $[\alpha]^{27} = +3.77°$), as described previously (2). The infrared spectrum of this substance (on KBr) was completely identical in the fingerprint region with that of the O-β-galactopyranosyl-(1 → 1)-D-glyceritol (m.p. 140.5°–141.5°, $[\alpha]^{20} = -7°$), synthesized by Wickberg (5). On the basis of this evidence it is concluded that the glycerol residue in the original lipid has the D configuration. Although the natural and synthetic compounds have similar melting points and superimposable infrared spectra, they differ somewhat in rotations, which remains to be explained. A similar conclusion has been reached by Wickberg (6), employing a sample of our monogalactosylglycerol from wheat flour lipid.

DISCUSSION

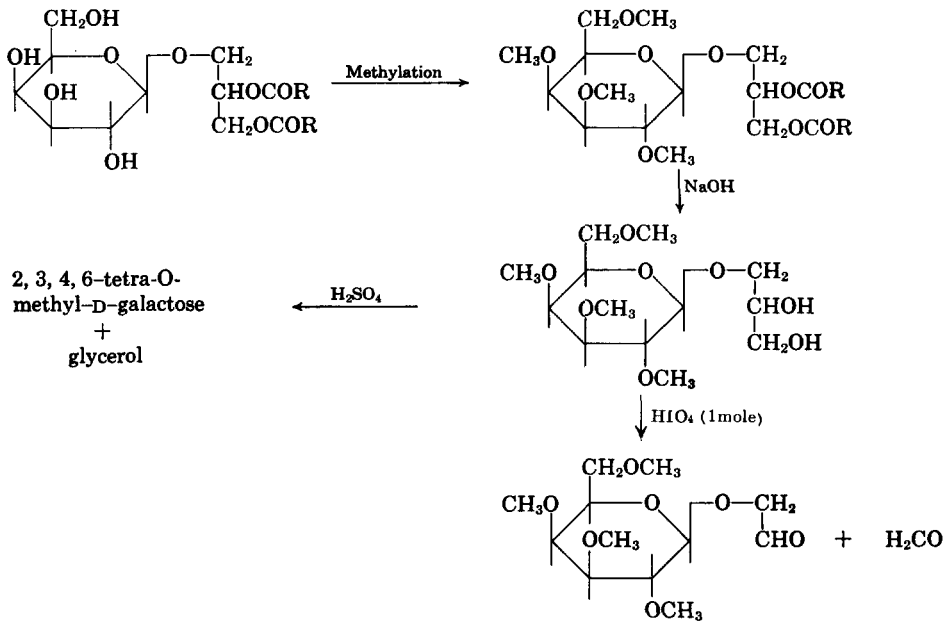
In 1956 we reported (2) fractionation studies on the benzene-extractable lipids of wheat flour, and described the partial separation of two galactosylglyceride components. Two problems arose from this study. The first concerned the identification of the galactosylglycerol moiety, obtained by alkaline hydrolysis of both lipids; the second concerned the structure of the intact lipids. In the initial communication (2) the carbohydrate moieties (obtained by alkaline hydrolysis of the lipids) were characterized as β-D-galactopyranosyl-1-glycerol, and α-D-galactopyranosyl-1,6-β-D-galactopyranosyl-1-glycerol. The second paper in this series (1) described the quantitative determination of the products of hydrolysis of both lipids, and the semiquantitative determination of the composition of the fatty acids. On the basis of these studies it was concluded that in both mono- and digalactosylglycerol lipids, 2 moles of fatty acids were present per mole of lipid. This fact should be noted, since a galactosylglycerol lipid from *Chlorella* was reported (7, 8) to have a monoglyceride structure. In the case of wheat flour glycolipids, final proof of the presence of 2 moles of fatty acids per mole of glycolipids is provided by the experiments reported in this paper.

Methylation, followed by alkaline deacylation, gave, in the case of both glycolipids, products that consumed 1 mole of periodate per mole with the formation of formaldehyde. Acidic hydrolysis of these products released only 2,3,4-tri-O-methyl-D-galactose and unmethylated glycerol, and 2,3,4-tri-O-methyl-D-galactose, 2,3,4,6-tetra-O-methyl-D-galactose and unmethylated glycerol, respectively. This evidence demands a structure with two vicinal esterified hydroxyls in the glycerol moiety of the lipid, i.e., the 2,3-diacyl-glyceryl-glycoside structure.

The reactions involved with monogalactosylglycerol lipid are shown in the equations on page 227.

It should be noted that the structure previously assigned to the digalactosylglycerol moiety is confirmed by the methylation studies, since the methylation product on acid hydrolysis gave essentially equimolar quantities of 2,3,4-tri-O-methyl- and 2,3,4,6-tetra-O-methyl-D-galactose.

Wickberg has isolated and characterized mono- and digalactosylglycerol from red algae, and for comparative studies has synthesized a series of 1-glycerol α- and β-galactosides, in both the D and L forms (5, 6). These synthetic glycosides have characteristic and



different absorptions in the frequency range 730 to 960 cm^{-1} (5). On the basis of physical properties and infrared spectra of the material isolated from red algae and synthetic samples, Wickberg was able to assign the D configuration to digalactosylglycerol from red algae, and to show its identity with our digalactosylglycerol from wheat flour. However, the monogalactosylglycerol isolated from red algae was found to be an isomorphous mixture of the D- and L-glycerol derivatives. Our monogalactosylglycerol, isolated from wheat flour, has an infrared spectrum identical with synthetic O-β-D-galactopyranosyl-D-glycerol, and therefore the glycerol residue has the D configuration. These data establish the D configuration of the glycerol residue in each of the galactosylglycerol lipids isolated from wheat flour.

In view of these experimental data, we assign to the monogalactosylglycerol lipid, isolated from unbleached wheat flour, the structure of 2,3-diacyl-1-β-D-galactopyranosyl-D-glycerol, and to the digalactosylglycerol lipid isolated from the same source, the structure of 2,3-diacyl-1-(α-D-galactopyranosyl-1,6-β-D-galactopyranosyl)-D-glycerol, with the gross composition of fatty acids described earlier (1).

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