# In Situ Incorporation of Fatty Acids into Lipids of the Outer and Inner Envelope Membranes of Pea Chloroplasts

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## ABSTRACT

When incubated with [1-$^{14}$C]acetate and cofactors (ATP, Coenzyme A, sn-glycerol-3-phosphate, UDPgalactose, and NADH), intact chloroplasts synthesized fatty acids that were subsequently incorporated into most of the lipid classes. To study lipid synthesis at the chloroplast envelope membrane level, $^{14}$C-labeled pea (Pisum sativum) chloroplasts were subfractionated using a single flotation gradient. The different envelope membrane fractions were characterized by their density, lipid and polypeptide composition, and the localization of enzymic activities (UDPgalactose-1,2 diacylglycerol galactosyltransferase, Mg$^{2+}$-dependent ATPase). They were identified as very pure outer membranes (light fraction) and strongly enriched inner membranes (heavy fraction). A fraction of intermediate density, which probably contained double membranes, was also isolated. Labeled glycerolipids recovered in the inner envelope membrane were phosphatidic acid, phosphatidylglycerol, 1,2 diacylglycerol, and monogalactosyldiacylglycerol. Their $^{14}$C-fatty acid composition indicated that a biosynthetic pathway similar to the prokaryotic pathway present in cyanobacteria occurred in the inner membrane. In the outer membrane, phosphatidylcholine was the most labeled glycerolipid. Phosphatidic acid, phosphatidylglycerol, 1,2 diacylglycerol, and monogalactosyldiacylglycerol were also labeled. The $^{14}$C-fatty acid composition of these lipids showed a higher proportion of oleate than palmitate. This labeling, different from that of the inner membrane, could result either from transacylation activities or from a biosynthetic pathway not yet described in pea and occurring partly in the outer chloroplast envelope membrane. This metabolism would work on an oleate-rich pool of fatty acids, possibly due to the export of oleate from chloroplast toward the extrachloroplastic medium. The respective roles of each membrane for chloroplast lipid synthesis are emphasized.

For almost 20 years, a number of studies have underlined the important role of the chloroplast envelope in lipid metabolism of photosynthetic cells (8, 18). Recently, developed techniques have allowed the isolation of i.e.m.[3] and o.e.m. from pea (12) and spinach chloroplasts (7). These have been further used to demonstrate the localization of several enzymic activities in the membranes of both plants (1–4, 7, 13, 27, 33, 35). The following enzymic activities dealing with lipid metabolism have been localized on the i.e.m.: (a) hydrolysis of the acyl-CoA or ACP thioesters (30, 36, 39); (b) acylation of 1-oleoyl-lysophosphatidic acid by palmitoyl-ACP (22); (c) dephosphorylation of phosphatidic acid to form DAG (23); (d) desaturation of oleoyl groups (42); (e) synthesis of glycolipids by either de novo galactosylation of DAG or transfer of galactose from one molecule of glycolipid to another (16, 44); and (f) synthesis of phosphatidylglycerol from phosphatidic acid with CDP-diacylglycerol as intermediate (3). On the other hand, galactolipid-galactolipid galactosyltransferase (27) and acyl-CoA synthetase (2) activities have been evidenced in the o.e.m. In contrast with the results obtained in spinach, the de novo galactosyltransferase activity was also evidenced in o.e.m. in pea (13).

However, these enzyme activities are not solely responsible for the synthesis of all chloroplast lipids. Taking into account the specificities of the molecular species of lipids (i.e. nature of the fatty acids esterified at positions 1 and 2 of the glycerol), one can classify the chloroplast lipids into two groups. The so-called "prokaryotic" lipids have a 1-$C_{18}$-2-$C_{16}$ fatty acid arrangement on carbons 1 and 2 of the glycerol, respectively. This is analogous to the structural arrangement of fatty acids in cyanobacterial lipids. PG, SQDG, and a variable proportion of MGDG, according to the plant considered, are the most representative of these prokaryotic lipids. They are entirely synthesized by the enzymes that have been localized in the i.e.m. (see above). The "eukaryotic" lipids have a 1-$C_{18}$-2-$C_{18}$ composition. This group consists of DGDG, the counter part of MGDG, and PC. Along with PC and DGDG, lipids present in the extrachloroplastic membranes (ER, mitochondria) are eukaryotic in composition. The eukaryotic lipids in chloroplasts were not found to be synthesized in chloroplasts, and it was subsequently proposed that the 1-$C_{18}$-2-$C_{18}$ backbones are imported from the ER (8). In such a scheme, both membranes of the chloroplast enve-

[3] Abbreviations: i.e.m., inner envelope membranes; o.e.m., outer envelope membranes; ACP, acyl carrier protein; G-3-P, sn-glycerol-3-phosphate; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; DAG, 1,2 diacylglycerol; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; triGDG, trigalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol.

lope would be involved in the lipid synthesis for the chloroplast, with the i.e.m. responsible for the prokaryotic pathway and the o.e.m. for the import of eukaryotic lipids from the ER. To clarify the nomenclature, it must be noted that because of their typical unsaturated fatty acid composition, comprising C18:3 and C16:3, plants whose chloroplast lipids are mostly prokaryotic, like spinach (i.e. containing a large proportion of prokaryotic MGDG), are called 16:3-plants. Conversely, those with almost exclusive eukaryotic MGDG, e.g. pea, which only contain C18:3 as triunsaturated fatty acid, are often called 18:3-plants.

In the present work, we aim to obtain a more complete understanding of chloroplast lipid biogenesis. We have investigated in organello the biosynthetic capacities of the inner and outer envelope membranes of pea chloroplasts fed with [1-$^{14}$C]acetate. We then looked for the incorporation of these newly synthesized fatty acids into each lipid class of the envelope membranes. Because we needed accurate and rapid techniques to isolate the envelope membranes after the labeling experiments, we used a single flotation gradient to fractionate the chloroplast envelope instead of two successive gradients as described by Cline et al. (12). For the metabolic studies, we incubated intact chloroplasts with [1-$^{14}$C]acetate along with other cofactors and precursors. Thus, fatty acids synthesised in situ in the chloroplasts, mainly oleic and palmitic acids (41, 43), were labeled. Finally, we analyzed the incorporation of the newly synthesized, labeled fatty acids into lipids of chloroplast envelope membranes.

The isolation method carefully separated the envelope membranes, as demonstrated by reference to the expected lipid fatty acid composition. The membranes incorporated labeled fatty acids into most of their glycerolipids, with a composition that reflected the pool of available fatty acids for each membrane. This allows us to discuss the utility of the modified technique and the respective roles of the envelope membranes in chloroplast lipid synthesis.

## MATERIALS AND METHODS

### Materials

[1-$^{14}$C]Acetate (1,998 MBq mmol$^{-1}$) and UDP-[$^{14}$C]galactose (11,433 MBq mmol$^{-1}$) were purchased from CEA (France). ATP, CoA, G-3-P (all disodium salts), UDPgalactose (sodium salt), and NADH were purchased from Sigma Chemical Co. All other chemicals were reagent grade. TLC plates (Kieselgel 60) were purchased from Merck.

### Isolation and Purification of Chloroplasts

Chloroplasts were isolated from 9-d-old pea seedlings (*Pisum sativum* var Petit Provençal; Vilmorin, France). Pea shoots (600 g) were homogenized in 2.4 L of medium A (330 mM sorbitol; 50 mM Tricine-KOH, pH 7.9; 1 mM EDTA; 1 mM MgCl$_2$; 0.1% BSA). All operations were carried out at 4°C. The homogenate was filtered through eight layers of cheesecloth and two layers of nylon cloth. Chloroplasts were spun down at 1000g for 1 min (International B-20, 6 × 250 mL rotor). Each pellet was resuspended in 10 mL of medium A and layered over 14 mL of 40% Percoll (v/v; Pharmacia) in medium A. After 2.5 min at 2500g (swinging buckets), intact

chloroplasts were pelleted at the bottom of the tube, and broken chloroplasts, which formed a broad band above the pellet, were discarded. Intact chloroplasts were carefully washed three times successively into an excess of medium A without BSA and recovered by pelleting at 2000g for 7 min. Photosynthetic oxygen evolution dependent on ferricyanide reduction (31) indicated that chloroplast intactness ranged from 86 to 95%. The absence of enzymic activities such as choline phosphotransferase (EC 2.7.8.2), ethanolamine phosphotransferase (EC 2.7.8.1), NADH dehydrogenase (EC 1.6.99.3), and succinate dehydrogenase (EC 1.3.99.1) indicated that there was no detectable contamination by microsomal or mitochondrial membranes.

### Incorporation of [1-$^{14}$C]Acetate

After purification through a layer of Percoll solution (see above), intact chloroplasts were incubated in the presence of [1-$^{14}$C]acetate in the medium as described by Roughan et al. (40). The 250-$\mu$L reaction mixture contained a volume of chloroplast suspension (250 $\mu$g of Chl), 55 nmol of sodium [1-$^{14}$C]acetate (1998 MBq mmol$^{-1}$), and 1 mM of the following cofactors: ATP, CoA, G-3-P, UDPgalactose, and NADH. Incubations were carried out at 25°C in an illuminated Warburg apparatus with constant shaking (50 strokes·min$^{-1}$) for 5 to 60 min. After each incubation period, 750 $\mu$L of cold (4°C) incubation medium was added and the tubes were centrifuged at 2000g for 1 min. The supernatant was removed and the pellet of chloroplasts suspended in 1 mL of incubation medium. The labeled chloroplasts (250 $\mu$g of Chl) were mixed with nonradioactive purified chloroplasts (750 $\mu$g of Chl) to allow the isolation of the envelope membranes and further analysis.

### Fractionation of Chloroplasts

Purified intact chloroplasts (50–70 mg of Chl) were suspended in 16 mL of 600 mM sucrose in medium B (10 mM Tricine-KOH, pH 7.9, 2 mM EDTA) and incubated on ice at 0°C for 20 min. The suspension was then frozen at −20°C for 1 h and thawed at room temperature. The ferricyanide-dependent oxygen evolution method indicated that more than 60% of the chloroplasts were broken at this step. The molarity of the suspension was adjusted to 1.3 M with a 2 M sucrose solution and poured at the bottom of 34-mL cellulose nitrate tubes (Beckman, No. 0344063). The different membrane fractions were then separated in a flotation gradient that constituted an 11-mL cushion of the chloroplast suspension (about 20 mg of Chl), overlayered by 9, 6, and 3 mL of sucrose solutions, 0.9, 0.6, and 0.3 M, respectively. All solutions were in medium B. The gradients were centrifuged at 73,000g for 16 h at 4°C in a Beckman SW25.1 rotor. Three bands were visible after the centrifugation: (a) an opalescent band, fraction 1 (d = 1.07); (b) a discrete and pale yellow band, fraction 2 (d = 1.09); (c) a yellow band, fraction 3 (d = 1.12); and a pellet of thylakoids and unbroken chloroplasts. The gradient was collected in 0.3-mL fractions using an Isco gradient fractionator and the $A_{280}$ was recorded. The contents of collected tubes corresponding to a single band were pooled. Membrane fractions were then washed in an excess of me-

dium B and pelleted at 100,000g for 1 h. The resulting membrane pellets were suspended in a minimal volume of medium B.

## Analysis of the Lipid Composition

Total membrane lipids were extracted according to Bligh and Dyer (6) and separated by TLC. Polar lipids were chromatographed using the solvent system: chloroform:acetone:methanol:acetic acid:water (50:20:10:10:5, v/v). Neutral lipids were chromatographed using the solvent system petroleum ether (boiling point 60–70°C):diethyl ether:acetic acid (70:30:0.4, v/v). Individual lipids were located under UV light after spraying the TLC plates with 0.001% primulin. Areas containing each radioactive lipid class were located by autoradiography, and then scraped off. Lipids were eluted and radioactivity was measured by scintillation counting of aliquots and corrected for quenching.

Fatty acids from total lipids or individual lipid classes were transmethylated (10) and analyzed by GLC using a fused silica capillary column of PEG 20 M (25 m long, helium as carrier gas). Quantitation was performed using a FID detector and heptadecanoic acid as standard. Radioactive fatty acid methyl esters were prepared from each lipid class as above and analyzed either in a radiogas chromatography system (Barber Colman 500, Nuclear, Chicago) or by TLC on silver nitrate impregnated silica gel plates with the solvent system of petroleum ether (boiling point 60–70°C):diethyl ether:acetic acid (70:30:0.4, v/v). These fatty acid methyl esters were located by autoradiography, scraped off the plates, and counted as above.

## Electrophoresis of Membrane Polypeptides

Polypeptides were analyzed by SDS-PAGE. Electrophoresis was performed at 4°C in gels containing 0.1% SDS with gradients of acrylamide from 7.5 to 15% and sucrose from 5 to 17% (11). Each sample contained 200 μg of protein. Protein bands were visualized by Coomassie blue (Sigma) staining and quantified by densitometry tracing.

## Enzyme Assays

Standard assays for UDPgalactose-1,2 diacylglycerol galactosyltransferase (EC 2.4.1.46) were performed according to Douce and Joyard (17) by the incorporation of UDP-[$^{14}$C] galactose into membrane lipids. The reaction mixture contained: 400 mM UDPgalactose with 0.18 nmol of UDP-[$^{14}$C] galactose (11,433 MBq mmol$^{-1}$); 4 mM MgCl$_2$; 10 mM Tricine-NaOH, pH 7.5; and 50 to 100 μg of protein in a final volume of 400 μL. After 30 min at 25°C, the reaction was stopped by lipid extraction. Mg$^{2+}$-dependent ATPase (EC 3.6.1.3) was measured as described by Nguyen et al. (35). The reaction mixture contained: 300 mM sucrose; 0 to 10 mM MgCl$_2$; 0 to 10 mM ATP; 50 mM Tris-HCl, pH 7.8; and 10 to 15 μg of protein in a final volume of 400 μL. After 15 min at 37°C, 50 μL of 60% TCA were added. The content of Pi in the supernatant fraction was determined after discarding the precipitated proteins (35). Acyl-CoA synthetase (EC 6.2.1.3) was assayed as in Andrews and Keegstra (2).

## EM

Membrane fractions isolated from pea chloroplast envelopes were fixed in 2% glutaraldehyde and 0.3 M sorbitol in 0.2 M phosphate buffer (pH 6.8), postfixed with 1% osmium tetroxide, and embedded in Araldite. Thin sections were stained with uranyl acetate and lead citrate and viewed with a Philips 300 electron microscope.

## Other Assays

Chl content was determined spectrophotometrically in 80% acetone cleared by a 1-min centrifugation at 2000g (5). Protein content was determined by the method of Lowry et al. (32) using BSA as standard.

## RESULTS

### Characterization of the Membrane Fractions Isolated from the Chloroplast Envelopes

The isolation method enabled us to obtain two distinct non-green membrane fractions from pea chloroplast (Fig. 1A). We identified band 1 as o.e.m. and band 3 as primarily i.e.m.s. This identification was based on polypeptide and lipid composition of the bands and localization of enzymic activities. It was in agreement with the data of Cline et al. (12) and Block et al. (7).

Figure 2 shows typical electrophoretic profiles of fractions 1 and 3. More than 30 polypeptides are visible. The polypeptides of 55 and 16 kD, which are abundant in both fractions, are the two subunits of Rubisco. According to Cline and Keegstra (13), the 154- and 76-kD polypeptides and the 104-, 34-, 23.6-kD polypeptides are unique to fractions 1 and 3, respectively, and the degree of cross-contamination between fractions can thus be estimated. Using this technique, we estimate that less than 5% of the proteins in fraction 1 were derived from fraction 3 membranes and about 10% of the proteins in fraction 3 were derived from fraction 1 membranes. A possible explanation for this slight overlap is the existence of zones of junction between the two envelope membranes as previously described by Cline et al. (14). The existence of such junctions could also be the main reason for the presence of band 2 in our gradients (Fig. 1A). The density of band 2 as well as its lipid and polypeptide composition is intermediate between that of the two other envelope membrane fractions. Moreover, electron micrographs of this fraction (Fig. 1B) reveal the presence of thicker, electron-dense zones between membranes that look like the junctions or fused membrane zones observed by Cline et al. (14). Thus, we conclude that band 2 is composed of fragments of envelope or "double membranes" that did not separate from each other during centrifugation.

The lipid composition (Table I) reveals a high content of glycolipids—essentially MGDG and DGDG—in the inner membrane compared to the outer one. The latter is characterized by an abundance of the phospholipid PC. The fatty acid composition (Table II) shows an enhancement in linolenic acid for the i.e.m. when compared to the o.e.m. This enrichment occurs at the expense of palmitic and linoleic acids, which are the two other major fatty acids. The meas-
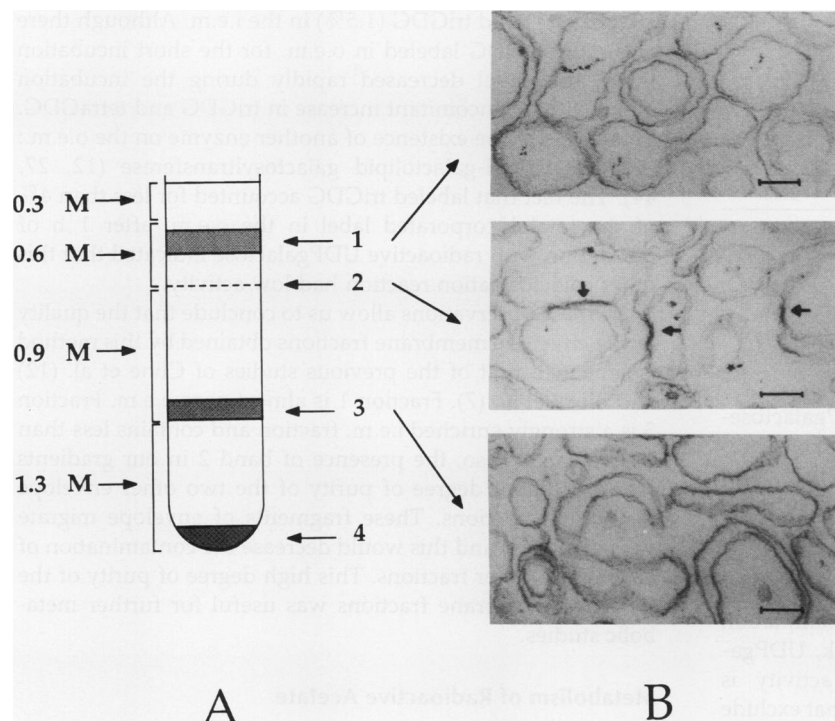
**Figure 1.** Isolation of envelope membrane fractions from pea chloroplasts using a flotation gradient. A, Freeze-thawed chloroplasts (50–70 mg of Chl) were overlayered by sucrose solutions of decreasing density. The gradient was centrifuged at 73,000g for 16 h at 4°C. After centrifugation, three bands were visible above a pellet of unbroken chloroplasts and thylakoids: 1, opalescent band (d = 1.07); 2, pale yellow band (d = 1.09); 3, yellow band (d = 1.12); 4, pellet of unbroken chloroplasts and thylakoids. B, Micrographs of the membrane fractions from pea chloroplast envelopes (fixation with glutaraldehyde-osmic acid; counter staining with uranyl acetate-lead citrate; ×80,000). The electron-dense zones between membranes are indicated by arrows. Bar = 0.14 μm.
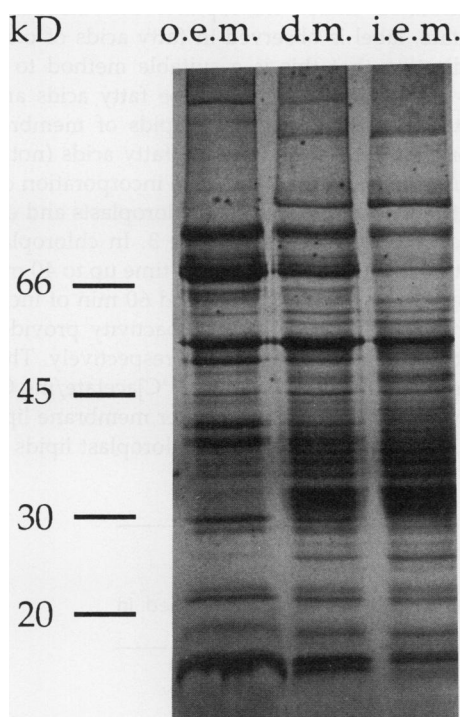


**Figure 2.** Electrophoretic patterns of envelope membrane fractions isolated from pea chloroplasts. The polypeptide composition of the envelope membranes was analyzed by SDS-PAGE. The amount of SDS was 0.1%. The amount of protein for envelope membrane fractions was 200 μg. Molecular mass standards: trypsin inhibitor (20 kD), carbonic anhydrase (30 kD), ovalbumin (45 kD), and BSA (66 kD). d.m., Double membranes.

urement of enzymic activities such as acyl-CoA synthetase and $Mg^{2+}$-dependent ATPase activities (Table III) was in agreement with the results of Andrews and Keegstra (2), Block et al. (7), and McCarthy et al. (33) on pea and spinach chloroplast envelope membranes, which localized these activities on the outer and inner envelope membranes, respectively. The fact that the only ATPase activity found in the o.e.m. is not $Mg^{2+}$-dependent and represents only 9% of the total ATPase activity (35) also confirmed that band 1 was very pure outer membrane.

**Table I.** *Lipid Composition of the Membrane Fractions Isolated from Pea Chloroplast Envelopes*

The membrane fractions were isolated as described in "Materials and Methods." Fraction 1 represents the o.e.m., and fraction 3 the i.e.m. The lipid contents of the fractions 1 and 3 were 3 and 1 mg lipid/mg protein, respectively. The values are expressed as weight percent of the total lipids (mean of three independent experiments).

| Lipid | Fraction 1 | Fraction 3 |
|---|---|---|
| | % | |
| MGDG | 2.5 | 29.8 |
| DGDG | 38.4 | 31.3 |
| SGDG | 6.7 | 5.4 |
| Total glycolipids | 50.6 | 70.7 |
| PC | 33.8 | 11 |
| PG | 4.8 | 5.4 |
| PI | 2.5 | 2.7 |
| PA | 2.8 | 3.8 |
| PE | 0.7 | 0.2 |
| Total phospholipids | 44.6 | 23.1 |
| Polar lipids | 95.2 | 93.8 |
| Neutral lipids | 4.8 | 6.2 |

**Table II.** *Overall Fatty Acid Composition of Membrane Fractions Isolated from Pea Chloroplast Envelopes*

The membrane fractions were isolated and their lipids were analyzed as described in "Materials and Methods."

| | Fatty Acid Composition | | | | | |
| | 16:0 | 16:1 | 18:0 | 18:1 | 18:2 | 18:3 |
|---|---|---|---|---|---|---|
| | | | mol % | | | |
| Fraction 1 | 21.6 | 0.9 | 6.4 | 2.7 | 22.8 | 45.6 |
| Fraction 2 | 18.5 | 0.8 | 5.7 | 2.4 | 20.2 | 52.5 |
| Fraction 3 | 13.0 | 1.7 | 3.5 | 1.9 | 12.1 | 67.9 |

Previous reports on the localization of the UDPgalactose-1,2 diacylglycerol galactosyltransferase activity have presented conflicting data. Cline and Keegstra (13), using pea chloroplasts, have reported its occurrence in the outer membrane, whereas Block et al. (7) and Heemskerk et al. (26) have shown that in spinach chloroplasts this activity is located in the i.e.m. In both cases, the specific activities of the enzyme were corrected for the assumed cross-contamination between membrane fractions. In the present work, UDPgalactose-1,2 diacylglycerol galactosyltransferase activity is present in each membrane with specific activities that exclude the possibility that cross-contamination might be responsible for such a distribution (Table III). Using UDPgalactose concentrations up to 400 $\mu$M, the apparent $K_m$ and $V_{max}$ were 167 $\mu$M and 150 nmol/h$^{-1}$ mg$^{-1}$, respectively, for the o.e.m., and 58 $\mu$M and 526 nmol h$^{-1}$ mg$^{-1}$, respectively, for the i.e.m. These values are close to those found for the spinach chloroplast envelope membranes (7), but differ strongly from the outer membrane values found by Cline and Keegstra (13) in pea chloroplast envelope membranes. Although the highest specific activity was associated with the outer membrane and the activity in the inner could be accounted for by outer membrane contamination, these authors could not be certain of the absence of activity in the inner membrane (13).

Analysis of the different galactolipids synthesized after incubation of each membrane fraction with radioactive UDPgalactose agrees with previous results (26, 27). MGDG was the main galactolipid labeled in all membrane fractions (85% of the radioactivity in the o.e.m., 94% in the i.e.m.). A small amount of [$^{14}$C]galactose was also incorporated into

DGDG (4%) and triGDG (1.5%) in the i.e.m. Although there was more DGDG labeled in o.e.m. for the short incubation times, this label decreased rapidly during the incubation along with a concomitant increase in triGDG and tetraGDG. This is due to the existence of another enzyme on the o.e.m.: the galactolipid-galactolipid galactosyltransferase (12, 27, 44). The fact that labeled triGDG accounted for less than 4% of the total incorporated label in the o.e.m. after 1 h of incubation with radioactive UDPgalactose indicated that this other galactosylation reaction had low activity.

All these observations allow us to conclude that the quality of the envelope membrane fractions obtained by this method is similar to that of the previous studies of Cline et al. (12) and Block et al. (7). Fraction 1 is almost pure o.e.m. Fraction 3 is a strongly enriched i.e.m. fraction and contains less than 10% o.e.m.s. Also, the presence of band 2 in our gradients favors a higher degree of purity of the two other envelope membrane fractions. These fragments of envelope migrate very distinctly, and this would decrease the contamination of each of the other fractions. This high degree of purity of the envelope membrane fractions was useful for further metabolic studies.

## Metabolism of Radioactive Acetate

*Incorporation of [1-$^{14}$C]Acetate into the Lipids of Chloroplasts*

When isolated intact chloroplasts are incubated with [1-$^{14}$C]acetate, label is observed in fatty acids of o.e.m. and i.e.m., indicating that this is a suitable method to observe fatty acid metabolism in vitro. These fatty acids are either incorporated into the constitutive lipids of membranes or, mainly for oleic acid, found as free fatty acids (not shown) as previously demonstrated (19). The incorporation of radioactive acetate into lipids of whole chloroplasts and envelope membranes only is shown in Figure 3. In chloroplasts, the incorporation increased linearly with time up to 40 min, then it decreased slightly. After 20, 40, and 60 min of incubation, 16, 29, and 34% of the initial radioactivity provided were incorporated into chloroplast lipids, respectively. The incorporation at 60 min was 149 nmol [1-$^{14}$C]acetate/mg Chl. The label recovered in the inner and outer membrane lipids was 4.4 and 1.8% of the label in total chloroplast lipids after 60

**Table III.** *Distribution of Enzymic Activities Associated with Chloroplast Envelope in Membrane Fractions*

The membrane fractions were isolated and the enzymic activities measured as described in "Materials and Methods." Activities are expressed as initial velocities.

| Enzyme | Fraction 1 | Fraction 3 | Ratio: Activity in Fraction1/Activity in Fraction 3 |
|---|---|---|---|
| Acyl-CoA synthetase ($\mu$mol [$^{14}$C]oleoyl-CoA formed h$^{-1}$ mg$^{-1}$ protein) | 21.5 | 1.44 | 14.9 |
| Mg$^{2+}$-dependent ATPase ($\mu$mol Pi released h$^{-1}$ mg$^{-1}$ protein) | 0.3 | 6.6 | 0.05 |
| UDP-galactose diacylglycerol galactosyltransferase (nmol [$^{14}$C]galactose incorporated h$^{-1}$ mg$^{-1}$ protein) | 150.0 | 526.3 | 0.29 |

min, respectively. The newly synthesized fatty acids were mainly palmitic and oleic acids. Less than 6% of the label was detected in myristic and linoleic acids.

The kinetics of incorporation of [1-$^{14}$C]acetate into the fatty acids of total chloroplast and envelope membranes lipids are shown in Table IV. The ratio of oleate plus linoleate to palmitate is 4.7 for the chloroplast, 4.8 for the inner membrane, and 12.7 for the outer membrane after a 60-min incubation. The intact chloroplast and the i.e.m. have similar ratios, whereas the o.e.m. differs noticeably.

### Acylation with the Newly Synthesized Fatty Acids

The label distribution in lipids of each membrane was also studied (Fig. 4). The unesterified fatty acids were the most labeled species and accounted for 34 to 48% of the radioactivity in the total fatty acids of the o.e.m. and 18 to 25% for the i.e.m. This indicates a significant acyl-ACP thioesterase activity even if all the cofactors, particularly G-3-P, required for the glycerolipid biosynthesis were present in the incubation. Indeed, oleate accounts for more than 50% of the unesterified fatty acids (not shown). A higher amount of oleoyl-ACP than of palmitoyl-ACP and the greater specificity of the acyl-ACP thioesterase for the oleoyl-ACP (36) would be responsible for such a situation.

In the i.e.m., most glycerolipids were labeled. PA, PC, and DAG were the most radioactive lipids, but PG and MGDG also were labeled along with traces of DGDG (Fig. 4). During the incubation, the proportion of radioactivity decreased in PA, whereas it increased in the DAG and MGDG. A slight decrease in the proportion of label was also observed in PG. In the outer membrane, the label in PC was more than 50% of the radioactivity in the glycerolipids. There was a much

**Table IV.** *Distribution of [1-$^{14}$C]Acetate Incorporated into Fatty Acids of Chloroplast and Envelope Membrane Fractions*

The chloroplasts were incubated with [1-$^{14}$C]acetate, ATP, CoA, UDP-galactose, and NADH as described in the legend of Figure 3; the envelope membrane fractions were then isolated (see "Materials and Methods").

| | Radioactive Fatty Acids | | | | | |
| --- | --- | --- | --- | --- | --- | --- |
| | Chloroplast | | o.e.m. | | i.e.m. | |
| | 16:0 | 18:1[a] | 16:0 | 18:1[a] | 16:0 | 18:1[a] |
| | mol % | | | | | |
| 5 min | 46.9 | 53.1 | 23.1 | 76.9 | 33.2 | 66.8 |
| 20 min | 36.9 | 63.1 | 15.5 | 84.5 | 36 | 64 |
| 40 min | 27 | 73 | 5.9 | 94.1 | 29.7 | 70.3 |
| 60 min | 19.5 | 80.5 | 8.1 | 91.9 | 19 | 81 |

[a] Myristate (14:0) and linoleate (18:2) account for less than 6% of the total radioactive fatty acids for both chloroplast and membrane fractions.

lower proportion of label in PA, MGDG, and PG of the outer membrane than those in the inner membrane (Fig. 4).

The distribution of in vitro synthesized fatty acids in the different lipid species (Table V) showed differences between the species in each membrane fraction. After an incubation time of 20 min with [1-$^{14}$C]acetate, the PA, PG, and MGDG in the inner membrane contained about the same proportion of labeled oleate and palmitate. Even though the small size of the samples did not allow an analysis of the molecular species, this suggests that PA, PG, and MGDG are 1-C$_{18}$-2-C$_{16}$ species. Indeed, it is well known that the acylation of G-3-P in the chloroplast (22, 28) leads almost exclusively to the
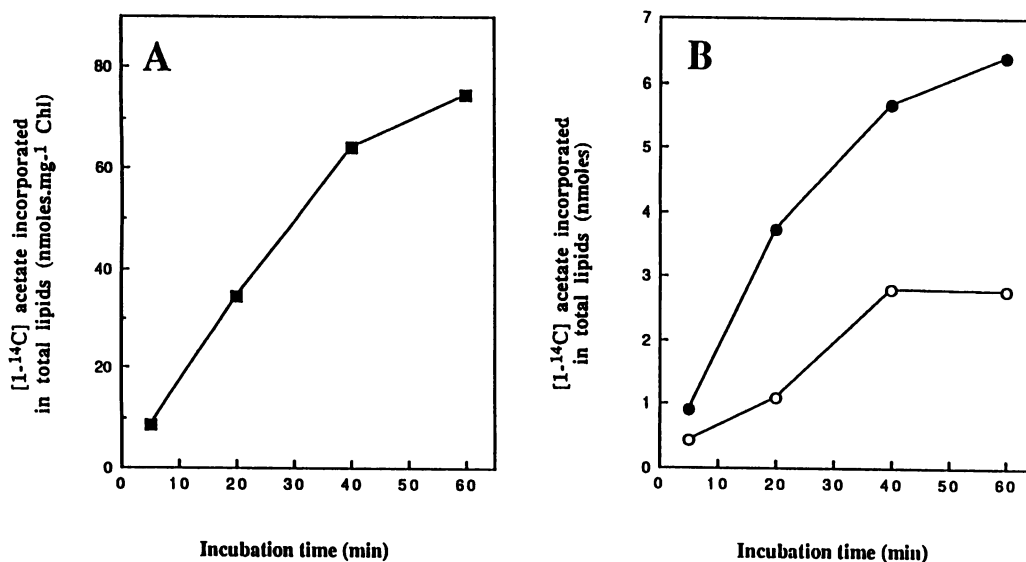


**Figure 3.** Incorporation of [1-$^{14}$C]acetate into total lipids of intact pea chloroplasts and envelope membrane fractions. A, Intact pea chloroplasts. B, Envelope membrane fractions. ●, i.e.m.; ○, o.e.m. The 250-μL mixture contained a volume of chloroplast suspension (250 μg of Chl), 55 nmol of sodium [1-$^{14}$C]acetate (1988 MBq nmol$^{-1}$), ATP, CoA, G-3-P, UDPgalactose, and NADH (each 1 mM final) in the same medium described by Roughan et al. (40). The incubations were carried out at 25°C in the light and with constant shaking (50 strokes min$^{-1}$) for 5 to 60 min.
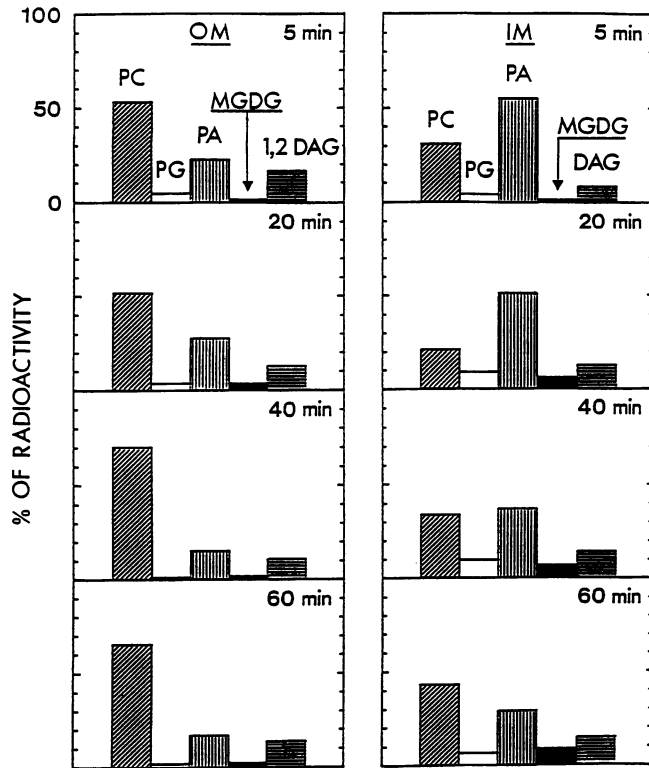
**Figure 4.** Distribution of in vitro synthesized fatty acids into lipid classes of both envelope membranes as function of the incubation time. OM, Outer envelope membrane; IM, inner envelope membrane. The incorporation of labeled acetate into chloroplast lipids is described in the legend to Figure 3. The isolation of envelope membranes after incubating chloroplasts with radioactive acetate and the purification of lipids were carried out as described in "Materials and Methods." The results are expressed as percent of label incorporated into each lipid class to the total lipid label.

$1\text{-}C_{18}\text{-}2\text{-}C_{16}$ species of PA by the intrachloroplastic pathway. Also, desaturation of only 18-carbon fatty acids was observed, as previously described (1). In PC of inner membrane, however, oleic acid represented 90% of the radioactive fatty acids. A contrasting situation was observed in the outer membrane, although there was still an enrichment of labeled oleate in PC of the outer membrane up to 98%. This enrichment in oleate was also observed, but to a lesser extent, in the PA, MGDG, and PG fractions. The DAG seemed to be an exception in both membranes, with a distribution of the label in palmitate and oleate that was intermediate between those of PA and PC, although the enrichment in oleate of DAG was still observed in the outer membrane.

## DISCUSSION

### Identification of the Isolated Fractions

The analytical results reported above are in general agreement with most data on envelope membranes. The overlapping of the o.e.m. fraction into the i.e.m. was estimated at 10%. This fits very well with previously published data (13). Concerning the lipid composition, the low ratios of glycolipids/phospholipids and MGDG/DGDG in o.e.m. are noticeable. They are characteristic of envelope membranes not treated with thermolysin (7). According to Dorne et al. (15), who demonstrated that PC is mostly localized on the cytosolic leaflet of the o.e.m. in spinach, some of the PC present in the i.e.m. would therefore originate from the cross-contamination of this membrane by the o.e.m. As a consequence of the higher content in glycolipids in i.e.m., this fraction is richer in linolenic acid, whereas linoleic acid is concentrated in the phospholipids of o.e.m. Enzymic activities such as those of $Mg^{2+}$ ATPase and acyl-CoA synthetase are also in the range of those found in other works. EM clearly shows the two types of envelope membranes, but no differences were evidenced at this level except the remarkable "thick zones" that appeared in the intermediary fraction. Taken all together, these lipid and polypeptide compositions of individual chloroplast envelope membranes, along with the lo-

**Table V.** *Distribution of the [1-$^{14}$C]Acetate Incorporated into the Fatty Acids of Each Lipid from Envelope Membranes*

The chloroplasts were incubated for 20 min with [1-$^{14}$C]acetate, ATP, CoA, UDP-galactose, and NADH as described in the legend of Figure 3. The isolation of envelope membranes and the purification of lipids were carried out as described in "Materials and Methods."

| | | Radioactive Fatty Acids | | | | | |
|---|---|---|---|---|---|---|---|
| | | PC | PA | PG | DAG | MGDG | Total Fatty Acids |
| | | *mol %* | | | | | |
| o.e.m. | 16:0 | 0.4 | 28.8 | 47.8 | 34.3 | 26.7 | 15.5 |
| | 18:1 | 99.6[a] | 71.2 | 52.2 | 65.7[a] | 73.3 | 84.5 |
| i.e.m. | 16:0 | 12.2 | 45.6 | 51.6 | 16.5 | 49.7 | 36 |
| | 18:1 | 87.8[a] | 54.4[a] | 48.4[a] | 83.5[a] | 50.3[a] | 64[a] |

[a] Linoleate (18:2) accounts for less than 6% of the total radioactive fatty acids for each lipid.

calization of enzymic activities (other than the UDPgalactose-1,2 diacylglycerol galactosyltransferase), have confirmed the purity of the envelope membrane fractions isolated.

## Localization of Galactolipid Synthesis

Such results that verify the quality of the purified i.e.m. and o.e.m. enable us to confirm the presence of a UDPgalactose-1,2 diacylglycerol galactosyltransferase activity in both envelope membranes of pea chloroplasts. The major activity was found in the i.e.m., as previously reported for spinach (7, 26), but we have also found significant activity associated with the o.e.m. (Table III). Our findings differ from those of Cline and Keegstra (13), who found the UDPgalactose-1,2 diacylglycerol galactosyltransferase activity predominantly associated with the o.e.m. of pea chloroplasts.

Because DAG is one of the substrates for the biosynthesis of MGDG and DGDG, the present evidence for galactosyl-transferase activity in each of the envelope membranes raises the question of the availability of DAG for the synthesis of chloroplast glycerolipids. Up to now, two main metabolic pathways have been proposed to provide the chloroplast with DAG (8). The prokaryotic intrachloroplastic pathway uses the de novo synthesized fatty acids directly to form 1-$C_{18}$-2-$C_{16}$ glycerolipid species in the i.e.m. Meanwhile, in parallel, the eukaryotic extrachloroplastic pathway works with phospholipid molecule backbones or DAG moieties originating from the ER and consequently leads to 1-$C_{18}$-2-$C_{18}$ glycolipid species (21, 37). Using Arabidopsis, Browse et al. (9) have confirmed the coexistence of two distinct sites of synthesis for the chloroplast DAG. If one takes into account the three following arguments: (a) spinach, which contains both 1-$C_{18}$-2-$C_{16}$ and 1-$C_{18}$-2-$C_{18}$ MGDG, is known for the very high activity of its prokaryotic pathway; (b) pea, which contains only 1-$C_{18}$-2-$C_{18}$ MGDG, is in the opposite situation (i.e. mainly eukaryotic); and (c) the results for the UDPgalactose-1,2 diacylglycerol galactosyltransferase localization are different in pea and spinach, one can conclude that there are two sites of galactolipid synthesis both on the o.e.m.s and i.e.m.s, the relative activity on the o.e.m. or i.e.m. depending on the species and probably the status of the plant (e.g. age). Because there is no firm evidence that such an activity is restricted to either the o.e.m. or the i.e.m., the postulated existence of two sites on each of the envelope membranes is a useful way to explain the synthesis of both chloroplast lipids in different proportions in 18:3 and 16:3 plants.

## Lipid Synthesis in i.e.m.

When intact chloroplasts were incubated with [1-$^{14}$C]acetate, fatty acids were synthesized and envelope membrane lipids were labeled. The incorporation rate is similar to those found in other studies (3, 19) but lower than the initial velocities reported by Gardiner et al. (24). It must be noted that the radioactivity recovered in lipids of envelope membranes accounts for only 4 to 5% of the radioactivity incorporated into the entire chloroplast (20, our results). However, other workers (20, 29) have shown that thylakoid membranes of isolated pea chloroplasts were labeled after only 30 s of incubation with UDP-[$^{14}$C]galactose and that the radioactivity

of thylakoids accounted for more than 50% of the incorporated radioactivity. Although thylakoid preparations have been shown to contain extensive contamination by envelope membranes (2, 4, 13), the low percentage of label remaining in those nevertheless suggests an active synthesis in the envelope membranes and does not preclude a very rapid transfer from the i.e.m. to the thylakoids, considering the well-admitted absence of UDPgalactose galactosyltransferase in thylakoids. The acylation of newly synthesized fatty acids in PA and PG in the i.e.m. agrees with the results of Andrews et al. (3, 4), who showed that the last step of PA synthesis (1-acylglycerolphosphate acyltransferase activity) and the entire synthesis of PG from PA occurred in the i.e.m. of pea chloroplast. Moreover, in our work, the absence of CTP in the reaction mixture could explain the low amount of labeled PG obtained (3, 38). The localization of the UDPgalactose-1,2 diacylglycerol galactosyltransferase activity suggests that substrates, namely the DAG moieties, are available in this membrane. Although the phosphatidate phosphatase had a very low level of activity and produces only a small pool of DAG in pea chloroplast (23), this did not prevent a measurable synthesis of MGDG in our experiments.

Finally, the equivalent proportions of labeled $C_{16}$ and $C_{18}$ fatty acids in PA, PG, and MGDG in the i.e.m. suggest that these lipids are 1-$C_{18}$-2-$C_{16}$ species.

## Lipid Synthesis in o.e.m.

In the outer membrane, the situation is more puzzling. First, we observed that label accumulated in a higher proportion in oleate than palmitate. This became more evident with time, while the lipids in the i.e.m. became slightly enriched in oleate to about the same degree as was observed for the entire chloroplast. These observations correspond to the selective export of oleic acid by chloroplasts (19). These differences between the two envelope membranes (2, 7), as compared with intact chloroplasts (19), and several studies on enzymic activities in envelopes (30, 43) suggest that the inner membrane constitutes a selective barrier to palmitic acid between the stroma and the intermembrane space of chloroplast envelopes. Consequently, the selective export of oleate occurred through the inner membrane. The oleate-rich pool would then be available for outer membrane metabolism, either in the intermembrane space or in the extrachloroplastic medium (assuming free permeability). However, the export mechanism remains unclear. Such a selective export and the accumulation of unesterified fatty acids fit very well with the localization of the acyl-CoA thioesterase activity on the i.e.m., which would release free fatty acids into the intermembrane space from the acyl-ACP thioesters synthesized in the stroma. Additionally, it can be related to the existence of ACP isoforms in plastids (25). Such isoforms were suggested as factors that would direct fatty acids toward different pathways. However, there is not yet any demonstration of the existence of such ACP isoforms in pea. The present study and those of Gardiner et al. (24) also show that chloroplasts of 18:3 plants such as pea, which synthesize their chloroplast lipids essentially by the extrachloroplastic pathway, synthesize high levels of unesterified fatty acids and acyl-CoA moieties and low levels of glycerolipids as

compared to chloroplasts of 16:3 plants like spinach, which uses both pathways. This also fits with the evidence of an acyl-CoA synthetase activity in the o.e.m., which would provide the membrane with acyl-CoA moieties. These acyl groups would be in the same proportions as the free fatty acids arising from export (i.e. rich in oleic acid).

Second, we observed that the labeling of PC was important and occurred almost exclusively in oleic acid. Labeling of MGDG in the o.e.m. was very low when compared to the i.e.m. It is concluded from other work that the synthesis of PA and PG does not occur in the o.e.m. when isolated envelope membranes are incubated with appropriate precursors (3, 4), and that the synthesis of PC is an extrachloroplastic process (15). Therefore, the labeling pattern obtained under our conditions can only be attributed to either transacylation activities (40) or a very weak de novo biosynthetic activity not yet described. These pathways would occur in o.e.m. and would use a fatty acid pool enriched in oleate. As a hypothesis, we can propose that in this latter case it would give rise to $C_{18}$ lipids, and suggest that cooperation between o.e.m. and i.e.m. gives rise to all lipids present in the chloroplast membranes. This would appear to fit quite well with the possibility of two separate routes (or perhaps three) for the fatty acids synthesized in the chloroplasts: (a) the in situ incorporation in the i.e.m. lipids; (b) the export and further metabolism in the endoplasmic reticulum; and (c) perhaps, metabolism in the o.e.m. The third process operates to a lesser extent, at least in vitro, and may be due to microenvironment concentration of the necessary substrates.

## CONCLUSION

This work on pea chloroplasts shows that the i.e.m. seems to have a higher glycerolipid biosynthetic activity than the o.e.m. In i.e.m., the weak phosphatidate phosphatase activity results in accumulation of PA, which can be used to synthesise PG (3) with only a low synthesis of MGDG. The i.e.m. may also be the site of selective export of fatty acids. The o.e.m. can incorporate only a few of these fatty acids from the available pool, which is rich in oleic acid. However, as far as lipid biosynthesis is concerned, the most important event occurring in pea o.e.m. would be the recycling of DAG from the ER and its use to synthesize galactolipids, the major eukaryotic type of chloroplast lipid (34). Synthesis in the outer membrane would require more time to reach the end product due to the more extensive trafficking involved. It would include export of oleoyl-CoA (19), synthesis of PC in ER, and transfer of phospholipid molecules (34) or DAG moieties back to the chloroplast, then metabolism in the envelope membranes and export to the thylakoids. In good agreement with this scheme, it has been reported that 18:3 plants do require more time (3–6 h) to synthesize their lipids than 16:3–18:3 plants (8).

The results reported hereby support the suggestion that there are separate metabolic events occurring in i.e.m. and o.e.m. of the chloroplast envelope and, hence, provide further support for the model involving at least two pathways of chloroplast lipid biosynthesis in pea.

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