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BIOSYNTHESIS OF MONOGALACTOSYL DIGLYCERIDE BY CHLOROPLASTS FROM *SPINACIA OLERACEA* AND FROM SOME GRAMINEAE

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Abstract—Synthetic diglycerides which differed in unsaturation of fatty acids gave the same incorporation of [^{14}C]galactose from UDP-[^{14}C]galactose when added to acetone powders of spinach chloroplasts up to about 0·6 mg diglyceride/20 mg acetone powder. Diolein and the endogenous diglyceride isolated from the acetone extract of chloroplasts stimulated galactolipid biosynthesis to a similar extent. With all diglycerides used, monogalactosyl diglyceride was the main product with little accompanying synthesis of digalactosyl diglyceride. The radioactivity in the monogalactosyl diglyceride synthesized from UDP-[^{14}C]galactose by whole chloroplasts was distributed widely among the monogalactosyl diglycerides with different fatty acid composition. It is concluded that the enzyme which catalyses the transfer of galactose from UDP-galactose to diglyceride is not specific for polyunsaturated diglycerides and that the polyunsaturated monogalactosyl diglycerides arise either by desaturation of the fatty acyl residues after monogalactolipid synthesis or by transacylation. Acetone powders of chloroplasts prepared from several Gramineae did not exhibit transferase activity although whole chloroplasts were active.

INTRODUCTION

ISOLATED spinach chloroplasts[1,2] and soluble enzyme extracts of spinach chloroplasts[3] catalyse the synthesis of galactolipids from UDP-galactose. Acetone powders of spinach chloroplasts likewise actively synthesize galactolipids provided that the resuspended acetone powder is supplemented with diglyceride (DG).[2] The DG of highest unsaturation, namely dilinolenin, was the best acceptor for galactose,[4] which suggests that highly unsaturated galactolipids predominate in spinach leaves because of the specificity of the enzyme catalysing the transfer of galactose from UDP-galactose for polyunsaturated DG acceptors. It is possible that in higher plants glycerophospholipids contribute unsaturated species to a DG pool. Phosphatidylcholine (PC) was the lipid component in pumpkin leaves most rapidly labelled following the application of [1-^{14}C]acetate to the leaf surface and the changes in the labelling of the fatty acid residues with time suggested that PC is the primary site of linolenic acid biosynthesis.[5]

However, the significant changes in the fatty acid composition of the monogalactosyl diglyceride (MGDG) fraction of *Chlorella vulgaris* which occur following *de novo* synthesis,[6] together with the variation in specific activity of a single fatty acid among species of MGDG of different fatty acid composition[7] are consistent with desaturation occurring after biosynthesis of MGDG.

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In the present investigation we have considered that two alternative pathways for the biosynthesis of MGDG in higher plants might be (a) the specific selection of the more highly unsaturated DG's for final galactose addition and (b) a non-specific selection of DG's from a DG pool with subsequent desaturation of the fatty acyl residues. Accordingly the substrate requirements for galactosylation and the distribution of ^{14}C-label in the MGDG species of different fatty acid composition have been examined using preparations of spinach chloroplasts. The study of DG requirements extends and complements the work of Mudd *et al.*[4] but unlike these workers we have not found a higher rate of incorporation of the highly unsaturated DG's.

This inconsistency between our own results and those of other workers with chloroplast preparations from spinach, has led to experiments with other higher plants. Four gramineae species were selected because their constituent galactolipids provides the grazing ruminant with its most abundant source of dietary fatty acids. Chloroplast preparations from the grasses actively synthesized galactolipid but the specificity of the galactolipid synthetase with respect to DG composition could not be tested because the acetone powders of these chloroplasts were inactive.

RESULTS

Comparison of Galactolipid Biosynthesis by Whole Chloroplasts of Different Species

The incorporation of radioactivity from UDP-D-[^{14}C]galactose into lipids by chloroplasts of various leaf tissues prepared by method B was highest with spinach. Of the Gramineae, fescue and ryegrass chloroplasts also gave appreciable incorporation after a 2-hr incubation (Table 1).

TABLE 1. THE INCORPORATION OF RADIOACTIVITY FROM UDP-[^{14}C]Gal (U) INTO THE LIPIDS OF CHOLOROPLASTS ISOLATED FROM DIFFERENT PLANT SPECIES

	Chloroplast source*				
	Spinach	Fescue	Ryegrass	Fog	Phalaris
Incorporation (%)	53·2	25 7	15·8	8·7	3·6

Reaction mixture: 0·2 ml chloroplast suspension (0·1 M Tris-HCl buffer, pH 7 4); 2 μl UDP-D-[^{14}C]-galactose (22,200 dis/min, 0 04 μmole).
Incubation: 30° for 2 hr in room light with constant shaking.
* Equivalent to 1 4 g leaf tissue.

Fescue chloroplasts were selected for further studies of galactolipid biosynthesis in grasses. Incorporation of galactose from UDP-Gal into galactolipids increased linearly up to a chlorophyll content of 0·26 mg. Chloroplast concentrations at a level of 0·51 mg chlorophyll was inhibitory (Table 2).

Radioscans of thin-layer chromatograms of the lipid extracts from each plant source gave a very similar distribution of radioactivity. Most of the radioactivity was in MGDG with some radioactivity corresponding to acyl MGDG and sterol glycoside. There was very little radioactivity ($<5\%$ of the total) in DGDG, but in some subsequent chloroplast preparations from spinach up to about 20% of the radiocarbon was in DGDG.

TABLE 2. THE EFFECT OF CHLOROPLAST CONCENTRATION ON THE INCORPORATION OF RADIOACTIVITY FROM UDP-[^{14}C]Gal (U) INTO THE LIPIDS OF FESCUE (*Festuca elatior*)

Chlorophyll content (mg)	Incorporation (%)
0·03	1·8
0·05	4·4
0·13	9·6
0·20	14·6
0·26	18·6
0·38	20·6
0·51*	11·9

Reaction mixture: 0·2 ml chloroplast suspension (0·1 M Tris-HCl buffer, pH 7·4); 8 μl UDP-[^{14}C] (88,800 dis/min, 0·16 μmole).
Incubation: 30° for 40 min.
* Contained chloroplasts equivalent to 3·0 g leaf tissue.

Effect of Procedure of Isolation of Chloroplasts and Subsequent Treatment on the Incorporation of Galactose from UDP-Gal into Galactolipid

The incorporation of galactose from UDP-Gal into lipids by chloroplasts obtained by the more vigorous grinding procedure (Method A) was about twice that of chloroplasts obtained using the milder methods of disruption of cells (Method B) (Table 3). In the latter method little or no chlorophyll was present in the supernatant fractions obtained during preparation of the chloroplasts, indicating that chlorophyll-containing membranes were essentially intact. Radioscans of thin-layer chromatograms of lipid extracts gave major peaks corresponding to MGDG and acyl MGDG when chloroplasts prepared by Method B were used but the acyl compound was virtually absent from chloroplasts prepared by the alternative method. Consequently the chloroplasts prepared by method A gave a

TABLE 3. THE INCORPORATION OF RADIOACTIVITY FROM UDP-[^{14}C]Gal (U) INTO LIPIDS BY CHLOROPLASTS PREPARED BY TWO METHODS OF ISOLATION

Leaf source	Method of isolation*	Chlorophyll content of the reaction mixture (mg)	Incorporation into galactolipid (%)
Fescue	A	0·15	35·9
	A	0·15	35·8
	B	0·25	19·8
	B	0·25	18·5
Spinach	A	0·20	30·8
	A	0·20	30·4
	B	0·19	11·9
	B	0·19	12·2

Reaction mixture and conditions of incubation: See Table 1.
* See Experimental.

relatively greater incorporation of galactose into MGDG than indicated by values for the incorporation of radioactivity into total lipid given in Table 3. The DGDG fraction was not significantly labelled using either method.

Five min sonication of both spinach and fescue chloroplast suspensions prepared by method B gave approximately 20 per cent increases in the incorporation of galactose into lipid from UDP-Gal. There was a reduction in the amount of acyl-MGDG and no DGDG was formed.

Two per cent (w/v) deoxycholate was added to the reaction mixture in the hope that this surface-active compound would facilitate the entry of UDP-Gal into chloroplasts. However, galactolipid biosynthesis was completely inhibited which could be due to binding of DG acceptors.

Galactolipid Biosynthesis by Freeze-dried Chloroplasts and Acetone Powders of Chloroplasts

Acetone powders and freeze-dried preparations of fescue chloroplasts showed no activity in synthesizing lipids from UDP-Gal. Biosynthesis was not stimulated either by addition of DG or sonication. In contrast, spinach chloroplasts reconstituted from acetone powders or from the freeze-dried state actively synthesized galactolipid. The freeze-dried preparations gave superior levels of incorporation and unlike the acetone powders the incorporation was not further enhanced by the addition of diolein. MGDG was the major galactolipid formed along with small amounts of sterol glycoside, acyl MGDG and an unidentified component. MGDG was the only galactolipid containing significant amounts of radioactivity when acetone powders were incubated with diolein and UDP-[^{14}C]Gal.

TABLE 4. THE STIMULATORY EFFECT OF DIFFERENT *rac*-1,2-DG'S ON THE INCORPORATION OF GALACTOSE FROM UDP-[^{14}C]Gal (U) INTO LIPIDS BY RECONSTITUTED ACETONE POWDERS OF SPINACH CHLOROPLASTS

Lipid added	Incorporation of radioactivity (%) (duplicate incubations with each substrate)
None	3·4
	3·4
1,2-Dioleoyl glycerol	22 5
	17·3
1-Oleoyl,2-linoleoyl glycerol	17·8
	18·2
1-Linoleoyl,2-oleoyl glycerol	11·5
	13·1
1,2-Dilinoleoyl glycerol	9·9
	11·5
1-γ-Linolenoyl,2-linoleoyl glycerol	11 9
	11·0
Acetone extract of chloroplasts	20·7
	18·7

Reaction mixture. 0·7 ml of 0·1 M Tris-HCl buffer, pH 7·4 containing a suspension of 14·0 mg acetone powder and lipid (1·2 mg synthetic DG or 3 4 mg of acetone extract of chloroplasts), 4 μl UDP-[^{14}C]Gal containing 44,400 dis/min (0·08 μmole).

Incubation conditions: 30° for 90 min with shaking.

Response of Galactolipid Biosynthesis to Added Diglyceride

When 1·2 mg of different racemic 1,2-diacyl glycerols were emulsified with 14 mg reconstituted acetone powders of chloroplasts and incubated with UDP-[^{14}C]Gal, the DG's containing an oleoyl residue in the 1-position gave the highest incorporation (Table 4) —approximately equivalent to that obtained by adding back the endogenous DG's removed during preparation of the acetone powders. The level of incorporation of 1-linoleoyl,2-oleoyl glycerol (LOG) was 69 per cent of the incorporation obtained for the positional isomer 1-oleoyl,2-linoleoyl glycerol (OLG). Radioscans of the lipid extracts showed that MGDG was the only lipid component with appreciable labelling. Sterol glycoside migrated immediately behind MGDG and a greater proportion of the total incorporation was present as sterol glycoside when DG's which gave lower incorporation were added. None of the incubation mixtures synthesized DGDG. The incorporation of galactose into lipid after different times of incubation using LOG as acceptor occurred in a diphasic manner (Fig. 1) with the enzyme still active after 6 hr. A similar time-course response was obtained for OLG. Slightly greater amounts of sterol glycoside were synthesized at longer times of incubation and again diolcin gave a greater stimulation than LOG.

Increasing the amount of each synthetic DG in the incubation mixture gave approximately equivalent stimulation of net incorporation of radioactivity up to the level of about 0·57 mg DG/20·0 mg acetone powder. Further increases in diolein and OLG gave still higher galactolipid synthesis but increasing LOG and 1-γ-linolenoyl 2-linoleoyl (LLG) led to decreased synthesis (Fig. 2). Only very large amounts of OLG were inhibitory. From Table 4 and Fig. 2 it will be seen that the incorporation using 1·2 mg of each DG is comparable in

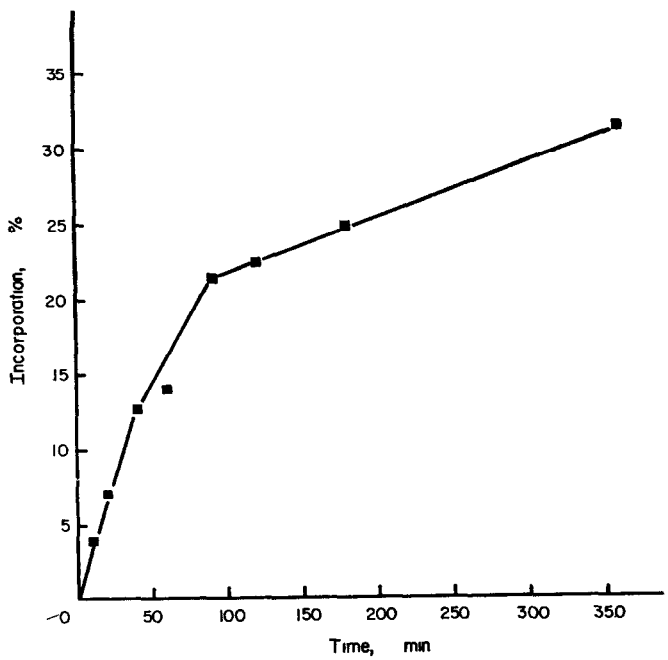

FIG. 1. DEPENDENCE OF SYNTHESIS OF GALACTOLIPID ON TIME WITH *rac*-1-LINOLEOYL,2-OLEOYL GLYCEROL AS DG ACCEPTOR.
Conditions were as indicated in Table 5.

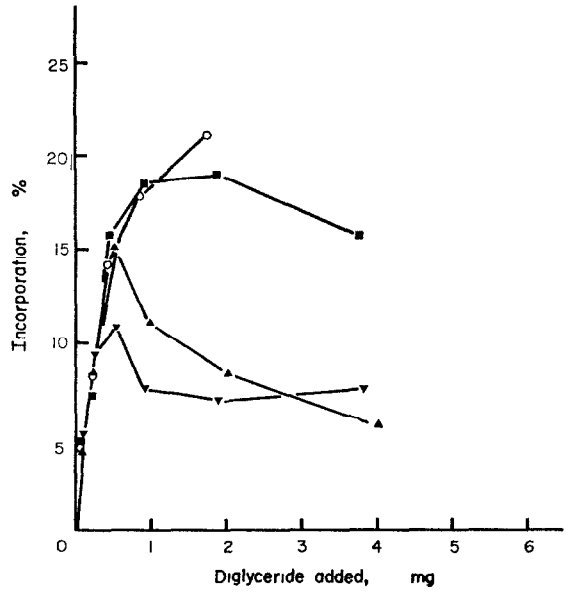

FIG. 2. DEPENDENCE OF GALACTOLIPID BIOSYNTHESIS BY ACETONE POWDER OF SPINACH CHLOROPLASTS ON ADDED DG.

Conditions were as indicated in Table 5.

Diglyceride acceptors (mg/20·0 mg acetone powder); ○———○ *rac*-1,2-dioleoyl glycerol; ■———■ *rac*-1-oleoyl,2-linoleoyl glycerol; ▲———▲ *rac*-1-linoleoyl,2-oleoyl glycerol, ▼———▼ *rac*-1-γ-linolenoyl,2-linoleoyl glycerol.

the two sets of experiments and several other acetone powder preparations gave results which were consistent with these observations.

Incubation of endogenous chloroplast DG with acetone powder gave a slightly higher incorporation into total lipid than diolein at the same DG/acetone powder ratio (Table 5) but radioscans showed a higher proportion of sterol glycoside when chloroplast DG was added so that MGDG synthesis was slightly less. Similarly, the radioactive lipid obtained when MGDG was added to the incubation mixture was very high in sterol glycoside (approx. 50 per cent). The small amount of endogenous DG available precluded a study of the effect of different levels on galactolipid biosynthesis. At the level used, egg yolk phospholipid inhibited incorporation when incubated alone with acetone powder preparations or with diolein.

Distribution of Radioactivity in MGDG Species after Incubation of Spinach Chloroplasts with UDP-[^{14}C]Gal (U)

The MGDG isolated from chloroplasts by preparative TLC after incubation of spinach chloroplasts with UDP-[^{14}C]Gal (U) for 1 hr at 30° gave one radioactive peak on TLC radioscans which corresponded to MGDG standard chromatographed simultaneously. The fatty acid composition was: 16:0 (4%), 16:3 (17%), 18:0 (1%), 18:1 (2%), 18:2 (9%), 18:3 (67%). When this MGDG was subjected to $AgNO_3$-TLC, each of the MGDG zones which separated contained appreciable radioactivity (Table 6). The radioactive zones on radioscans of TLC separations corresponded to bands which fluoresced after spraying with 2,6-dichlorofluorescein. Fractions 3 and 4 fluoresced strongly and 1 and 2 fluoresced weakly.

TABLE 5. THE EFFECT OF LIPID SUBSTRATES ON INCORPORATION OF RADIOACTIVITY FROM UDP-[^{14}C]Gal (U) INTO LIPID BY ACETONE POWDERS OF CHLOROPLASTS

Lipid added	Amount added (mg)		Incorporation (%)
	Diolein	Other lipid	
None	—	—	3·8
MGDG	—	1·36	11·5
			12·8
Chloroplast DG	—	1·61	18·7
			23·7
Egg yolk phosphatidylcholine (PC)	—	0·79*	1·0
Egg yolk PC and diolein	0·81	0·79*	3·6
Diolein	0·81	—	19·1
Diolein	1·61	—	17·3

Reaction mixture and incubation as for Table 5.
* Weight of DG obtained on hydrolysis of the PC added.

The four major radioactive peaks corresponded to groups of molecular species of MGDG containing on average approximately 2,3,5 and 4 double bonds. Fractions 3 and 4 comprised 30 and 41% respectively of the constituent fatty acids in the total MGDG recovered from the chromatograms (Table 6). The less abundant and least unsaturated fractions (1 and 2) contained about as much radioactivity as the more abundant and more unsaturated fractions. It is not understood why the R_f of fraction 4, with an average of 4·0 double bonds/molecule, is lower than that of fraction 5 which has an average of 5·2 double bonds/molecule. It will be noted that 16:3 is confined almost entirely to fraction 4 but fraction 3 contains about twice as much 18:3.

TABLE 6. FATTY ACID COMPOSITION OF GROUPS OF MOLECULAR SPECIES OF MGDG ISOLATED FROM SPINACH CHLOROPLASTS AFTER INCUBATION WITH UDP-[^{14}C]Gal (U) FOR 1 hr AT 30°

Fatty acid	Fraction				Total
	1	2	3	4	
14:0	2	2	1	2	2
16:0	25	16	5	8	10
16:1	4	2	1	1	2
16:2	8	4	0	0	2
16:3	0	0	2	24	10
18:0	4	9	2	6	5
18:1	47	14	7	13	16
18:2	10	23	2	10	9
18:3	0	30	80	36	44
Double/bonds molecule	1·7	3·0	5·2	4·0	
% of total fatty acids in fraction	14	14	30	41	
% of total radioactivity in fraction	33	21	26	20	

DISCUSSION

The major point of interest in the present results is whether they can differentiate between (a) the presence of one or more UDP-D-galactose: 1,2-di-*o*-acyl-*sn*-glycerol galactosyl transferases in spinach chloroplasts which exhibit specificity for DG's of particular fatty acid composition or (b) the presence of a non-specific transferase which synthesizes MGDG's to be further modified by desaturases. The results from both experimental approaches appear to favour the latter alternative. Firstly, the synthetic DG's which differed in fatty acid composition gave the same incorporation of galactose from UDP-galactose up to the level of approximately 0·6 mg DG/20 mg acetone powder. Secondly, when the MGDG synthesized from UDP-[^{14}C]Gal by whole chloroplasts was fractionated by TLC according to the nature of the fatty acids, it was found that the radioactivity was distributed widely among the different species of MGDG.

Speculation on the reasons for inhibition of synthesis of galactolipid at higher DG/acetone powder ratios is probably not justified; rather it is more appropriate to emphasize the similarities in the behaviour of the synthesizing system with respect to DG's of different composition at low DG/acetone powder ratios. Nevertheless, the nature of the response to different levels of DG indicate the dangers in choosing arbitrary substrate levels in comparative studies involving this system.

The unfractionated acetone extract and the DG isolated from the acetone extract of spinach chloroplasts gave approximately the same enhancement of MGDG synthesis as diolein. Whether the endogenous 1,2 DG, which contained over 40% linolenic acid, reflects the composition of the DG pool in the chloroplasts, or whether the DG was primarily formed by degradation of glycerolipids is uncertain. The linolenoyl residue in the synthetic DG containing five double bonds was the 6,9,12 isomer whereas the 9,12,15 isomer is the one which predominates in plant tissues. Since these isomers possess differing molecular conformations, the utilization of *rac* 1-γ-linolenoyl-2-linoleoyl glycerol to the same extent as other DG's tested would appear to confirm the non-specificity of the UDP-D-galactose: 1,2 di-*o*-acyl-*sn*-glycerol galactosyl transferase for acyl groups in the 1,2-DG's.

In similar studies using DG's prepared from spinach and egg phospholipids Mudd *et al.*[4] found that highly unsaturated DG's were preferred to the more saturated DG's as acceptors for galactose from UDP-Gal. A selectivity of this nature *in vivo* would lead directly to the formation of the dominant MGDG species present in spinach chloroplasts. The addition of DG to the acetone powders in organic solvent prior to the addition of the aqueous component of the incubation medium[2,8] was chosen in the present study in preference to the addition of DG as an emulsion[4,8–10] in buffer containing Tween 20 to the acetone powders, to reduce the possibility of solubility and emulsion differences with DG of different fatty acid composition. One factor which could have contributed to the levels of incorporation of galactose from UDP-Gal obtained by Mudd *et al.*[4] being lower than in previous studies[2] or in the present work was their use of 0·03% Tween 20 as an emulsifier. This level of Tween 20 inhibited phosphocholineglyceride transferase activity leading to the biosynthesis of phosphatidylcholine.[10]

With the predominance of 16.3 and 18:3 fatty acids in the MGDG of spinach chloroplasts it is of interest to find such a wide distribution of label between the MGDG's with different degrees of unsaturation. Since there is very little MGDG containing 2–4 double

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bonds in spinach molecule the specific activity of these MGDG's must be considerably higher than the more abundant 5 and 6 double bond species. Nichols and Moorhouse[7] also found that in *Chlorella vulgaris* incubated with [2-^{14}C]Na acetate, the specific activity of a single fatty acid varied considerably among the various MGDG species thus supporting earlier evidence[6,11] for changes in the fatty acid composition of MGDG following *de novo* synthesis.

If as indicated by this investigation, the transferase enzyme is non-specific, and the acceptor DG's have a different fatty acid composition from the endogenous MGDG, it is interesting to consider how the characteristic fatty acid composition of MGDG arises. This may occur if the MGDG itself is the substrate for the desaturase or if there is acyl migration of 18:3 and 16:3 residues from other complex lipids or by a combination of both mechanisms. Direct evidence for either of these mechanisms occurring in MGDG biosynthesis is lacking, but there is evidence for the desaturation of fatty acids in other glycerolipids. Gurr *et al.*[12] found that the conversion of oleic acid to linoleic acid in *C. vulgaris* is associated with the PC fraction. Similarly, following infusion of [^{14}C]acetate into pumpkin leaves, a rapid turnover of radioactive carbon occurred only in PC thus providing evidence for the sequences 18:1 → 18:2 → 18:3.[5] On the other hand, no evidence for acyl transfer between PC and galactolipids has been obtained.[5,6,12]

Only insignificant amounts of DGDG were present in the lipid products in most chloroplast preparations. Similarly Mudd *et al.*[4] obtained little DGDG synthesis at pH 7·4 in their chloroplast preparations, but in earlier work MGDG/DGDG ratios of 2:1 were obtained.[2] Extracts of *Euglena gracilis* catalysed the incorporation of acyl groups from ACP and CoA thioesters into MGDG without giving significant incorporation into DGDG.[13]

In view of the uncertainty whether desaturation of fatty acids occurs before or after biosynsthesis in spinach, it is desirable that this aspect of galactolipid biosynthesis in higher plants should be extended to other plants. Although we have found that isolated chloroplasts of some grasses actively synthesize MGDG, attempts to obtain synthesis in reconstituted chloroplast systems have not been successful and this has precluded comparative studies so far. Incorporation of galactose from UDP-Gal into MGDG was enhanced by sonication of chloroplast suspensions and by the use of more vigorous procedures for the disruption of leaf tissues. These aspects of MGDG biosynthesis are being investigated.

EXPERIMENTAL

Plant Tissues

Meadow fescue (*Festuca elatior*) and phalaris (*Phalaris arundinacea* × *tuberosa*) was gathered from clones grown in the open. Ryegrass (*Lolium perenne*) and Yorkshire fog (*Holcus lanatus*) were grown separately in fields. Field-grown spinach (*Spinacia oleracea*) was obtained from the local market.

Isolation of Chloroplasts

The leaves were washed with distilled water and chilled in a 3° cold room for 1–2 hr. Two methods were used.

A. Leaves were ground in a mortar with acid-washed sand in sucrose–phosphate buffer, pH 7·4 (0·5 M sucrose and 0·01 M phosphate). The homogenate was filtered through three layers of nylon stocking or two layers of Miracloth, centrifuged at 120 *g* for 2 min and the supernatant centrifuged at 3000 *g* for 10 min.

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B. The mincing technique developed by Spencer and Wildman[14] to give maximum preservation of organelle structures was used in the following medium: 25 mM Tris-HCl, pH 7·4, 250 mM sucrose, 1·0 mM Mg $(OAc)_2$, 4 mM mercaptoethanol, 2·5% Ficoll, 5% Dextran 40T. The brie was filtered through nylon stocking, centrifuged at 30 *g* for 2 min and the supernatant centrifuged at 3000 *g* for 10 min. The chloroplast pellets were suspended in 0·1 M Tris-HCl buffer, pH 7·4, to give about 0·4 mg chlorophyll/ml for incubations with substrate.

Preparation of Acetone Powder

Acetone powders were prepared according to the procedure of Ongun and Mudd,[2] dried in a vacuum desiccator at −20° and stored at 4°.

Preparation of Diglyceride Substrates

2-Monoacyl glycerol was prepared from 1,3-benzylidene glycerol and fatty acid chloride and then reacted with equimolar amounts of fatty acid chloride.[15] The DG's isolated by preparative TLC using hexane–Et_2O (2:3, v/v)[16] and silica gel G impregnated with 3% (w/v) boric acid contained little or no 1,3-diglyceride. The DG's were stored at −20° under N_2. The positional distribution[17] of the fatty acids in the synthetic rac-1,2-di-O-acyl glycerols was as follows.

Diglyceride	Fatty acid (moles %)		
	18:1	18:2	18 3
1,2-Dioleoyl glycerol	91·4	8·6	0
1-Oleoyl,2-linoleoyl glycerol			
Position 1	86 1	13 9	0
Position 2	2·2	97·8	0
1-Linoleoyl,2-oleoyl glycerol			
Position 1	7·7	92·3	0
Position 2	96·7	3·3	0
1,2-Dilinoleoyl glycerol	1·0	99·0	0
1-γ-Linolenoyl,2-linoleoyl glycerol			
Position 1	0	11·2	88 8
Position 2	0	100	0

Endogenous 1,2-diacyl-*sn*-glycerols of spinach chloroplasts were isolated by preparative TLC on silica gel G impregnated with 3% (w/v) boric acid, after the acetone extract had been passed through a carbon–Celite (1:1) column.[18] The fatty acid composition of the endogenous DG's was: 16:0 (16·8%), 16:1 (15·3%), 18:0 (1·6%), 18:1 + 16:3 (8·4%), 18:2 (14 0%), 18:3 (43·9%).

Incubation of Chloroplast Preparations with Substrates

Chloroplast suspensions were incubated with UDP-[^{14}C]Gal (U) with constant shaking at 30° in room light. The lipid substrates were added in acetone (0·2 ml solution to 20·0 mg powder) according to the procedure of Ongun and Mudd[2] and in addition the homogenate in Tris-HCl buffer was sonicated in an atmosphere of N_2 for 5 min. Preliminary experiments had shown that 3–5 min sonication of the homogenates before addition of UDP-[^{14}C]Gal gave about 30 per cent increase in galactolipid synthesis. When the suspensions of acetone powder and DG were held at 0° for 0, 1, 2 and 4 hr before addition of UDP-[^{14}C]Gal and incubated at 30° for 90 min the per cent incorporation was 20·3, 14·9, 14·0 and 13·5 respectively, and consequently incubations of each sample were started immediately after preparation of the emulsions.

Isolation of MGDG and Phosphatidyl Choline (PC)

MGDG was isolated by preparative TLC. PC was obtained from the yolks of fresh eggs by preparative TLC of the acetone insoluble fraction of the total lipid extract using $CHCl_3$–MeOH–HOAc–H_2O (85:15:10:4, v/v) as solvent. The fatty acid composition was: 16:0, 34 7%; 16:1, 20%; 18:0, 12 5%; 18·1, 39·8%; 18:2, 10·9%.

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Analytical Methods

Chlorophylls were measured by the method of Arnon.[19]

The incubations were stopped by adding sufficient $CHCl_3$–MeOH (1:2, v/v) to give one phase and heating to 60° for 5 min. After low speed centrifugation, the supernatant was decanted and the residue agitated with $CHCl_3$–MeOH (2:1, v/v) on a Vortex mixer and heated to boiling. The bulked extracts were washed three times with 0·6% aq. NaCl, the $CHCl_3$ layer evaporated to dryness and the total lipid extract redissolved in 0·5 ml $CHCl_3$. Suitable aliquots were dried in a counter vial for measurement of radioactivity by liquid scintillation (Packard Tri-carb Liquid Scintillation Spectrometer, model 3375). The nature of the radioactive products in the $CHCl_3$ extract was determined by scanning thin-layer chromatograms (Packard Radiochromatogram Scanner, model 7200) and radioautography.

TLC. The chromatograms were prepared on layers of silica gel G using toluene–EtOAc–95% EtOH (2:1:1 v/v). MGDG and digalactosyl diglyceride (DGDG) were isolated by two-stage preparative TLC using the above solvents in the first stage followed by rechromatography of the isolated lipids using $CHCl_3$–MeOH–HOAc–H_2O (200:20:5:2, v/v).[7] MGDG and DGDG were separated according to unsaturation on silica gel HR impregnated with 10% $AgNO_3$ using $CHCl_3$–MeOH–H_2O (60:22:4, v/v) and the fractions recovered from the silica gel layers by the procedure of Nichols and Moorhouse.[7] Galactolipids were detected on the chromatograms by spraying with 0·05% dichlorofluorescein in methanol or with diphenylamine reagent followed by heating at 110° for 10 min.[20]

Methyl esters. Fatty acids liberated from lipids by refluxing with methanolic KOH were treated with freshly prepared CH_2N_2, and then purified by preparative TLC using silica gel G and hexane–Et_2O (4:1, v/v). Determination of the relative amounts of each MGDG species separated by $AgNO_3$–TLC was facilitated by addition of a known amount of methyl pentadecanoate to hexane solutions of the methyl esters of the fatty acids. Peak areas of the chromatograms were measured by planimetry.

GLC. A Wilkens Aerograph model 1520 was used with a 6 ft × ⅛ in. column operated at 165° to separate methyl esters. Packing materials were 12% DEGS coated on 60–70 mesh Anakrom Q.

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[20] E. V. Levin, W. J. Lennarz and K. Bloch, *Biochim. Biophys. Acta* **84,** 471 (1964).