The Isolation and Characterization of Trigalactosyl Diglyceride from Potato Tubers

By T. GALLIARD

Agricultural Research Council Food Research Institute, Norwich NOR 70F

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Trigalactosyl diglyceride has been isolated from tubers of potato (*Solanum tuberosum*) by a combination of chromatographic methods. This galactolipid, which constitutes approximately 1% by weight of the total lipids, was characterized by analysis of the intact lipid and its deacylation product. The fatty acids:glycerol:galactose molar proportions were shown to be close to 2:1:3. Evidence was obtained that suggests that trigalactosyl diglyceride is a higher homologue of mono- and di-galactosyl diglycerides and contains an additional D-galactopyranosyl moiety that is linked α -(1 $\rightarrow$ 6) to the terminal galactose unit of digalactosyl diglyceride.

Carter, McCluer & Slifer (1956) identified β -D-galactopyranosyl-1-glycerol and α -D-galactopyranosyl-(1 $\rightarrow$ 6)- β -D-galactopyranosyl-1-glycerol in the products of mild alkaline hydrolysis of lipids from wheat flour, and the parent lipids were identified as MGDG* and DGDG respectively (Carter, Ohno, Nojima, Tipton & Stanacev, 1961a; Carter, Hendry & Stanacev, 1961b). MGDG and DGDG are the major lipid constituents of chloroplasts (Allen & Good, 1965; Mudd, 1967) and, although interest has recently been focused on the possible role of these lipids in photosynthetic processes (James & Nichols, 1966), both have also been found in the non-photosynthetic tissues of higher plants that have been studied; for example, in potato tubers (Lepage, 1964a; Galliard, 1968a), turnip root (Lepage, 1967), carrot root (T. Galliard, unpublished work), pea root, cauliflower florets and avocado fruit (Ongun & Mudd, 1968), apple fruit (Galliard, 1968b), bulbs (Nichols & James, 1964) and wheat flour (Carter *et al.* 1956).

The presence in plants of a higher homologue, TGDG, has also been indicated. Benson, Wiser, Ferrari & Miller (1958) administered [14 C]CO₂ to *Chlorella* and identified a glyceryl galactotrioxide in the deacylation products of the labelled lipids. Neufeld and Hall (1964) incubated spinach chloroplasts with UDP-D-[14 C]galactose and, after deacylating the labelled lipids, observed radioactive products that had the paper-chromatographic

properties of tri- and tetra-galactosylglycerol. Ongun and Mudd (1968) separated by t.l.c. the 14 C-labelled lipids obtained by incubating either spinach chloroplasts or a particulate fraction from cauliflower florets with UDP-D-[14 C]galactose. A lipid giving a radioactive spot on t.l.c. plates was tentatively identified as TGDG. Allen, Hirayama & Good (1966) described a galactolipid from spinach chloroplasts as trigalactosyl diglyceride but no results on its structural characterization were presented. Galliard (1968a) described a 'polygalactosyl diglyceride' among the lipids of the potato tuber, which forms the subject of the present paper.

EXPERIMENTAL

Potatoes (*Solanum tuberosum*, var. Orion) were grown locally; tubers were harvested in September and stored for 4 months at 6° before use. Leaves from spinach (*Spinacia oleracea*, var. Clean Leaf) were taken from 6-week-old plants grown in the greenhouse.

Enzymes used in analytical methods were obtained as follows: galactose dehydrogenase (EC 1.1.1.48), glycerokinase (EC 2.7.1.30), pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; β -D-galactoside galactohydrolase (EC 3.2.1.23) from Koch-Light Laboratories Ltd., Colnbrook, Bucks.; 'pancreatin' (a crude freeze-dried pancreatic extract) from British Drug Houses Ltd., Poole, Dorset; NAD⁺, NADH, ATP and phosphoenolpyruvic acid from C. F. Boehringer; neocuproine (2,9-dimethyl-1,10-phenanthroline) hydrochloride, produced by Fluka A.G., Buchs, Switzerland, from Ralph Emanuel Ltd., London S.E.1; heptadecanoic acid from Phase Separations Ltd., Queensferry, Flintshire. Solvents either were purified by distillation in glass, or were

* Abbreviations: MGDG, monogalactosyl diglyceride (2,3-di-O-acyl-1-O- β -D-galactopyranosyl-D-glycerol); DGDG, digalactosyl diglyceride (2,3-di-O-acyl-1-O- β -(6-O- α -D-galactopyranosyl-D-galactopyranosyl)-D-glycerol); TGDG, trigalactosyl diglyceride.

AnalaR grade (British Drug Houses Ltd.) and used as supplied.

Extraction of total lipids. Extraction and washing of the total lipid fraction from potato tubers were as described by Galliard (1968a). 2.2g. of lipid was obtained from 2kg. of tubers and was stored as a solution in benzene-ethanol (4:1, v/v) under N_2 at -20° .

Preliminary fractionation of lipids. A DEAE-cellulose column (acetate form; 3cm. $\times$ 30cm.) was prepared as described by Galliard (1968a). The total lipid extract (2.2g.) was dissolved in chloroform (25ml.) and applied to the column. A slow stream of oxygen-free N_2 was continuously passed through the eluting solvents. The following solvent mixtures (500ml. of each) were passed through the column at approximately 5ml./min.: *A*, chloroform; *B*, chloroform-methanol (49:1, v/v); *C*, chloroform-methanol (24:1, v/v); *D*, chloroform-methanol (9:1, v/v); *E*, chloroform-methanol (3:2, v/v); *F*, chloroform-methanol (1:2, v/v). The eluate from each solvent mixture was collected as a single fraction. The lipid composition of each fraction was determined by t.l.c. of suitable samples (Galliard, 1968a).

MGDG, which was eluted in fraction *B*, was separated from esterified sterol glucosides on a column of silicic acid (Lepage, 1964b). Fraction *D* contained DGDG, which was purified by chromatography on a column of Florisil (O'Brien & Benson, 1964) to remove phospholipids and by rechromatography on a DEAE-cellulose column to separate DGDG from glucocerebroside. MGDG and DGDG prepared by these procedures were shown by t.l.c. to be free from other lipids.

Isolation of TGDG. Fraction *E* contained most of the TGDG together with phosphatidylethanolamine and traces of DGDG, glucocerebroside and an unidentified lipid. Fraction *F* contained the remaining TGDG together with phosphatidylethanolamine. These fractions were recombined and evaporated to dryness *in vacuo*; the residue was dissolved in 10ml. of chloroform-2,2-dimethoxypropane (19:1, v/v) and the solution run on to a Florisil column (2cm. $\times$ 17cm. 60-100 mesh; British Drug Houses) with the anhydrous solvent systems described by O'Brien & Benson (1964). The column was washed sequentially with the following solvent mixtures: 1, 100ml. of chloroform-2,2-dimethoxypropane (19:1, v/v); 2, 200ml. of chloroform-methanol-2,2-dimethoxypropane (38:19:3, by vol.); 3, 200ml. of chloroform-methanol-2,2-dimethoxypropane (19:19:2, by vol.); 4, 800ml. of chloroform-methanol-2,2-dimethoxypropane (19:38:3, by vol.). Fractions (200ml.) were collected and analysed by t.l.c. Solvent system 3 eluted most of the TGDG together with traces of DGDG and the unidentified lipid; the first 200ml. of solvent mixture 4 eluted the remaining TGDG plus a small amount of phosphatidylethanolamine. The above fractions containing TGDG were combined, evaporated *in vacuo* and the residue was dissolved in 10ml. of benzene-ethanol (4:1, v/v).

For final purification t.l.c. on silica gel G (750 μ m. thick; E. Merck A.-G., Darmstadt, Germany) on 20cm. $\times$ 20cm. glass plates was used. The lipid mixture was applied to the plate under N_2 and marker spots were run on side lanes. The plates were developed in chloroform-methanol-acetic acid-water (85:15:10:3, by vol.). The area on the developed plate containing TGDG was located by: (a) spraying the plate with water, and (b) spraying the marker lanes with a 0.2% solution of 2,7-dichlorofluorescein in ethanol

and examination under u.v. light. The band containing TGDG was scraped into tubes to which was added 3ml. of chloroform-methanol-formic acid-water (97:97:4:2, by vol.) (Abramson & Blecher, 1964). The silica gel was extracted three times with the solvent mixture; the supernatants were combined, evaporated to dryness *in vacuo* and the residue was dissolved in benzene-ethanol (4:1, v/v) under N_2 and stored at -20° . T.l.c. confirmed the efficiency of the preparative t.l.c. isolation of TGDG.

Analytical t.l.c. and g.l.c. These were performed as described by Galliard (1968a).

Infrared spectra. I.r. spectra of galactolipids, as approx. 5% (w/v) solutions in carbon disulphide, were obtained by using a Unicam model 200G recording i.r. spectrophotometer (Pye-Unicam Ltd., Cambridge) fitted with a rock-salt micro-cell and reference cell.

Paper chromatography. Monosaccharides and glycerol were separated by descending chromatography on Whatman no. 1 paper in butan-1-ol-pyridine-water (7:3:1, by vol.). The water-soluble deacylation products of galactolipids were subjected to descending chromatography on Whatman no. 1 paper in both phenol-water (5:2, w/w) and butan-1-ol-propionic acid-water (151:75:100, by vol.). Polyhydroxyl compounds were detected with the $AgNO_3$ -NaOH reagents (Trevelyan, Procter & Harrison, 1950).

Hydrolytic procedures. Total hydrolysis of galactolipids to glycerol, galactose and fatty acids was carried out with 1M- H_2SO_4 as described by Galliard (1968a). Deacylation of the galactolipids was accomplished by the mild alkaline hydrolytic procedure of Benson & Maruo (1958). The deacylation products were further hydrolysed with 1M-HCl for 1hr. at 100° ; the hydrolysate was neutralized with NaOH before enzymic analysis of the liberated glycerol and galactose.

Analytical methods. Ethanolic solutions of galactolipids were assayed for total sugar content by the modification (Galanos & Kapoulas, 1965) of the phenol- H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956), with use of an ethanolic solution of galactose to construct a standard curve. The fatty acid content of lipids was quantitatively determined by g.l.c. analysis of fatty acyl methyl esters prepared from a mixture containing the galactolipid and heptadecanoic acid as an internal standard. Aqueous solutions of galactolipid-hydrolysis products were analysed for total sugar content by the phenol- H_2SO_4 method (Dubois *et al.* 1956), for reducing sugars by the neocuproine method (Dygert, Li, Florida & Thoma, 1965) and for D-galactose with D-galactose dehydrogenase (Eggstein, 1966; Eggstein & Kreutz, 1966). Glycerol was determined enzymically by using the glycerokinase-pyruvate kinase-lactate dehydrogenase system (Wallenfels & Kurz, 1961). The deacylation products from the mild alkaline hydrolysis of the galactolipids were treated with β -D-galactoside galactohydrolase, a sample of the deacylated lipid containing approximately 0.1-0.2 μ mole of sugar being incubated at 30° for 4hr. with a mixture containing 0.1M-potassium phosphate buffer, pH 7.0, 5mm-KCl and 0.1mg. of enzyme in a total volume of 0.2ml. The reaction was stopped by the addition of $Ba(OH)_2$ and $ZnSO_4$ (Hestrin, Feingold & Schramm, 1955) and after centrifugation and collection of the supernatant, the reducing sugar liberated by the enzyme was determined by the neocuproine method (Dygert *et al.* 1965). Control samples containing 0.1 μ mole of D-galactose and appropriate blanks were taken through

the above procedures. To investigate the action of pancreatin and β -D-galactoside galactohydrolase on the intact galactolipids, incubations were performed as described for pancreatin by Sastry & Kates (1964b). For periodate oxidation studies, the galactolipid-deacylation products (approx. 0.6 μ mole of sugar) were incubated in the dark at 35° with 6 mM-NaIO₄ in a volume of 0.5 ml. At intervals, samples (50 μ l.) of the mixture were withdrawn and diluted with water to 5 ml. and the extent of oxidation was measured spectrophotometrically (Hay, Lewis & Smith, 1965). When the oxidation was complete (after approx. 2 hr.), further samples (25 μ l.) were taken for determination of formaldehyde by a method based on that of de Freitas (1967). The samples were diluted to 5 ml. with water, 1 M-sodium arsenite (0.1 ml.) and then ammoniacal acetylacetone were added, followed by heating and fluorescence measurement with a spectrophotometer fitted with fluorescence-measurement facilities (Eppendorf model 1100M, Netheler and Hinz G.m.b.H., Hamburg, Germany). The primary filter transmitted light at 405 nm. and 436 nm. and the secondary filter absorbed light of wavelengths below 470 nm. A standard curve for formaldehyde concentration was constructed by taking a standard glycerol solution through the oxidation procedure.

RESULTS

The amount of TGDG recovered from the isolation procedure was approximately 20 mg., representing about 1% by weight of the total lipid from potato tubers.

Characterization of the intact TGDG. TGDG gave a single spot on t.l.c. in either chloroform-methanol-acetic acid-water (85:15:10:3, by vol.) or chloroform-methanol-7 M-NH₃ (65:3:4, by vol.). Colour reactions given by the lipid when the developed t.l.c. plate was treated with specific spray reagents were identical with those also given by MGDG and DGDG, i.e. 50% (v/v) H₂SO₄ and partial charring (pink), periodate-Schiff reagents (blue) and anthrone reagent (green). In the chloroform-methanol-acetic acid-water system, the R_F values relative to MGDG (R_{MGDG}) of DGDG and TGDG were 0.40 and 0.15 respectively.

The water-soluble products from acid hydrolysis of TGDG were analysed by paper chromatography and found to contain only galactose and glycerol. T.l.c. and g.l.c. of the lipophilic hydrolysis products showed that free fatty acids only were obtained by acid hydrolysis and fatty acid methyl esters by mild alkaline hydrolysis.

The i.r. spectra of the three galactolipids, MGDG, DGDG and TGDG in carbon disulphide solution were compared. All showed basically similar spectra and major absorption bands in each were obtained for the following functional groups: O-H (3400 cm.⁻¹, broad peak), CH₂ (2860 and 2490 cm.⁻¹), alcohol C-O (1080-1090 cm.⁻¹), ester C-O (1150-1160 cm.⁻¹), ester C=O (1750-1760 cm.⁻¹) and *cis* double bonds (1665 cm.⁻¹).

These results are in agreement with those of Sastry & Kates (1964a) for the i.r. spectra of MGDG and DGDG from leaves. The relative intensities of the hydroxyl absorptions (1080 and 3400 cm.⁻¹) increased in the order MGDG < DGDG < TGDG, as expected on the basis of carbohydrate content. The molar ratio of sugars to fatty acids in TGDG was 1.53 ± 0.06 , very close to the theoretical value for a trigalactosyl diglyceride (1.5). The fatty acid composition of the lipid characterized here as TGDG has been described previously (see data for 'polygalactosyl diglyceride'; Galliard, 1968a).

Characterization of the water-soluble deacylation products from MGDG, DGDG and TGDG. The aqueous extract obtained after mild alkaline hydrolysis of TGDG gave a single spot in each of two solvent systems used for paper chromatography. In Table 1 the R_F values for deacylation products from MGDG, DGDG and TGDG are compared with published figures for the corresponding galactosyl-glycerol derivatives (Ferrari & Benson, 1961). When a function of the R_F values in each of the two solvent systems was plotted against the number of galactose units/mole of deacylated lipid (French & Wild, 1953) a straight line was obtained, suggesting that the glycoside obtained by deacylation of TGDG is homologous with those from MGDG and DGDG and contains three galactose units/mole. The light-brown spots obtained on paper chromatography of the deacylated galactolipids MGDG, DGDG and TGDG all developed at equal slow rates, indicating the absence of reducing sugars.

The analyses of the deacylation products are summarized in Table 2. The results show that approx. 1 mole of formaldehyde was liberated/mole of each deacylated galactolipid, indicating free vicinal hydroxyl groups in the glycerol moiety of the galactosylglycerols. The periodate consumption was close to 3, 5 and 7 moles/mole of deacylated

Table 1. *Paper chromatography of galactolipid-deacylation products*

The samples were subjected to descending chromatography on Whatman no. 1 paper at 20°. Values in parentheses refer to corresponding R_F values taken from Ferrari & Benson (1961).

Parent galactolipid	Solvent system	
	Phenol-water (5:2, w/w)	Butan-1-ol-propionic acid-water (151:75:100, by vol.)
MGDG	0.65 (0.62)	0.24 (0.25)
DGDG	0.50 (0.46)	0.10 (0.13)
TGDG	0.35 (0.38)	0.04 (0.04)

Table 2. *Analysis of galactolipid-deacylation products*

Values in part *A* are expressed as moles/mole of deacylation product as determined by total sugar analysis. The molar ratio in part *B* was determined after acid hydrolysis of the deacylation products. For experimental details see the Experimental section.

	Parent galactolipid		
	MGDG	DGDG	TGDG
<i>(A)</i>			
Total sugar	1.00	2.00	3.00
Periodate consumed	3.24	5.00	6.60
Formaldehyde liberated	1.25	1.06	1.17
Reducing sugar released by β -D-galactoside galactohydrolase	1.0	0	0
<i>(B)</i>			
D-Galactose/glycerol molar ratio	0.99	2.04	2.90

MGDG, DGDG and TGDG respectively. These values would be expected for galactosylglycerol derivatives in which C-1 of glycerol was linked to one, two and three galactopyranosyl units respectively, the hexoses being linked through C-1 and C-6. Further hydrolysis of each deacylation product yielded D-galactose and glycerol in the molar ratios shown in Table 2.

To establish the configuration of the anomeric glucosidic bonds, the deacylated galactolipids were incubated with β -D-galactoside galactohydrolase, which released galactose from the deacylation product of MGDG but did not attack the DGDG or TGDG derivatives.

An attempt was made to find an enzymic system that would liberate the diglyceride from the galactolipids. Sastry & Kates (1964*b*) reported that a crude pancreatic extract liberated mono- and di-glycerides, in addition to free fatty acids, from MGDG. With a commercial pancreatin preparation on the galactolipids from potato, only MGDG was hydrolysed; free fatty acids (but not mono- or di-glycerides) were identified by t.l.c. of the hydrolysis products.

DISCUSSION

The results presented here support the conclusion that the trigalactosyl diglyceride isolated from potato tubers is a higher homologue of MGDG and DGDG and contains an additional D-galactopyranosyl unit linked α -(1 $\rightarrow$ 6) to the terminal galactose residue of DGDG. From the limited information given by Allen, Good, Davis, Chisum & Fowler (1966) the lipid that these authors identify as 'trigalactolipid' in spinach chloroplasts is, from the DEAE-cellulose-column elution pattern, almost certainly the same lipid as the TGDG described in the present work.

A ^{14}C -labelled lipid was tentatively identified as trigalactosyl diglyceride in the lipids formed when UDP- ^{14}C galactose was incubated with spinach chloroplasts (Neufeld & Hall, 1964; Ongun & Mudd, 1968). Repetition of the experiments of Ongun & Mudd (1968) and analysis of the labelled lipids has shown that their ^{14}C trigalactosyl diglyceride has chromatographic properties on DEAE-cellulose columns and on t.l.c. identical with those of the TGDG from potato tubers (T. Galliard, unpublished work). This is probably also the same lipid as that which, after deacylation, gave ^{14}C trigalactosylglycerol in the experiments with *Chlorella* (Benson *et al.* 1958).

The analysis of MGDG and DGDG from potato tubers has shown that these lipids have the same structures as the corresponding lipids previously characterized in wheat flour (Carter *et al.* 1961*a,b*) and green leaves (Sastry & Kates, 1964*a*). A question about the configuration of the glycosidic linkage of the terminal galactose unit in DGDG was posed by the results of Neufeld & Hall (1964) who found that, in the deacylation product of ^{14}C -labelled DGDG formed in spinach chloroplasts by incubation with UDP- ^{14}C galactose, 83% of the terminal galactose was released by β -D-galactoside galactohydrolase. In the present work with potato lipids and in the cited analyses (Carter *et al.* 1961*a,b*; Sastry & Kates, 1964*a*) of naturally-occurring DGDG from several sources, the glycosidic linkage of the terminal galactose unit of DGDG was in the α -configuration.

The results of Ongun & Mudd (1968) suggest that the enzymic formations of DGDG and TGDG from MGDG are closely related processes, whereas the formation of MGDG from diglyceride is catalysed by a different enzyme system. This might be expected if higher homologues of MGDG were formed by the sequential addition of D-galactopyranosyl

units in α -(1 $\rightarrow$ 6) linkages to the β -D-galactopyranosyl moiety of MGDG.

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