

## Galactolipid Synthesis in *Vicia faba* Leaves

### I. GALACTOSE, GLYCEROL, AND FATTY ACID LABELING AFTER $^{14}\text{CO}_2$ FEEDING<sup>1</sup>

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#### ABSTRACT

The galactose, glycerol, and fatty acids of mono- and digalactosyl diglycerides (MGDG and DGDG) have been separated and analyzed for  $^{14}\text{C}$  activity after  $^{14}\text{CO}_2$  feeding of *Vicia faba* leaf discs. Fully expanded and developing leaves were analyzed at time intervals following feeding during continuous illumination. In addition, fully expanded leaves were analyzed after similar times in complete darkness. In all cases,  $^{14}\text{C}$  was incorporated very rapidly into galactose, whereas glycerol and fatty acids were labeled much more slowly and over a longer period of time. The data are consistent with the galactosylation of a diglyceride to MGDG which is in turn galactosylated to DGDG. The data suggest that the formation of diglycerides suitable for galactosylation to MGDG is slow in comparison to the galactosylation process. It is also suggested that DGDG may be formed from more than one pool of MGDG. The complete analysis of the  $^{14}\text{C}$  incorporation into galactose appears to represent the only satisfactory method of comparing galactolipid synthesis by  $^{14}\text{C}$  incorporation. Estimates of comparative rates of synthesis of MGDG and DGDG have been made on this basis.

Monogalactosyl diglyceride and digalactosyl diglyceride are the major chloroplast lipids of most photosynthetic tissue and have been the subject of many investigations (see reviews 6, 8). These lipids are unique in that they contain high levels of unsaturated fatty acids, the biosynthesis of which has attracted much attention in both algae and higher plants. Few reports, however, have been published on the metabolism of the galactose and glycerol moieties of the lipids.

Ferrari and Benson (5) proposed that MGDG<sup>3</sup> is synthesized by galactosylation of a diglyceride utilizing UDP-galactose. A further galactosylation would result in the conversion of MGDG to DGDG. Since this proposal was made a number of reports have appeared which support it. Most of this evidence is based on *in vitro* experiments using isolated chloroplasts or extracts, and UDP- $^{14}\text{C}$  galactose (2, 4, 9, 11).

Williams *et al.* (17) reported *in vivo* studies in *Vicia faba* which also support the idea of galactosylation of MGDG to

DGDG. Lin and Chang (7), however, have presented evidence which may cast some doubt on this theory.

Eberhardt and Kates (3) were the first to separate highly labeled hexoses from water-soluble products of lipid-hydrolysis after feeding  $^{14}\text{CO}_2$  to runner bean leaves. Ferrari and Benson (5) reported a very high rate of incorporation of  $^{14}\text{C}$  into the galactose moiety of the lipids of *Chlorella*, while Roughan (13) indicated that the polar portions of these lipids from pumpkin leaves were highly labeled after  $^{14}\text{CO}_2$  feeding. Trémolières (15) has analyzed these lipids (in clover leaves) for radioactivity in the galactosyl glycerols and fatty acids with similar results. The general conclusion reached in these studies is that galactose is more rapidly labeled than the fatty acids in galactolipids. Little data, however, have been published on the glycerol labeling.

Until now galactolipid synthesis could be determined only by quantitative changes of the lipid measured over a period of time. This does not permit determinations over short periods of time or under differing environmental conditions where changes may be small. Estimates of synthesis using  $^{14}\text{C}$  precursors could be misleading if the different moieties of the lipid are labeled at different rates through different pathways. The purpose of the work reported here was to determine the labeling kinetics of the galactose, glycerol and fatty acids of galactolipids in *Vicia faba* leaf discs after  $^{14}\text{CO}_2$  feeding. From these data, it is hoped to clarify the proposed metabolic pathways of galactolipids, to determine whether galactose and/or fatty acids were turned over rapidly and to determine a method for the estimation of galactolipid synthesis by  $^{14}\text{C}$  incorporation. In a previous report (17), we determined the labeling patterns of the two galactoses of DGDG, the knowledge of which is necessary before the incorporation of activity into DGDG galactose can be fully assessed.

#### MATERIALS AND METHODS

Mature leaves were harvested from 3- to 4-week-old broad bean (*Vicia faba* L. Giant Windsor) plants grown in growth chambers at 21 °C under artificial illumination of 1100 ft-c. Leaf discs (20 mm) were taken from fully expanded leaves and weighed before feeding. For the longer period experiments (up to 72 hr), the discs were floated on distilled  $\text{H}_2\text{O}$  and allowed to assimilate 480  $\mu\text{Ci CO}_2$  (generated from  $\text{Na}_2^{14}\text{CO}_3$ ) for 30 min. In the shorter term experiment (up to 4 hr), the leaf discs were exposed to 600  $\mu\text{Ci }^{14}\text{CO}_2$  for 10 min.

Developing leaves were taken from plants which had been grown for 3 weeks in complete darkness and then exposed to 48 hr of continuous light. The leaves at this stage were expanding and contained chloroplasts with rapidly developing lamellar systems. They were de-ribbed, and the laminae (10–20 g) were allowed to assimilate 600  $\mu\text{Ci }^{14}\text{CO}_2$  for 7 min.

In all cases, the discs were spread on damp filter papers in

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<sup>3</sup> Abbreviations: MGDG: monogalactosyl diglyceride; DGDG: digalactosyl diglyceride.

Petri dishes and exposed during  $^{14}\text{CO}_2$  feeding to high intensity fluorescent light (1100 ft-c). The zero time sample was taken immediately after removal from the  $^{14}\text{CO}_2$  feeding chamber. The remaining discs were exposed to 1100 ft-c light or kept in complete darkness at 21 C and sampled at the time intervals indicated in the text.

The lipids were extracted from approximately 2 g of leaf material (weighed before feeding) by the method of Williams and Merrilees (18) and separated by TLC on Silica Gel G (Merck), with or without the addition of 5%  $(\text{NH}_4)_2\text{SO}_4$ , using the solvent acetone-benzene-water, 91:30:8 (12). The plates were sprayed with 0.05% dichlorofluorescein in methanol, and the bands corresponding to the galactolipids were scraped into tubes and methanolized with dry methanolic HCl (1.5 N).

The fatty acid methyl esters were extracted with hexane. The remaining methanolic HCl was then neutralized with silver carbonate, filtered, and dried, and the methyl galactoside and glycerol were derivatized in pyridine-hexamethyldisilazane-trimethylchlorosilane, 10:4:2 (14). The trimethylsilyl derivatives were separated by GLC on 61 cm  $\times$  4 mm i.d. glass columns packed with 3% SE-30 (Ultraphase) on Chromasorb W, H.P. (Chromatographic Specialties, Brockville, Ontario).

The glycerol and methyl galactosides were collected and their radioactivity determined according to the method of Watson and Williams (16).

Total activity of the fatty acid methyl esters was determined by scintillation counting of an aliquot of the hexane extract in toluene containing PPO (6 g/l) and POPOP (300 mg/l).

Chlorophyll was determined according to the method of Arnon (1).

## RESULTS

The galactolipid and Chl content of the mature leaf discs incubated in the light are listed in Table I. The results indicate that there was a significant increase in the total quantity of DGDG over the time course of the experiment. The MGDG and Chl contents did not change appreciably.

If DGDG is formed by galactosylation of MGDG, the rate of synthesis of this lipid would have to be equal to or greater than that of DGDG in order to maintain the level of MGDG in the leaf, assuming little or no degradation of either lipid. The quantitative data (Table I) indicate that during a long period of exposure to light net DGDG synthesis in mature tissue exceeded net MGDG synthesis, resulting after 72 hr in the greater quantity of DGDG than MGDG in the leaf discs.

Our data from the  $^{14}\text{CO}_2$  feeding experiments of mature

leaves (Table I) indicate that during the feeding periods more activity was incorporated into the galactose of DGDG than that of MGDG. In view of our previous findings (17), that less than 10% of the activity in the galactose was derived from MGDG at this time, the total activity incorporated into MGDG and DGDG galactose may be used as an estimate of the amount of each galactolipid synthesized. The rate of synthesis of DGDG, under the conditions in these experiments, must have been equal to or greater than the rate of MGDG synthesis.

In the present study nearly all of the labeling of the galactose moieties occurred during the  $^{14}\text{CO}_2$  feeding periods, and estimates of galactolipid synthesis using galactose labeling can be made only for these times. Net synthetic rates determined from the labeling of galactose would be greatly affected by a high turnover of the galactose, if this occurs. Turnover would be reflected by a decrease in activity of the galactose after the removal of  $^{14}\text{CO}_2$ . The activity did not decrease in either MGDG or DGDG, indicating no evidence for rapid turnover of the galactoses of these lipids. In fact, in the 72 hr following the removal of  $^{14}\text{CO}_2$ , the radioactivity in DGDG more than doubled that at zero time in mature leaf tissue, while the level of activity in MGDG did not increase significantly. The gradual increase in the activity of DGDG up to 72 hr was probably due to the galactosylation of radioactive MGDG (17) and to the recycling of respired  $^{14}\text{CO}_2$  by photosynthesis into UDP-galactose (Williams and Leung, unpublished results). The level of activity in MGDG was probably the result of a balance between a loss by galactosylation to DGDG or degradation and an increase by  $^{14}\text{C}$ -recycling similar to that found in DGDG.

To clarify the labeling patterns of galactose, glycerol, and fatty acids in the early period after feeding, experiments were conducted with the feeding time reduced from 30 min to 10 min and with samples being taken at shorter times after feeding (Fig. 1). Even at zero time (after the 10-min feeding period) the activity of galactose was relatively high, while at 15 min the activity had already leveled off. Clearly the radioactive UDP-galactose pool was rapidly diluted by  $^{14}\text{CO}_2$  resulting in little increase in radioactivity once the  $^{14}\text{CO}_2$  had been removed. As in the previous experiments, the activity in DGDG was higher than in MGDG indicating a higher rate of synthesis of DGDG than MGDG in the light.

The incorporation of  $^{14}\text{C}$  into glycerol and fatty acids followed an entirely different pattern (Table I, Fig. 1). At zero time the level of activity in both glycerol and fatty acid in both lipids was very low in comparison with that in galactose. The activity, however, increased steadily with time, even to 72 hr

Table I. Chlorophyll and Galactosyl Diglyceride Content of Mature Leaves

The radioactivity recovered from the galactose, glycerol, and fatty acid moieties of the galactosyl diglycerides at time intervals in the light after  $^{14}\text{CO}_2$  feeding are shown.

Time	Chl a + b	Radioactivity							
		Monogalactosyl diglyceride				Digalactosyl diglyceride			
		Quantity	Galactose	Glycerol	Fatty acid	Quantity	Galactose	Glycerol	Fatty acid
hr	$\mu\text{mole/g fresh wt}$	$\mu\text{mole/g fresh wt}$	$\text{dpm/g fresh wt} \times 10^{-3}$			$\mu\text{mole/g fresh wt}$	$\text{dpm/g fresh wt} \times 10^{-3}$		
0	2.70	3.82	282.2	2.5	20.4	2.72	345.1	0.7	6.6
2	2.68	3.62	200.9	4.8	132.5	2.59	349.9	1.1	23.2
6	2.71	3.98	352.5	20.8	291.8	2.37	343.3	3.2	62.5
12	2.64	4.06	254.8	18.7	505.9	2.97	370.0	5.6	136.5
24	2.63	3.87	280.4	26.4	818.2	3.60	508.3	13.0	336.2
48	2.51	3.60	198.1	31.4	920.6	3.51	586.2	22.5	502.2
72	2.57	4.08	318.7	97.4	1,542.9	4.29	735.1	62.6	1,150.1

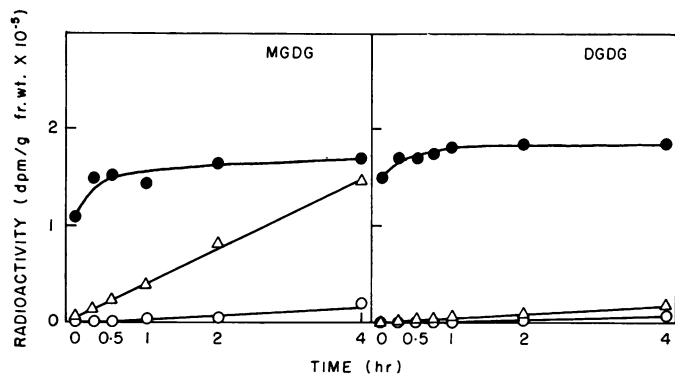


FIG. 1. Radioactivity recovered from the galactose, glycerol, and fatty acid moieties of the galactosyl diglycerides after incubation for short time intervals in the light following  $^{14}\text{CO}_2$  feeding. Galactose (●); glycerol (○); fatty acids (△).

after the removal of  $^{14}\text{CO}_2$  in mature tissue. There is clearly a fundamental difference between the rapid incorporation of activity into galactose and the slower but continuous increase in glycerol and fatty acid. Calculations of the galactose, glycerol, and fatty acid results in terms of specific radioactivity of their carbon atoms (Fig. 2) indicate more clearly the differences and similarities in the labeling patterns of the carbons of the three moieties. The more or less parallel increase in activity in glycerol and fatty acids suggests that they are incorporated simultaneously as diglycerides into the galactolipids. We believe this difference in the rate of incorporation of activity in galactose from the glycerol and fatty acids is due to the time taken for the formation and desaturation of the long chain fatty acids.

In mature tissue (Table I), the glycerol and fatty acids of DGDG contain less radioactivity than those of MGDG, unlike the labeling in the galactose. This is consistent with the view that DGDG is formed from MGDG and not from a common precursor diglyceride pool. If the latter were the case, the activity in DGDG glycerol and fatty acid would be equal to or greater than that in MGDG (in view of the estimates of lipid synthesis determined by incorporation of  $^{14}\text{C}$  into the galactose).

Even though DGDG was synthesized at a rate equal to or greater than MGDG, the majority of the MGDG formed from  $^{14}\text{CO}_2$  remained as part of the total cell MGDG and was not converted to DGDG, even after 72 hr. If galactosylation of MGDG to DGDG occurs utilizing a pool of newly formed MGDG, then the major part, if not all, of this pool would have been converted fairly rapidly to DGDG and very little radioactivity would have remained in the galactose, glycerol, or fatty acids of MGDG. On the other hand, if galactosylation occurs using only an existing pool of nonradioactive MGDG (such as in the lamellae), little or no activity would have appeared immediately in the glycerol or fatty acids of DGDG. As neither of these situations arose, it would appear that DGDG is formed both from an existing nonradioactive pool of MGDG and from a pool of MGDG containing a high level of the fed  $^{14}\text{C}$ . These results support our previous suggestion (17) that conversion of MGDG to DGDG may occur from two pools: galactosylation of part of the pool of newly formed MGDG and galactosylation of existing MGDG in the cell.

In developing tissue where net synthesis of both lipids and Chl was clearly taking place (Table II), the incorporation of activity into the galactose of MGDG was higher than that of DGDG. The galactose in both lipids followed a similar labeling pattern to that found in the mature tissue becoming actively labeled very rapidly. Once again there was no indication of rapid turnover or degradation of the galactose moieties of the lipids.

In developing tissue (Table II), similar trends in the labeling patterns of glycerol and fatty acids were found to those in mature tissue. In this tissue, however, a higher proportion of the activity was found in the glycerol and fatty acids of MGDG than of DGDG. This is partially the result of a higher synthetic rate of MGDG which, from the incorporation of activity into galactose, appears to be nearly double that of DGDG.

In the dark-incubated discs (Table III) the quantities of MGDG and DGDG did not appear to change significantly for 48 hr. A slight increase in MGDG and a small decrease in DGDG was observed resulting in an increase in the MGDG/DGDG ratio from 1.24 at zero time to 1.61 at 48 hr. At 72 hr, however, breakdown of both lipids was apparent although there did not appear to be any significant loss of Chl.

In the dark there was a continued increase in the specific radioactivities of the carbons of both MGDG and DGDG galactose (Fig. 2). Unlike in the light, the activity of the galactose continued to increase rapidly, the precursor pool was apparently being replenished from a radioactive carbohydrate source in the leaf. Because of this, it is not possible from the galactose data to compare rates of synthesis of these lipids in light and dark. It is possible, however, to compare the synthetic rates of DGDG and MGDG from the same tissue in either light- or dark-incubated samples.

Although the initial level of activity of DGDG galactose following the feeding period in the light was higher than that of MGDG, after 24 hr in the dark the situation was reversed. This suggests that the rate of synthesis of MGDG was greater than

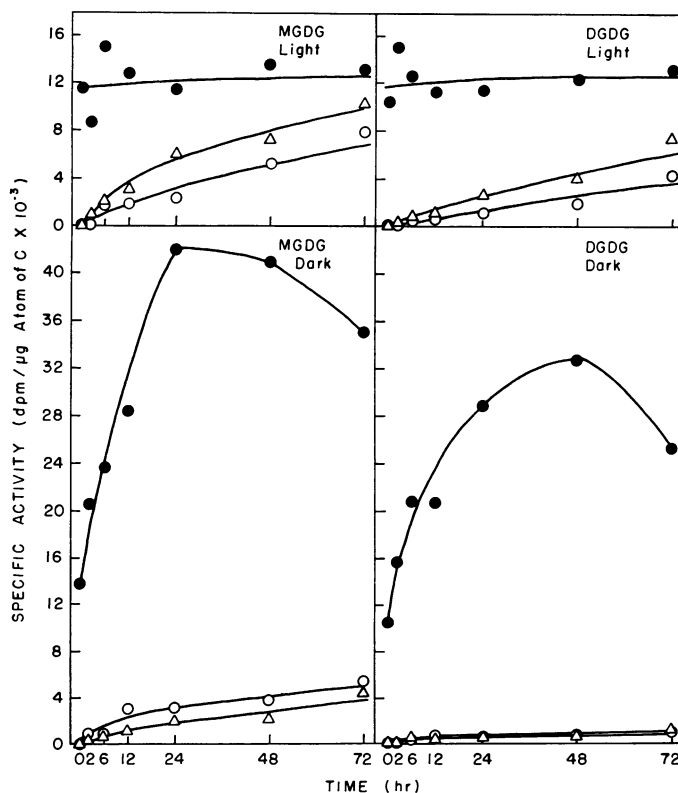


FIG. 2. Specific radioactivities (dpm/ $\mu\text{g}$  atom of C) of galactose, glycerol and fatty acid carbon of mono- and digalactosyl diglycerides (MGDG and DGDG) after  $^{14}\text{CO}_2$  feeding of mature leaf tissue and incubation in light or dark. The specific radioactivities were calculated by dividing the specific radioactivity in dpm/ $\mu\text{mole}$  for galactose in MGDG by 6 and in DGDG by 12; for glycerol by 3 and for fatty acid by 17.875 (the average number of C in galactolipid fatty acids). Galactose (●); glycerol (○); fatty acids (△).

Table II. *Chlorophyll and Galactosyl Diglyceride Content of Developing Leaves*

The radioactivity recovered from the galactose, glycerol, and fatty acid moieties of the galactosyl diglycerides at time intervals in the light after  $^{14}\text{CO}_2$  feeding are shown.

Time	Chl a + b	Radioactivity							
		Monogalactosyl diglyceride				Digalactosyl diglyceride			
		Quantity	Galactose	Glycerol	Fatty acid	Quantity	Galactose	Glycerol	Fatty acid
hr	$\mu\text{mole/g fresh wt}$	$\mu\text{mole/g fresh wt}$	$\text{dpm/g fresh wt} \times 10^{-3}$			$\mu\text{mole/g fresh wt}$	$\text{dpm/g fresh wt} \times 10^{-3}$		
0	1.44	3.93	51.8	2.4	17.5	2.70	29.1	trace	6.3
0.25	2.02	3.80	98.5	6.1	54.6	2.77	58.9	0.5	10.5
0.5	2.60	3.94	104.7	6.0	84.9	2.72	43.0	0.6	13.1
1	2.13	4.12	109.8	9.5	128.3	2.70	63.1	0.8	16.5
2	2.28	4.12	90.5	8.5	166.5	2.75	52.5	1.5	20.1
4	2.25	4.42	92.7	11.4	220.7	2.77	51.5	2.9	30.5
6	2.20	4.40	102.1	12.2	239.1	2.78	59.2	2.9	37.4
8	2.34	5.05	76.8	12.2	289.9	3.25	53.8	3.3	53.0

Table III. *Chlorophyll and Galactosyl Diglyceride Content of Mature Leaves at Time Intervals in Dark after  $^{14}\text{CO}_2$  Feeding*

Time	Chl a + b	Monogalactosyl Diglyceride	Digalactosyl Diglyceride
hr		$\mu\text{mole/g fresh wt}$	
0	2.71	3.47	2.79
2	2.68	3.72	2.77
6	2.74	4.04	2.23
12	2.56	3.81	2.59
24	2.74	3.98	2.64
48	2.63	4.06	2.52
72	2.66	3.02	1.82

DGDG in the dark, in agreement with the quantitative results. This is in contrast to what was found in the light.

Incorporation of activity into glycerol in MGDG does not appear to be drastically affected by darkness, the levels of activity in the dark being generally higher than in the light at the same time intervals. The fatty acids, however, at most time intervals, contained approximately 30% of the activity found in the light.

The  $^{14}\text{C}$  activities in both glycerol and fatty acid moieties of DGDG in the dark were very low in comparison with the results from light-treated tissue. Presumably the combined effect of lower activities in the MGDG precursor (fatty acids) and probably a lower rate of galactosylation of MGDG to DGDG resulted in very little activity in both glycerol and fatty acids in DGDG, in contrast to the high levels of activity in the galactose.

## DISCUSSION

Our results are consistent with the metabolic pathway proposed by Ferrari and Benson (5) for the galactosylation of MGDG to DGDG. The data indicate that there is a more rapid incorporation of  $^{14}\text{C}$  activity from  $^{14}\text{CO}_2$  into the galactose moieties of both galactolipids than into the glycerol and fatty acids. In this study however, little or no turnover was detected in the galactose of MGDG or DGDG. Our results suggest that the high incorporation of activity into galactose may be due to rapid synthesis but not turnover. The apparently slower metabolism of glycerol and fatty acid could be explained by a slower incorporation of activity into a diglyceride pool suitable for galactosylation to MGDG. This would explain the results ob-

tained by Ferrari and Benson (5) without requiring a high rate of turnover of galactose in the lipid.

Trémolières (15) indicated a similarly high rate of incorporation of activity into the galactosyl glycerols of the galactolipids of clover leaves. The maximum levels of activity, however, were obtained after 4 to 8 hr and the fatty acids were only weakly labeled after 12 hr. In pumpkin leaves, Roughan (13) found very high levels of activity (>90%) in the polar moieties (containing galactose and glycerol), 1 hr after  $^{14}\text{CO}_2$  feeding, which did not change significantly over 48 hr. In neither study, however, was the labeling of galactose and glycerol determined separately or the different labeling patterns of the galactose established in DGDG.

As there appears to be little turnover or degradation of the galactolipids, the labeling of the galactose moiety may be used as an estimate of galactolipid synthesis using  $^{14}\text{CO}_2$  as a precursor. The lower  $^{14}\text{C}$  activities found in the glycerol and fatty acid of DGDG than in MGDG would be expected if MGDG is the precursor of DGDG. Glycerol and fatty acids cannot be used to determine comparative rates of synthesis as the  $^{14}\text{C}$ -labeling kinetics appear to confirm that DGDG is a product of the galactosylation of MGDG. The glycerol and fatty acids of DGDG are, therefore, secondary products formed from MGDG and cannot be used to estimate rates of synthesis. Under the conditions used in the experiments on mature tissue the rate of DGDG synthesis appeared to be equal to or greater than MGDG. In developing tissue, however, MGDG synthesis was approximately double that of DGDG. Factors affecting the relative rates of synthesis of these two lipids are presently under investigation in our laboratory. It is possible that the light conditions used in these experiments during feeding of  $^{14}\text{CO}_2$  favored the net synthesis of the DGDG over MGDG in mature leaves. Under less favorable conditions (e.g. darkness or lower light intensity) the reverse may be the case.

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