Comparison of Lipid Biosynthesis of Normal and Dark Kept Spinach Leaves in Photosynthetically Active Light

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Lipid Biosynthesis, Dark Starvation

Upon illumination, dark starved spinach leaves show a 50% reduction of ^{14}C-incorporation capacity into lipids. If ^{32}P-incorporation is measured, the reduction is 85% compared to controls. The relative labelling of the fatty acid residues in total lipids of normal leaves was 80–90% compared to 30–50% in the darkened material. The kinetics of ^{14}C-incorporation into lipids show that in the controls the majority of ^{14}C was located in the monogalactosyl diglyceride fraction while in the dark kept material phosphatidyl glycerol and phosphatidyl choline were more highly labelled. Corresponding with the changed incorporation capacity a decrease in fatty acid concentration in the glycolipid fraction and a simultaneous increase in the phosphatide fraction in dark pretreated leaves is observed. An illumination period of 30–60 minutes at 2×10^4 lx brings these inverse values back to normal. The most rapid labelling occurred in a glycolipid fraction lacking galactose and having a slightly more hydrophilic character than monogalactosyl diglyceride. The molar ratio of sugar, glycerol and fatty acids in this fraction is 1 : 1 : 1–2. The label in the fatty acid residues of this lipid fraction was approximately 10% in dark kept but 90% in control leaves. From ^{14}C-incorporation behavior it is suggested that this lipid is a precursor of monogalactosyl diglyceride. The changes of the labelling pattern of lipids in leaves kept in prolonged darkness are similar to those obtained with 3-(3,4-dichlorophenyl)-1,1-dimethylurea treated *Chlorella*. A close connection of these effects to the decrease of photosynthetic capacity is therefore suspected. Also the observation that the concentration of α-linolenic acid, bound in the glycolipids, is reduced in dark kept material and increases upon subsequent light exposure, fits into this context. A possible explanation of these labelling shifts seems to be a pH shift in the various cell-compartments induced by light/dark treatment.

Introduction

A considerable turnover of lipids has been observed through studies of diurnal periodic changes in their pool concentrations in spinach leaves [1].

Fluctuations have also been observed in the concentration of phospholipids in hypocotyl hooks of *Phaseolus* [2]. These were accompanied, however, by linear incorporation of ^{32}P into the same phosphatide fraction. We analyzed such changes in lipid composition of dark-starved leaves. Dark treatment of spinach leads to a decreased ability for incorporation of $^{14}CO_2$, a decline of malate and aspartate labelling [3] and other differences in photosynthetic capacity [4]. Changes in the lipid composition of dark starved leaves and in the pattern of ^{14}C-incorporation into their lipids during CO_2 assimilation should give some information about the importance of lipids in light dependent membrane changes.

Abbreviations: ACP, acyl carrier protein; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DGDG, digalactosyl diglyceride; FFA, free fatty acids; GGL and GL, unidentified glycolipids, characteristics see Table I; LPC, lysophosphatidyl choline; MGDG, monogalactosyl diglyceride; NL, neutral lipid; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PEP, phosphoenolpyruvate; PGA, phosphoglyceric acid; PG*, phosphatidylglycerol with traces of other acid phosphatides and SL; POPOP, 2,2′-*p*-phenylenbis-(5-phenyloxazol); PPO, 2,5-diphenyloxazol; PA, phosphatidic acid; SL, sulphoquinovosyl diglyceride; UDP, uridine diphosphate.

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Material and Methods

Spinach plants were grown under constant conditions as described previously [1].

8–10 weeks old plants were transferred into a dark room and kept at 18 °C and a relative humidity of 60% for 4–5 days.

For the direct analysis the leaves were frozen in liquid nitrogen, ground in a mortar and extracted according to Bligh and Dyer [5].

$^{14}CO_2$-fixation was measured with leaf sections whose lower epidermis had been removed. They were vacuum infiltrated with 0.33 M sorbitol, 42 mM Hepes (pH 7.2 or pH 8.4) and subsequently illuminated for 15 minutes. In some experiments 42 mM MES, pH 6.3 was used. In the next step they were reincubated in the same medium con-

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taining 18 mM $H^{14}CO_3^-$ (spec. activity 1.1 μCi/μmol) and illuminated with white light of 1.2×10^5 ergs $\times$ cm^{-2} $\times$ sec^{-1} at 20 °C. Samples were removed after 2, 5, 15, and 30 minutes. In pulse-chase experiments the samples were immediately after a pulse of two minutes washed free of label using the same buffer medium and illuminated under the same conditions in the presence of 18 mM HCO_3^- for the time indicated. After illumination the samples were immediately frozen in liquid N_2 and analysed according to Krapf [3]. Lipid extraction and analysis is described by Heise and Jacobi [6].

^{32}P incorporation was measured with leaf sections which were incubated 5 h in a medium of 0.33 M sorbitol – 42 mM MES pH 6.3 – 1 mM $^{32}PO_4^{3-}$ (1 μmol = 2.163×10^6 cpm), washed afterwards with 0.33 M sorbitol – 42 mM Hepes, pH 7.8, illuminated in the presence of 18 mM HCO_3^- for the time indicated, and analysed as described above.

Results

The main pool of fatty acids in normal spinach leaves was localized in the MGDG-fraction, whereas in dark starved spinach leaves the relative distribution of lipid bound fatty acids was significantly displaced in favour of PC (Table I). The same effect was observed for the main fatty acids of these

Table I. Relative distribution of fatty acids between the different lipid classes of 5 days darkened spinach leaves and its change after subsequent illumination at 2×10^4 lx.

Lipid		Fatty acids	Control	Dark pretreated leaves Time of illumination [min] 0′	15′	30′	60′
NL	(R_F: 0.6–1.0)		0.3	0.7	0.4	0.3	0.3
MGDG	(R_F: 0.56)	$C_{16:3}$	10.3	5.1	10.7	10.4	8.4
		$C_{18:3}$	27.5	15.3	32.6	30.4	24.3
		Σ	39.0	20.8	44.1	41.3	33.0
GL*	(R_F: 0.44)	Σ	0.6	0.3	0.8	0.3	1.1
GGL**	(R_F: 0.30)	Σ	1.2	0.2	0.7	1.4	1.3
DGDG	(R_F: 0.25)	$C_{16:3}$	0.7	0.5	0.5	0.6	1.2
		$C_{18:3}$	13.5	10.9	12.9	13.2	17.3
		Σ	16.8	13.1	15.0	15.9	20.9
SL+PG	(R_F: 0.1–0.15)	$C_{16:0}$	5.8	4.4	5.6	5.4	5.4
		$C_{16:1(3t)}$	2.5	1.3	2.4	2.3	1.8
		$C_{18:2}$	3.9	3.4	2.8	3.4	4.1
		$C_{18:3}$	8.0	6.7	9.4	9.1	8.0
		Σ	21.6	17.3	21.5	21.5	21.0
PC	(R_F: 0.02)	$C_{16:0}$	3.3	6.7	2.9	2.9	3.2
		$C_{18:1}$	4.1	4.4	1.5	1.5	2.3
		$C_{18:2}$	6.3	12.0	3.8	4.4	5.5
		$C_{18:3}$	6.5	22.7	8.9	9.9	10.9
		Σ	20.5	47.6	17.5	19.3	22.4

The total amount of free and lipid bound fatty acids was taken as 100%. From fatty acid patterns of the individual lipids only the dominant ones are listed. The data are based on three experiments. The lipids were separated on silica gel G plates [solvent system: acetone–benzene–H_2O (91 : 30 : 8 v/v)]. GL* and GGL** presumably are glycolipids which lack galactose. They are composed of sugar, glycerol and fatty acids in a molar ratio of 1 : 1 : 1–2 for GL and 2 : 1 : 1–2 for GGL respectively. Relative fatty acid patterns [%]:

	$C_{14:0}$	$C_{16:0}$	$C_{16:3}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
GL	2–5	4– 9	13–26	1–4	3– 7	1– 2	56–72
GGL	1–3	8–14	1– 3	1–2	12–18	26–32	34–50

fats, especially for α-linolenic acid. This inverse fatty acid distribution between glyco- and phospholipids could be compensated for by illumination of dark kept leaves (Table I). During this illumination period a relative increase of fatty acid concentration, especially that of α-$C_{18:3}$, in the glycolipid fractions and a simultaneous decrease in PC was measured. In spite of its relative low content in spinach leaves, also the light dependent increase in fatty acid content of GL, an unidentified glycolipid fraction (see Table I), seems to be of interest for glycolipid synthesis. This was derived from its labelling pattern in pulse-chase experiments (Fig. 2). Simultaneously the fatty acid concentration in the neutral lipid fraction of dark starved leaves decreased after illumination.

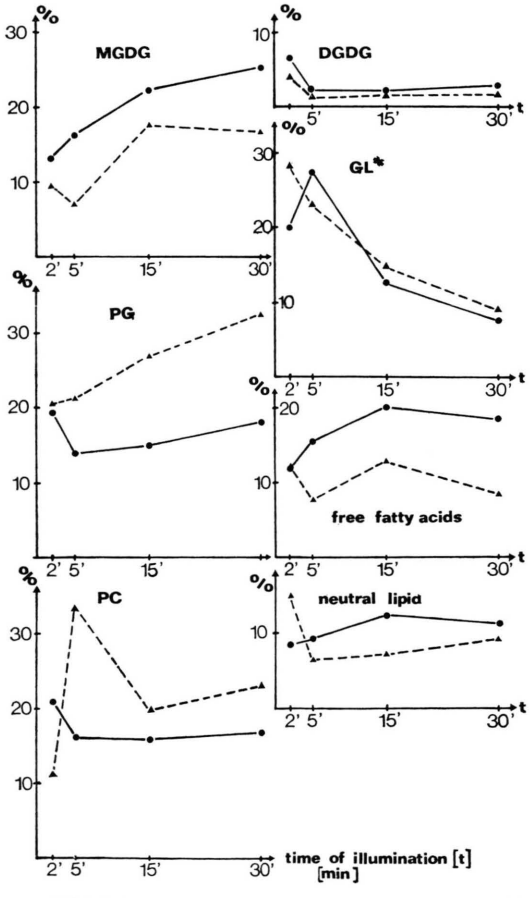

Fig. 1. ^{14}C-labelling kinetics in the lipids of normal (—●—) and four days darkened (—▲——▲—) spinach leaves at pH 7.2 in relation to the time of illumination. The sum of the incorporated ^{14}C of the analyzed lipids was taken as 100%. The data are mean values of four experiments. * GL see Table I.

Table II. Relative amount of ^{14}C-incorporation into the fatty acid fraction of lipids.

	Control leaves [%]	Dark pretreated leaves [%]
neutral lipids + free fatty acids	75—95	70—90
MGDG	25—70	25—60
GL*	90—95	6—12
DGDG	10—40	10—30
phosphatides	50—80	30—50
total lipids	80—90	30—50

The amount of ^{14}C in the entire molecule of the lipid investigated is arbitrarily set as 100%. The data are based on three experiments.

Table III. Comparison of relative ^{14}C-uptake into the lipids of leaf sections of normal (a) and dark treated (b) spinach in relation to illumination time and pH of incubation medium.

		Time of fixation in the light [min]			
		a		b	
	pH	5′	30′	5′	30′
free fatty acids (FFA)	6.3	11.1	11.8	4.7	5.4
	7.2	15.4	18.4	7.7	8.4
	8.4	17.6	22.1	10.9	14.3
neutral lipids	6.3	9.0	10.4	8.5	5.1
	7.2	9.1	11.2	6.6	9.1
	8.4	10.1	12.0	7.4	15.3
MGDG	6.3	12.4	27.2	6.7	14.2
	7.2	16.2	25.3	7.0	16.7
	8.4	15.8	21.9	6.3	16.8
GL	6.3	23.5	6.7	36.2	7.1
	7.2	27.2	7.5	23.0	9.0
	8.4	31.4	16.6	16.7	18.4
DGDG	6.3	0.9	1.3	0.8	1.2
	7.2	2.2	2.9	1.3	1.6
	8.4	2.1	1.3	2.6	1.5
acid phosphatides (PG)*	6.3	8.4	13.9	14.9	33.8
	7.2	13.8	18.0	21.1	32.3
	8.4	4.1	9.7	17.2	15.4
PC	6.3	34.7	28.7	28.2	33.2
	7.2	16.1	16.7	33.3	22.9
	8.4	18.9	16.4	38.9	18.3

* The acid phosphatide fraction contained mostly phosphatidyl glycerol (PG).
In order to overcome the lag in the rate of CO_2 assimilation, the leaf sections were freed of the lower epidermis, infiltrated with the incubation medium (absence of $H^{14}CO_3^-$) and preilluminated for 15 min.
The sum of the incorporated ^{14}C was taken as 100%.
The data are based on three experiments.

A short incubation of control leaf sections with $H^{14}CO_3^-$ in light leads to the appearance of approximately 2% of the label in the lipid soluble phase. Similar results were reported by Murphy and Leech[7] for $^{14}CO_2$-incorporation into leaves still attached to spinach plants. In contrast, the rate of label incorporation into lipids in dark kept material is only half of that or about 1%. For comparison of the light driven ^{14}C-incorporation kinetics in control leaves and leaves kept in darkness for four days the material was preilluminated for 15 min to overcome the lag in CO_2 assimilation. The following differences were observed (Fig. 1, Tables II and III) under these conditions:

1. The relative label of the fatty acid residues in total lipids of control leaves was much higher than that of the darkened material (Table II).
2. The most rapidly labelled product was always the glycolipid fraction GL. This lipid fraction shows, however, a significant difference in amount of incorporated label between normal and dark kept leaf sections. In normal leaves, GL was mainly labelled in the fatty acid residues (90 – 95%, Table II), in dark treated material in the glycerol and sugar residues (90%).
3. In control leaf sections the relative ^{14}C-incorporation into MGDG and free fatty acids dominated showing a linear increase and saturation in its kinetics. In dark kept leaves, on the other hand, a relatively higher label was observed in PC and PG, again predominantly in the polar moieties of these molecules (Table II).
4. The following pattern of ^{14}C-incorporation was determined from the degree of labelling (Fig. 1 and Table III):
   a) in control leaf sections (5 min illumination): GL > PC > MGDG = FFA > NF > PG* > DGDG;
   after 30 min illumination:
   MGDG > PC > FFA > PG* > NF > GL > DGDG;
   b) in dark kept leaf sections (5 min illumination):
   PC > GL > PG* > FFA = NF > MGDG > DGDG;
   after 30 min light:
   PG* > PC > MGDG > GL > NF = FFA > DGDG.
5. The rate of incorporation was dependent on the pH of the incubation medium. This pH dependence manifests itself as an increased ^{14}C-label in the free fatty acids (FFA) under alkaline conditions. Incorporation into PC and GL shows an inverse behaviour with shifting pH (Table III).

From opposite ^{14}C-incorporation behavior of MGDG and GL in pulse-chase experiments (Fig. 2) a precursor function of GL for MGDG-synthesis is suggested. The relative amount of GL was very small (Table I). Therefore the high ^{14}C-incorporation rates after short illumination periods point to a high specific activity and an intensive turnover in this lipid fraction.

The light induced ^{32}P-incorporation in dark kept leaves was reduced to approximately 85% compared to that of the control (Table IV) confirming the decrease of ^{14}C-label in phosphatides.

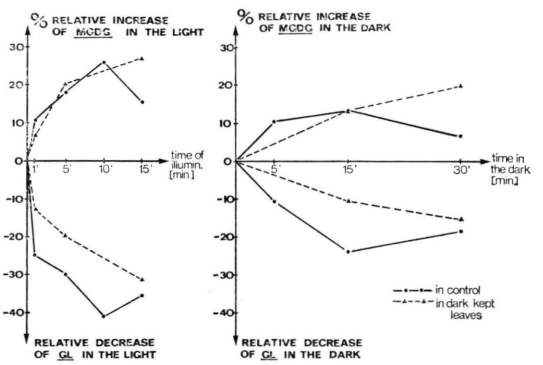

Fig. 2. Relative variation of ^{14}C-incorporation in MGDG- and GL-fractions of normal (—●——●) and dark starved (—▲——▲—) spinach leaves after a ^{14}C-pulse of 2 min in the light at pH 7.8. The ^{14}C-incorporation into the total lipid fraction was taken as 100%. The ^{14}C-label after the pulse of 2 min was arbitrarily set as starting point (○) of the incorporation kinetics. The data are mean values of two experiments.

Table IV. ^{32}P-incorporation into the phosphatides in sections of normal (a) and dark treated (b) spinach leaves.

	cpm × 10^5 / g fresh weight					
	after 5 h of ^{32}P-incorporation in the dark		Time of CO_2 assimilation [min] in the light (absence of ^{32}P)			
			2′		15′	
	a	b	a	b	a	b
PG	10.6	1.5	16.2	2.6	26.4	3.3
PC	6.4	1.2	9.6	1.4	12.8	2.0
total phosphatides	17.0	2.7	25.8	4.0	39.2	5.3

The data are mean values of two experiments.

Discussion

The following considerations point out the difficulties for an interpretation of the observed data.

- Lipids and their site of synthesis are found in various compartments.
- Lipids and their subcomponents can be transferred between the different cell organelles [8, 9].
- ^{14}C is incorporated into the subcomponents of complex lipid molecules with different rates [1, 7, 10, 11].

The different fluctuations of label in the polar and apolar subcomponents of lipids in spinach leaves correspond to the last notion. An "acyl-transfer" between different lipids during the daily light/dark cycle was observed [1]. A connection between the stimulated ^{14}C-incorporation into the polar moieties of the lipids on the one hand and into the sucrose molecule on the other [3] in dark kept spinach leaves is suggested. GL (Table I) shows the ^{14}C-incorporation kinetics of a precursor (Fig. 1). Since opposite ^{14}C-incorporation kinetics are found between MGDG and GL in pulse-chase experiments (Fig. 2), GL seems to be a direct precursor of MGDG. In the controls, GL shows already after short illumination the highest label especially under alkaline conditions (Table III).

The decreased light-dependent pool size in fatty acid content of MGDG, free fatty acids and neutral lipids as well as the ^{14}C-incorporation into these lipids of dark kept leaves is possibly due to changes in the photosynthetic apparatus of chloroplasts. Similar results were described by Sumida *et al.* [12] after poisoning of photosynthesis with DCMU. Nakamura and Yamada [13] also reported the inhibition of fatty acid synthesis in the light by DCMU.

Poisoning of electron transport by DCMU should inhibit the function of stearyl-ACP-desaturase. This enzyme, being responsible for the introduction of the first cis-double bond into the hydrocarbon chain of fatty acids at the 9,10-positions, requires two electrons for the activation of molecular oxygen. According to Stumpf [14] these electrons are obtained from reduced ferredoxin via photosystem I.

According to Douce [15], galactolipid synthesis is localized in the chloroplast envelope. Joyard and Douce [16] gave direct evidence that isolated fractions of the chloroplast envelope are able to synthesize MGDG very rapidly by galactosylation of a large endogenous pool of diglycerides. The main activity of fatty acid synthesis (about 87%) is supposed to be located in the chloroplast [17]. The authors suggested from their data the following sequence of events:

PGA → PEP → pyruvate → acetyl-CoA → FFA.

Spinach leaves kept in prolonged darkness showed a shift in their main pool of lipid-bound fatty acids from glyco- to phospholipids (Table I). Additionally, a relative increase of ^{14}C-incorporation into the phosphatide fraction as well as an increased level of fatty acids in the glycolipid fractions of dark starved leaves was observed during short term photosynthesis. This increase of glycolipid-bound fatty acids was obviously connected with an increase of those fatty acids, showing a higher degree of desaturation.

Sumida *et al.* [12] reported that light dependent incorporation of ^{14}C-acetate in *Chlorella* favours PG and PE in DCMU treated specimen. This suggests an intimate correlation between changes of photosynthetic capacity and the ^{14}C-incorporation pattern of lipids in dark treated spinach leaves. The site of phosphatide synthesis is supposed to be located in the mitochondrial and microsomal fraction [9, 16]. Short term ^{14}C-incorporation takes place mainly in the fatty acid chains of these lipids [1, 7, 11]. Therefore a direct "transfer" of fatty acids to precursors of phosphatides, for example the lyso compounds of these lipids, is suggested to take place outside of the chloroplast. This correlates with the light dependent formation of lyso-PC (LPC) in isolated chloroplasts[18]. As shown in Fig. 1, next to GL the most rapidly labelled lipid in darkened material during short illumination (5 min) was phosphatidyl choline. The fatty acid moieties were the main site of label [1]. As suggested by some authors the chloroplast is not able to desaturate completely the galactolipid-bound fatty acids [7, 11, 19]. These authors attribute a possible "carrier" function to PC for fatty acid synthesis of MGDG and DGDG.

On the basis of our experiments we propose an additional "carrier" function for GL, serving as precursor for MGDG. This is concluded from the following observations:

1. The relatively high specific activity in the fatty acid fraction of GL after short illumination periods (Fig. 1, Tables II and III) under ^{14}C-fixating conditions.
2. The following decrease of this specific activity under continued illumination in favour of increasing ^{14}C-incorporation into MGDG (Fig. 2).

3. This decrease was reduced, if the ^{14}C-pulse was followed by a dark period.
4. The ^{14}C-incorporation into the fatty acid fraction of GL in dark kept leaves (Table II) was significantly reduced.

A possible explanation for the differences in the fixation pattern of complex lipids between dark and light kept leaf materials are pH-shifts in the different cell compartments (Table III).

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