Galactolipid Synthesis in Vicia faba Leaves

II. FORMATION AND DESATURATION OF LONG CHAIN FATTY ACIDS IN PHOSPHATIDYLCHOLINE, PHOSPHATIDYLGLYCEROL, AND THE GALACTOLIPIDS

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ABSTRACT

The labeling kinetics of the fatty acids of phosphatidylcholine (PC), phosphatidylglycerol (PG), monogalactosyldiglyceride (MGDG), and digalactosyldiglyceride (DGDG) were examined after 14CO2 feeding and incubation of leaf discs of Vicia faba over 72 hours in continuous light. The results indicate a rapid accumulation and turnover of radioactivity into PC and PG fatty acids (oleic acid in PC and oleic and palmitic acids in PG). Radioactivity accumulates in MGDG and DGDG fatty acids much more slowly and continuously over 72 hours. Most of this activity is found in linoleic and linolenic acids; very little activity is found in the more saturated fatty acids. Little or no desaturation occurs in situ in conjunction with the galactolipids. The results suggest that PC and PG may act as “carriers” for MGDG and DGDG fatty acid synthesis. Analyses of the labeling patterns of the molecular species of MGDG after 14CO2 and 14C-acetate feeding confirm that MGDG is formed by galactosylation of a preformed diglyceride containing predominantly unsaturated fatty acids.

The major chloroplast glycerolipids, MGDG2 and DGDG, contain high concentrations of long chain unsaturated fatty acids (mainly linolenic acid, 18:3). The mechanisms by which these high levels of unsaturated fatty acids are formed and maintained are key problems of lipid metabolism in algae and higher plants.

James (9) suggested that fatty acids in plant glycerolipids are divided into two pools: one of saturated fatty acids (mainly palmitic and stearic), the other a series of unsaturated fatty acids (mainly oleic, linoleic and linolenic). He showed that the saturated fatty acids once incorporated into glycerolipids were not desaturated. Oleic acid in leaves was readily desaturated to linoleic and linolenic acids and this is believed to be the major synthetic pathway in many plants (2, 6). Recently, Jacobson et al. (8) have shown that in spinach, linolenic acid is formed by chain elongation of hexadecatrienoic acid, 16:3.

Smirnov (18), Mudd and McManus (12), and Stumpf and James (19) suggested that the major site of fatty acid synthesis in leaves is the chloroplast. They obtained chloroplast preparations which could synthesize saturated and monoenoic fatty acids. It has not been possible in these or later attempts (7, 10, 11) to obtain high rates of synthesis of unsaturated fatty acids in isolated chloroplasts proportionate to the quantities found in the galactolipids of chloroplast lamellae.

1 The research was supported by a grant from the National Research Council of Canada.
2 Abbreviations: MGDG: monogalactosyldiglyceride; DGDG: digalactosyldiglyceride; PC: phosphatidylcholine; PG: phosphatidylglycerol.

Three possible mechanisms have been proposed by which plants may attain high levels of unsaturated fatty acids in their galactolipids. Gurr et al. (5) and Gurr and Brawn (4) in Chlorella, and Roughan (15) in pumpkin, have suggested that PC may act as a “carrier molecule” involved in the desaturation of fatty acids which may then be transferred to the galactolipids. Appleby et al. (1), however, concluded that in Chlorella both PC and MGDG were involved in fatty acid synthesis and desaturation. Nichols and Moorhouse (14) and Safford and Nichols (16) proposed that desaturation in Chlorella occurs in situ after the formation of MGDG and hence in conjunction with MGDG, not PC. The third mechanism for obtaining high levels of unsaturated fatty acids in MGDG would be by galactosylation of specific diglycerides containing unsaturated fatty acids. Mudd et al. (13) showed that, in chloroplast preparations, diglycerides containing unsaturated fatty acids were preferentially galactosylated to MGDG. Eccleshall and Hawke (3), have indicated no such preference in chloroplast extracts of spinach. Trémolières and Mazliak (20) proposed that the diglyceride utilized in the production of MGDG may be produced by removal of the phosphoryl group of a phospholipid containing unsaturated fatty acids.

There is conflicting evidence for the participation of PC in the production and desaturation of galactolipid fatty acids, and confusion as to whether desaturation occurs before or after incorporation into galactolipids.

This study was undertaken to clarify the metabolic systems involved in the fatty acid synthesis of galactolipids in Vicia faba leaves. We report a detailed analysis of the labeling kinetics of the fatty acids of PC, PG, MGDG, and DGDG over a period of 72 hr in continuous light after 14CO2 feeding. Although the separation of molecular species of Chlorella MGDG by argentation TLC has been reported (14), we are not aware of any attempts to use this technique in conjunction with 14C feeding for the investigation of galactolipid metabolism.

MATERIALS AND METHODS

Plant material was grown, fed 14CO2, and incubated in light as previously described (24).

Leaf discs were fed with [1-14C]acetate (16 µCi/g fresh weight of discs) by infiltration under reduced pressure and then incubated in the same way as the 14CO2-fed leaf discs. Lipids were extracted, chromatographed by TLC, and methanolyzed as previously described (23, 24).

Methyl pentadecanoate (0.083 µmole) was added as internal standard. The fatty acid methyl esters were separated by GLC on 122 cm × 4 mm I.D. glass columns packed with 15% EGS on Chromosorb W, 100/120 mesh; or 10% EGSS-X on Gas-Chrom P, 100/120 mesh; or 10% Silar 10C on Gas-Chrom Q, 100/120 mesh (Applied Science Laboratories Inc.). Each fatty acid methyl ester was collected and the radioactivity determined according to the method of Watson and Williams (21).
The distribution of radioactivity in the galactose, glycerol, and fatty acids, and the total radioactivity of the fatty acids were determined as previously described (24).

**Argonation Chromatography.** The molecular species of MGDG were separated on Silica Gel G plates (0.25 mm) (E. Merck) impregnated with 20% silver nitrate (w/w). The plates were activated for 1 hr at 95 C and, when cool, were stored in dark boxes maintained at 30% humidity. The plates were developed in chloroform-methyl alcohol-water (65:35:4, v/v/v) and, after drying, were pressed firmly against x-ray films (Kodak, No-Screen) for 7 days. The x-ray films were washed with H2O to remove AgNO3 before being developed in the usual way.

To identify the molecular species, similar plates were developed and the bands visualized with 0.05% dichlorofluorescein in methyl alcohol. Each band was scraped off into tubes and the lipid extracted first with chloroform-methyl alcohol (2:1) and then with chloroform-methyl alcohol (1:2). The silver nitrate was removed from the combined extract by several washings with distilled H2O. The fatty acid composition of each band was determined by GLC as described above.

**RESULTS**

Quantitative changes in PC, PG, MGDG, and DGDG are shown in Table 1. Significant increases were found only in PC and DGDG. The fatty acid compositions of the lipids changed only slightly during the experiment, the most significant difference being an increase in the levels of linoleic acid and a drop in linolenic acid in all four lipids. The changes in mole percent appear to represent increases in the quantities of linoleic acid rather than a decrease in other fatty acids, most of which increased slightly throughout the experiment.

The specific radioactivities of PC, PG, MGDG, and DGDG total fatty acids (Fig. 1) indicate that the incorporation of radioactivity into all four lipids occurred in different ways. Radioactivity was incorporated most rapidly into PC and reached a peak in this lipid at about 2 hr after feeding. The specific radioactivity then decreased, indicating depletion of the fatty acid 14C precursors and turnover of the fatty acids. Since no further decrease in specific radioactivity was apparent after 24 hr of illumination, only part of the PC in the leaf was turning over. The fatty acids of PG also demonstrated a rapid incorporation of radioactivity, which reached a peak at a similar time to those of PC. A significantly smaller proportion of the PG fatty acids appeared to turn over.

A more detailed analysis of the fatty acids of PC (Fig. 2) indicates that the turnover was almost entirely due to turnover of oleic acid. This may be partially due to desaturation in the pool of PC but must also be due to loss of radioactivity either by oxidative breakdown or by transfer of fatty acid, possibly to other glycerolipids, because there was no corresponding increase in 18:2 and 18:3. A similar situation existed with the fatty acids of PG (Fig. 3) except that the loss of radioactivity was mainly from palmitic acid.

The maximum specific radioactivities reached by both palmitic and oleic acids were higher in PG than in PC. However the total radioactivity in palmitic and oleic acids in PC was higher than in PG, suggesting that PC was the major site of esterification of these fatty acids.

After 72 hr of incubation the specific radioactivities of all the fatty acids in PC and PG reached similar levels, with the exception of linolenic acid in PC which was consistently lower throughout the experiment. The incorporation of radioactivity into linolenic acid in PC was considerably lower than that found later in linolenic acid in MGDG despite the higher initial rate of fatty acid synthesis in PC.

The fatty acids of MGDG and DGDG incorporated radioactivity much more slowly than the phospholipids (Fig. 1), and there is little indication of turnover from these data. The radioactivity in MGDG and DGDG fatty acids increased at least to 72 hr, despite the depletion of fatty acid precursors indicated by the

**Table 1. Quantity and Fatty Acid Composition of PC, PG, MGDG and DGDG from Leaf Discs Incubated in Continuous Light after 14CO2 Feeding**

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Time (hr)</th>
<th>Quantity (umole fresh wt)</th>
<th>16:0</th>
<th>16:1</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>18:3</th>
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<td>22.1</td>
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<td>3.3</td>
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<tr>
<td></td>
<td>24</td>
<td>2.4</td>
<td>21.2</td>
<td>tr</td>
<td>4.2</td>
<td>11.9</td>
<td>40.4</td>
<td>21.4</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>2.5</td>
<td>21.5</td>
<td>tr</td>
<td>5</td>
<td>7.9</td>
<td>43.6</td>
<td>21.6</td>
</tr>
<tr>
<td>PG</td>
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<td>0.2</td>
<td>21</td>
<td>31.8</td>
<td>1.6</td>
<td>6.1</td>
<td>15.7</td>
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<td>28.5</td>
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<td>2.1</td>
<td>6.8</td>
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<td>9.1</td>
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</table>

**Fig. 1.** Specific activities of the total fatty acid moieties of PC (Δ), PG (●), MGDG (○) and DGDG (□) after 14CO2 feeding (30 min) and incubation in continuous light.
DGDG is a product of the galactosylation of MGDG.

Determination of the molecular species of MGDG first formed by galactosylation should indicate the degree of unsaturation of the diglyceride precursors to which galactose was added. Leaf discs were fed $^{14}$CO$_2$ and $^{14}$C-acetate separately (in the light) for 10 min and 120 min, respectively. MGDG was isolated and purified by TLC and the distribution of activity in the galactose, glycerol, and fatty acids was determined. In the $^{14}$CO$_2$-fed MGDG, 93.3% of the radioactivity was found in the galactose moiety as compared with 0.7% in the glycerol and 6% in the fatty acids. Consequently the molecular species containing this radioactivity would represent the molecular species of diglyceride to which newly formed galactose was attached. After $^{14}$C-acetate feeding, 96.7% of the radioactivity was found in the fatty acid fraction, 0.6% in the glycerol, and 2.7% in the galactose. The distribution of radioactivity in the molecular species of MGDG after $^{14}$C-acetate feeding would, therefore, indicate the distribution of newly formed fatty acids in the diglyceride substrate for MGDG synthesis.

The molecular species of MGDG were separated by argentation chromatography and the fatty acids were identified by GLC analysis (Table II). Autoradiographs of the molecular species of MGDG separated on similar plates are presented in Figure 6.

In MGDG from $^{14}$CO$_2$-fed tissue the major part of the radioactivity was found in the molecular species containing five and six double bonds (linoleyl-linolenoyl and dilinolenoyl species, respectively). The radioactivity from $^{14}$C-acetate was distributed among the more saturated fatty acid-containing species. The results indicate that the species of diglyceride to which galactose was added contained highly unsaturated fatty acids, mainly linoleic and/or linolenic acids. These results could not

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**Fig. 2.** Radioactivities and specific activities of the individual fatty acids of PC after $^{14}$CO$_2$ feeding and incubation in continuous light. Palmitic ($\Delta$), stearic (●), oleic (□), linoleic (▲), and linolenic (○) acids.

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**Fig. 3.** Radioactivities and specific activities of the individual fatty acids of PG after $^{14}$CO$_2$ feeding and incubation in continuous light. Palmitic ($\Delta$), palmitoleic (▲), stearic (●), oleic (□), linoleic (▲), and linolenic (○) acids.
have been due to rapid desaturation of fatty acids after MGDG synthesis. If this had been the case, the radioactivity after 14C-acetate feeding would have been distributed in a similar pattern to that found after 14CO2 feeding. The 14CO2 results could be interpreted to indicate turnover of the galactose moiety of the lipid. In two previous reports we showed that in the galactose moiety of MGDG of Vicia faba, rapid turnover does not occur (22, 24).

**DISCUSSION**

The labeling patterns of the fatty acids of PC and MGDG support the idea that fatty acid lost from PC is accumulated in MGDG in a more unsaturated form. Our data do not support the conclusion of Roughan (15) that linolenic acid is transferred by acyl transfer from PC to MGDG. Acyl transfer of fatty acids from PC to MGDG and DGDG could occur in two ways: exchange of fatty acids between PC and the galactolipids, or acylation of galactosylmonoglycerides. Only by synthesis of more saturated fatty acid in MGDG or lysomonogalactolipid is it possible to exchange or transfer the unsaturated fatty acids at later times. In both cases we would expect to see, during de novo synthesis, an early peak of radioactivity in the saturated fatty acids or oleic acid in MGDG. Acyl transfer or exchange may be possible for repair and maintenance of lipid molecules in our tissue but not for de novo synthesis.

Our labeling data for MGDG and DGDG gave no indication of desaturation of saturated fatty acid or oleic acid to linoleic or linolenic acids. Insufficient label was accumulated in these fatty acids at early times to account for the large quantities of radioactivity found later in the unsaturated fatty acids. Our results rule out two possible mechanisms for obtaining unsaturated fatty acids in galactolipids in Vicia faba leaves: synthesis and desaturation in PC followed by acyl transfer, and desaturation after the formation of MGDG. The third possibility that of galactosylation of a diglyceride containing unsaturated fatty acids, appears to be the only possible mechanism for obtaining high levels of unsaturated fatty acids in galactolipids consistent with our results.

Our data on the labeling of molecular species appear to indicate that the major form of diglycerides used in the galactosylation to MGDG contained highly unsaturated fatty acids. These results are consistent with the experiments of Mudd et al. (13) who showed a specificity of the galactosylating enzymes for diglycerides containing unsaturated fatty acids. On the other hand, some radioactivity (from 14C-acetate) in the form of the more saturated fatty acids was found in MGDG, suggesting that the galactosylating enzyme was not entirely specific for unsaturated fatty acids.

The origins of the more saturated fatty acids (oleic, palmitic, and stearic) in MGDG are less clear. The labeling kinetics of palmitic acid in PG suggest that PG may be a source of palmitic acid for MGDG.

Many in vitro attempts have been made to obtain chloroplast preparations which synthesize and desaturate long chain fatty acids. Most of the attempts have been successful in synthesizing saturated and monoenoic fatty acids (palmitic and oleic) but few have been successful in obtaining significant quantities of the more unsaturated fatty acids (7, 10, 11). This may be explained if much of the saturated and monoenoic acids are synthesized inside the chloroplast, perhaps in conjunction with PG. As PC is predominantly found in the cytoplasm this might indicate that the dienoic and trienoic unsaturated fatty acids are synthesized in the cytoplasm and not in the chloroplasts. These

**FIG. 4.** Radioactivities and specific activities of the individual fatty acids of MGDG after 14CO2 feeding and incubation in continuous light. Symbols as in Fig. 2.

**FIG. 5.** Radioactivities and specific activities of the individual fatty acids of DGDG after 14CO2 feeding and incubation in continuous light. Symbols as in Fig. 2.
conclusions are supported by further work in our laboratory on cellular fractions after 14CO2 feeding of leaves (17), and by the results of Hawke et al. (7) in which the addition of mitochondrial and microsomal fractions increased the level of desaturase activity in the chloroplast fractions.

Our experiments were conducted over an extended period of time and indicated that radioactivity was continuously incorpo-

rated into the galactolipids for at least 72 hr. Experiments performed by others over much shorter periods of time have failed to show the high rates of 14C incorporation that were found in the unsaturated fatty acids in this study. Our data also suggest that the time taken for the incorporation of unsaturated fatty acids in MGDG is sufficiently long to predict that similar experiments using shorter periods of time or in vitro experiments would be unsuccessful in attaining high levels of labeled unsaturated fatty acids. Coupled with the fact that the chloroplast may lack the ability to desaturate the fatty acid that it does form, it would seem that in vitro experiments aimed at obtaining high levels of desaturation in chloroplast may continue to prove unproductive.

LITERATURE CITED


