

# ***In vivo* Synthesis of [<sup>14</sup>C]Dilinoylgalactosylglycerol by Gametophytes of the Fern *Anemia phyllitidis***

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Blue light-grown gametophytes of *Anemia phyllitidis* were used for the *in vivo* synthesis of radioactively labeled dilinoylgalactosylglycerol, a potential intermediate in the synthesis of linolenic acid. Optimal yield was obtained by incubating gametophytes with [<sup>14</sup>C]linoleate first for 2 h under anaerobic conditions followed by 46 h under aerobic conditions. As product [<sup>14</sup>C]dilinoylgalactosylglycerol was identified.

## **Introduction**

$\alpha$ -Linolenic acid is the most abundant fatty acid of the membrane lipids in higher-plant chloroplasts and is also a significant component of seed oils, for example in linseed, soybean, and rapeseed.

From tracer and kinetic studies there is evidence for a sequential desaturation of oleic acid *via* linoleic acid to linolenic acid. Previous studies have indicated that in plants not the free but the lipid-linked fatty acids are desaturated (*cf.* reviews [1–3]).

Depending on the type of tissue, different substrates and subcellular compartments appear to be involved in the sequential conversion of oleic acid into linolenic acid. Phosphatidylcholine is believed to be the substrate of the microsomal desaturation system which mainly is operating in seed tissue [4, 5] while diacylgalactosylglycerol is thought to be involved in the chloroplast system [6–8]. Recently [9, 10] it has been shown that, in a so-called “cooperative” pathway, both substrates contribute to the synthesis of diacylgalactosylglycerol enriched in linolenic acid.

The *in vitro* demonstration of n-3-desaturase, that catalyzes the final step of linolenic acid biosynthesis, failed hitherto because of the unavailability of appropriate substrates.

Recently, we found that fern gametophytes accumulate linoleic acid instead of linolenic acid when being cultured in continuous blue light [11]. Here we describe that this system is useful for a comparatively economic preparation of labeled dilinoylgalactosylglycerol as an important potential precursor for further studies of linolenic acid biosynthesis.

## **Material and Methods**

### *Chemicals and plant material*

[1-<sup>14</sup>C]Linoleic acid (2.19 GBq/mmol) were obtained from New England Nuclear, Dreieich, W. Germany. Lipase from *Rhizopus arrhizus* was supplied by Boehringer, Mannheim.

Spores of the fern *Anemia phyllitidis* (L.) Sw. were from plants grown in the greenhouse of the University of Ulm. Spores were sterilized with sodium hypochlorite and sown aseptically in Fernbach flasks on mineral salt medium as described previously [11]. The spores were induced to germination by red light irradiation and cultures were grown at 21 °C under continuous blue light as described [11].

### *Incubation of gametophytes*

Gametophytes were harvested by filtration and resuspended into new medium containing <sup>14</sup>C-labeled linoleate. Usually 14.8 KBq were fed to that amount of gametophytes which emerged from 100 mg spores, in the large scale experiments 370 KBq were used per 10 g fresh weight.

### *Extraction and analysis of lipids*

After incubation for 48 h, gametophytes were recovered from the cultures by filtration and transfer-

**Abbreviations:** PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; FFA, free fatty acid; DG, diacylglycerol; TG, triacylglycerol; MGD, diacylgalactosylglycerol; DGD, diacyldigalactosylglycerol; TLC, thin layer chromatography.

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red to boiling 2-propanol. Gametophytes were homogenized in a mortar and lipids were extracted with chloroform/methanol (2:1, v/v) and purified according to Folch *et al.* [12].

Lipid classes were separated on silica gel columns (5 × 1 cm, Kieselgel MN 60, 70–130 mesh, Macherey Nagel, Düren, W. Germany) under reduced pressure using dichlormethan and diethyl ether to elute neutral lipids and pigments followed by acetone and methanol to elute polar lipids. The polar lipid fraction was further purified by preparative TLC on silica gel with acetone/benzene/water (35:14:3, by vol.). The band corresponding to MGD was scraped off and extracted with chloroform/methanol (2:1) for further analysis. Molecular species of MGD were separated by 10% AgNO<sub>3</sub>-silica gel TLC with chloroform/methanol/water (60:21:4, by vol.) according to [13]. The positional specific distribution of fatty acids at the glycerol backbone was determined with lipase from *Rhizopus arrhizus* according to ref. [14]. Separation of free fatty acids from lysogalactolipid and the uncleaved galactolipid was performed by TLC on silica gel using a one-dimensional sequential system of ether/ethyl ether/acetic acid (50:50:4, by vol.) followed by chloroform/methanol/water (140:30:2, by vol.). The bands were scraped off and methylated. Methyl esters of free fatty acids and of lipids were prepared using the boron trifluoride method [15]. Fatty acid methyl esters were analyzed by radio-gas chromatography on a 10% DEGS column. The amount of fatty acids was determined by the addition of penta-decanoic acid as internal standard.

Radioactivity of the individual lipid classes was monitored by a Berthold TLC-Linear Analyzer LB 282 equipped with an integrator.

## Results and Discussion

### *Fatty acid composition of blue light-grown gametophytes*

Analysis of the fatty acid composition of diacylgalactosylglycerol from gametophytes grown under continuous blue light confirmed the previously [11] reported high amount of linoleic acid (Table I). As compared to the fatty acids already present in the spore, it appears that during growth under blue light linoleic acid accumulates while the amount of linolenic acid remains unaltered low. This might be

Table I. Fatty acid composition of diacylgalactosylglycerol from spores and gametophytes of *Anemia phyllitidis* grown at various times under continuous blue light (mol%, mean of three experiments).

| Fatty acid | Spores* | Days under blue light |    |    |
|------------|---------|-----------------------|----|----|
|            |         | 7                     | 13 | 21 |
| 16:0       | 19      | 13                    | 6  | 6  |
| 18:0       | 3       | 9                     | 2  | 1  |
| 18:1       | 27      | 17                    | 17 | 18 |
| 18:2       | 47      | 57                    | 70 | 70 |
| 18:3       | 2       | 3                     | 3  | 2  |
| others     | 1       | 1                     | 2  | 1  |

\* Data from [11].

indicative for a block of linoleic acid desaturase catalyzing the last reaction in linolenic acid biosynthesis. Taking advantage of this finding it is intended to synthesize *in vivo* labeled dilinoylgalactosylglycerol by adding labeled linoleate to gametophytes growing in blue light.

### *Incorporation of [ $^{14}\text{C}$ ]linoleate into lipids*

[ $^{14}\text{C}$ ]Linoleate added to the culture medium was rapidly incorporated into gametophytes, and after 48 h of incubation about 50% of the administered radioactivity was found in the lipid fraction. Incubations for various times revealed a gradual movement of radioactivity from free fatty acids to diacylglycerol to triacylglycerol and to the phospholipids (Fig. 1). Label associated with diacylgalactosylglycerol occurred 3 h after feeding and steadily increased with increasing times of incubation. Labeled diacyldigalactosylglycerol could be detected 24 h after feeding. From these data it appears that a 48 h incubation time is optimal for high yield labeling of diacylgalactosylglycerol.

To determine whether the developmental stage of the gametophytes might play a role, gametophytes of various ages were incorporated for 48 h. The results (Table II) show that in young cultures the amount of total radioactivity is low and preferential labeling of phospholipids occurs. With time the level of radioactivity in the lipid fraction increases gradually and diacylgalactosylglycerol becomes the major labeled lipid. The highest specific activity was found at day 13. Thus, for further high yield preparation cultures of 11–13 days are appropriate.

Table II. Incorporation of [ $^{14}\text{C}$ ]linoleate into lipids by gametophytes grown for various times under blue light.

| Days of culture | dpm $\times 10^3$<br>Total lipids | Distribution of label in lipids<br>% of total |    |     |     |    |    |     |
|-----------------|-----------------------------------|-----------------------------------------------|----|-----|-----|----|----|-----|
|                 |                                   | PC                                            | PE | MGD | DGD | TG | DG | FFA |
| 6               | 53                                | 30                                            | 25 | 14  | 4   | 19 | 8  | 1   |
| 9               | 86                                | 19                                            | 13 | 30  | 5   | 25 | 7  | 1   |
| 11              | 88                                | 19                                            | 14 | 29  | 5   | 26 | 6  | 1   |
| 13              | 91                                | 22                                            | 14 | 29  | 5   | 22 | 4  | 1   |
| 16              | 89                                | 23                                            | 17 | 28  | 4   | 23 | 4  | 1   |

Note. Incubation time was 48 h.

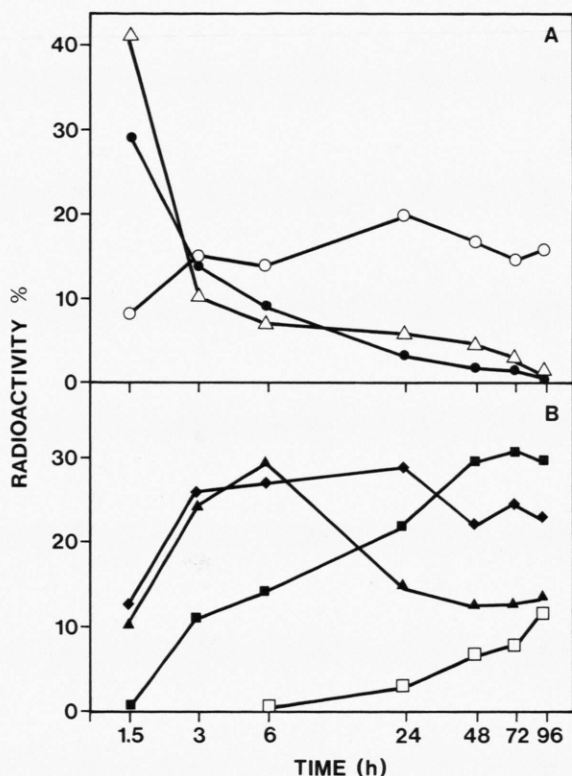


Fig. 1. Incorporation of [ $^{14}\text{C}$ ]linoleate into lipids by gametophytes of *Anemia phyllitidis* grown for 13 days under continuous blue light. A, neutral lipids; B, polar lipids.  $\triangle$  Diacylglycerol,  $\circ$  triacylglycerol,  $\bullet$  free fatty acid,  $\blacklozenge$  phosphatidylcholine,  $\blacktriangle$  phosphatidylethanolamine,  $\blacksquare$  diacylgalactosylglycerol,  $\square$  diacyldigalactosylglycerol.

#### Improvement of labeling rate of diacylgalactosylglycerol

Because about the half of the initial label was lost mainly by degradation, experiments were performed

to improve the labeling rate of diacylgalactosylglycerol. Several inhibitors of lipoxygenase were assayed and cerulenin was applied to inhibit *de novo* synthesis of fatty acids [16] but no promotive effect resulted. Only growth under anaerobic conditions had some positive effects. Thus, in order to inhibit the aerobic reaction of lipoxygenase [17], gametophytes were kept for various times under nitrogen atmosphere during 48 h incubation. It was found that 2 h under anaerobic conditions at the beginning of the incubation increased the label in diacylgalactosylglycerol to 40–50%, longer times, however, resulted in a marked increase in labeled triacylglycerol concomitant with a decrease in labeled diacylgalactosylglycerol. Summarizing, it resulted that optimal yield can be obtained by incubating 11–13 days old gametophytes with [ $^{14}\text{C}$ ]linoleate first in the presence of nitrogen for 2 h followed by 46 h under aerobic conditions. Under these conditions about 14% of the administered  $^{14}\text{C}$  was incorporated into diacylgalactosylglycerol and a specific activity of 167 MBq/mmol was obtained. Likewise, the specific activities found for the other lipids were 203 MBq/mmol for phosphatidylcholine, 160 MBq/mmol for phosphatidylethanolamine, 39 MBq/mmol for diacyldigalactosylglycerol, and 29 MBq/mmol for phosphatidylglycerol, respectively.

#### Analysis of the product

Radio-gas chromatographic analysis of the distribution of labeled fatty acids among lipid classes indicated that radioactivity was confined solely to linoleic acid (Table III). Treatment of diacylgalactosylglycerol with *Rhizopus* lipase and positional specific analysis showed an approximately equal distribution of fatty acids at position C-1 and C-2 of the

Table III. Fatty acid composition of major polar lipids (% of total) and distribution of label from [ $^{14}\text{C}$ ]linoleate.

| Fatty acid | MGD   |                   |     |     | PC |                   | PE |                   |
|------------|-------|-------------------|-----|-----|----|-------------------|----|-------------------|
|            | total | % $^{14}\text{C}$ | C-1 | C-2 | %  | % $^{14}\text{C}$ | %  | % $^{14}\text{C}$ |
| 16:0       | 8     | 0                 | 13  | 6   | 27 | 0                 | 25 | 0                 |
| 18:0       | 2     | 0                 | 8   | 4   | 3  | 0                 | 4  | 0                 |
| 18:1       | 17    | 0                 | 20  | 20  | 27 | 0                 | 19 | 0                 |
| 18:2       | 64    | 100               | 47  | 64  | 40 | 100               | 44 | 100               |
| 18:3       | 7     | 0                 | 8   | 4   | 1  | 0                 | 0  | 0                 |
| others     | 3     | 0                 | 4   | 2   | 3  | 0                 | 8  | 0                 |

Note. Incubation time was 48 h, 2 h nitrogen atmosphere. C-1, C-2; position of the glycerol backbone.

glycerol backbone, with a slight preference of linoleic acid to C-2 (Table IV). The radioactive linoleic acid, however, was found esterified at equal proportions at C-1 and C-2. The fact that the separa-

Table IV. Stereospecific distribution of fatty acids and label at the glycerol backbone of diacylgalactosylglycerol (%).

| Fatty acid | C-1 |                 | C-2 |                 |
|------------|-----|-----------------|-----|-----------------|
|            | %   | $^{14}\text{C}$ | %   | $^{14}\text{C}$ |
| 16:0       | 13  | 0               | 6   | 0               |
| 18:0       | 8   | 0               | 4   | 0               |
| 18:1       | 20  | 0               | 20  | 0               |
| 18:2       | 47  | 100             | 64  | 100             |
| 18:3       | 8   | 0               | 4   | 0               |
| others     | 4   | 0               | 2   | 0               |

tion of molecular species by argentation chromatography yielded one labeled spot further confirms the synthesized molecule to be dilinoylgalactosylglycerol.

In summary, it was clearly shown that fern gametophytes provide an useful system for preparation of radioactive labeled lipids, in particular of diacylgalactosylglycerol. The described *in vivo* method is superior to the chemical synthesis and the semisynthetic method [18] in producing radioactively labeled substrates of relatively high specific activity at reasonable costs and in short time. In contrast to other systems [4, 19, 20] which obtained only 2–5% incorporation of linoleate into diacylgalactosylglycerol, with the fern system reported here as much as 14% incorporation were attained. One major reason for this high yield may be that the desired substrate represents an end product in lipid metabolism, in particular under the applied conditions.

Considering recent proposals for the biosynthesis of linolenic acid [2, 3] our results are consistent with the eukaryotic pathway [3]. It is hoped that our product can be utilized for *in vitro* assays to clarify the question whether dilinoylgalactosylglycerol represents a substrate for the n-3-desaturase.

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