Preparation and Characterization of Envelope Membranes from Nongreen Plastids

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ABSTRACT

We have developed a reliable procedure for the purification of envelope membranes from cauliflower (Brassica oleracea L.) bud plastids and sycamore (Acer pseudoplatanus L.) cell amyloplasts. After disruption of purified intact plastids, separation of envelope membranes was achieved by centrifugation on a linear sucrose gradient. A membrane fraction, having a density of 1.12 grams per cubic centimeter and containing carotenoids, was identified as the plastid envelope by the presence of monogalactosyldiacylglycerol synthase. Using antibodies raised against spinach chloroplast envelope polypeptides E24 and E30, we have demonstrated that both the outer and the inner envelope membranes were present in this envelope fraction. The major polypeptide in the envelope fractions from sycamore and cauliflower plastids was identified immunologically as the phosphate translocator. In the envelope membranes from cauliflower and sycamore plastids, the major glycerolipids were monogalactosyldiacylglycerol, digalactosyldiacylglycerol, and phosphatidycholine. Purified envelope membranes from cauliflower bud plastids and sycamore amyloplasts also contained a galactolipid:galactolipid galactosyltransferase, enzymes for phosphatidic acid and diacylglycerol biosynthesis, acyl-coenzyme A thioesterase, and acyl-coenzyme A synthetase. These results demonstrate that envelope membranes from nongreen plastids present a high level of homology with chloroplast envelope membranes.

The feature shared by all plastids (proplastids, leucoplasts, amyloplasts, chromoplasts, etioplasts, and chloroplasts) is a pair of outer membranes, known as the envelope (12). Together, these membranes provide a flexible boundary between the plastid and the surrounding cytosol. Unfortunately, work on the envelope membranes from nongreen plastids has lagged far behind that conducted on their chloroplast counterpart mostly because it is very difficult to prepare large amounts of intact nongreen plastids free from contaminating membranes and mitochondria. Among nongreen plastids, chromoplasts and etioplasts have been the main sources for investigations on the structure, chemical composition, and functions of envelope membranes (for a review, see Douce et al. [12]). However, except for the work of Fishwick and Wright (14) on envelope membranes from potato tuber amyloplasts, no studies on starch-containing plastids are available, mainly because the presence of starch grains makes the preparation of intact plastids, and consequently of envelope membranes, difficult.

Recently, Journet and Douce (17) developed a procedure for the preparation of large amounts of intact and highly purified starch-containing plastids from cauliflower buds. These plastids, limited by the two envelope membranes, contain almost no internal membranes; when present, these membranes appear to be connected to the inner envelope membrane (see Fig. 1 from Ref. 17). The starch grains present in the stroma have a rather small size and therefore have only a limited effect on the plastid envelope integrity during centrifugation. In addition, Journet et al. (18) have developed a procedure for the preparation of intact and highly purified amyloplasts from sycamore cells. In contrast to cauliflower buds, sycamore amyloplasts are almost devoid of internal membranes. Thus, these two types of nongreen plastids are highly suitable for the preparation of almost pure envelope membranes.

MATERIALS AND METHODS

Plant Material. Cauliflower buds (Brassica oleracea L.) were purchased from local markets. Sycamore (Acer pseudoplatanus L.) cells were grown in a suspension in a liquid medium according to Biligyn (4).

Preparation of Cauliflower Bud Plastids. The top parts of 15 kg of cauliflower inflorescences (corresponding to about 4–5 kg of tissue) were disrupted with a 1-gallon Waring Blender at low speed for 3 s in chilled extraction medium (1 L/kg tissue) containing: 0.3 M mannitol, 20 mM tetra-sodium pyrophosphate; 4 mM cysteine, 1 mM EDTA, 0.1% (w/v) defatted bovine serum albumin; pH was adjusted to 7.6 with HCl. The plastid material was homogenized in three successive batches. All operations were carried out at 0 to 4°C. A crude plastid pellet was obtained as described by Journet and Douce (17) and was then purified twice by isopycnic centrifugation in Percoll gradients (17). After this double purification, the plastid preparation was almost devoid of extraplantidial (cytosol, peroxisomes, and mitochondria) contaminants (17). For instance, less than 1% of the protein of the plastid pellet could be attributed to mitochondrial membranes. Starting from 4 to 5 kg of material, the yield of highly purified intact plastids was 50 to 80 mg protein.

Preparation of Sycamore Cell Plastids. Sycamore plastids were prepared from protoplasts. Sycamore cells (150–200 g) were harvested during exponential growth (3–5 d after the beginning of the culture). Fifteen h prior to the experiment, the cells were transferred to a sucrose-free medium in order to decrease the amount of starch within amyloplasts. This step was essential to improve the yield of intact plastids at the end of the preparation. The events which trigger starch breakdown under these culture conditions have been described by Journet et al. (18). The cells were washed twice in culture medium and 0.5 M mannitol. Protoplasts were then prepared as described by Journet et al. (18). The protoplast pellet was then washed once and resuspended in 150 ml of the following medium: 0.5 M mannitol, 20
mm MOPS-NaOH (pH 7.5), 2 mm EDTA, 1 g/L bovine serum albumin, 0.4 mm spermidine, 7 mm β-mercaptoethanol, 1 mm PMSF, 10 μM leupeptin, 1 mM benzamidine- HCI, 5 mM ε-aminocaproic acid, 1% (w/v) insoluble polyvinylpyrrolidone (K c.a. 25, Serva). Rupture of the protoplasts was achieved by two successive filtrations on a 20 μM-nylon blutex. A crude plastid preparation was then obtained by fractionation of the whole homogenate by differential centrifugation as described by Journet et al. (18). Aliquots (2 mL) of the crude plastid pellet were then layered on top of 8 mL of 50% (v/v) Percoll containing: 0.5 mM mannitol, 10 mM MOPS-NaOH (pH 7.5), 2 mM EDTA, 10 μM leupeptin, 1 mM PMSF, 1 mM benzamidine-HCl, 5 mM ε-aminocaproic acid. After 10 min centrifugation at 3000 rpm (Sorvall, HB 4), purified plastid pellets were recovered. The plastids obtained, loaded with large starch grains, were almost devoid of contamination by extraplasmidial membranes and cytosol, as judged by the absence of marker enzymes for these compartments. For instance, contamination by mitochondrion membranes represented about 1% of the protein. From 150 to 200 g of sycamore cells, the yield of purified intact plastid was about 1 mg protein.

**Thermolysin Treatment of Purified Intact Cauliflower Plastids.** In some experiments, thermolysin, a protease isolated from *Bacillus thermoproteolyticus* (from Calbiochem), was used to destroy the galactolipid:galactolipid enzyme which in chloroplasts is accessible to the cytosolic side of the outer envelope membrane (8). Proteolytic digestion of intact, purified cauliflower plastids (final concentration: 10 mg protein/mL) was done as described by Dorne et al. (8). After digestion, the plastids were purified again with 35% (v/v) Percoll containing: 0.3 M sucrose, 10 mM tricine-NaOH (pH 7.8), and protease inhibitors (1 mM PMSF, 1 mM benzamidine-HCl, and 5 mM ε-aminocaproic acid). This purification step removed thermolysin and broken plastids. The treated and control intact plastids were then used for envelope preparation and lipid determination.

**Electrophoretic Analyses of Envelope Proteins.** Polypeptides were analyzed by PAGE, in a slab gel containing 7.5 to 15% linear acrylamide gradient and in presence of LDS, at 4°C, as described by Joyard et al. (22).

**Immunoblotting Studies of Envelope Membranes.** Antibodies raised against E24 and E30 from the spinach chloroplast envelope were used to characterize envelope membranes from nongreen plastids. Preparation and characterization of rabbit antibodies to E24 and E30 was as previously described by Joyard et al. (22). The presence of antigens corresponding to envelope polypeptides in the different fractions separated from cauliflower plastids was determined with the dot-blot method, using a microfiltration apparatus (Bio-Dot, Bio-Rad), following the procedure described by the manufacturer. The antigen-antibody complexes were revealed using goat anti-rabbit IgG-horseradish peroxidase conjugate and 4-chloro-1-naphthol (Bio-Rad). Western blotting experiments were performed after electrophoretic transfer of envelope polypeptides from polyacrylamide gels to nitrocellulose sheets, as described previously by Block et al. (5).

**Analyses of Envelope Lipids.** Envelope lipids were extracted with a chloroform/methanol mixture (1:2, v/v) according to the method of Bligh and Dyer (3). Quantitative analyses of envelope glycolipids were made according to Douce and Joyard (10).

**Measurements of Enzymatic Activities.** The activities were determined in 100 μL aliquots of the fractions obtained on the sucrose gradients. Phosphoglucone isomerase was assayed as described by Simcox et al. (27). MGDG synthase was measured by following the incorporation of galactose from UDP-[14C]galactose into MGDG, as described by Douce and Joyard (10). Acyl-CoAACP:sn-glycerol 3-phosphate acyltransferase and acyl-CoAACP:monoaacyl-sn-glycerol 3-phosphate acyltransferase were measured as described by Joyard and Douce (19) by following the incorporation of sn-[14C]glycerol 3-phosphate, respectively, into lysophosphatidic acid and phosphatidic acid. Acyl-CoA synthetase was measured using [14C]oleic acid as described by Joyard and Stumpf (20), whereas acyl-CoA thioesterase was measured using [14C]palmitoyl-CoA, as described by Joyard and Stumpf (21). Marker enzymes from mitochondria, endoplasmic reticulum, peroxisomes, and cytosol were measured as described by Douce et al. (11) and Journet and Douce (17); the following enzymes have been assayed in purified envelope fractions: alcohol dehydrogenase, nitrite reductase, catalase, fumarase, NAD(P)H:Cyt c- and cytochrome C oxidoreductase activities.

**Other Assays.** Density of linear sucrose gradients was determined using a Bausch and Lomb refractometer. Proteins were determined by the method of Lowry et al. (25) using bovine serum albumin as a standard. The radioactivity was determined using a scintillation counter (Intertechique) after addition of 10 mL ACS medium (Amersham) to the samples to be analyzed.

**RESULTS**

**Fractionation of the Ruptured Cauliflower Bud Plastids.** A gentle osmotic shock, which is the most efficient method for the rupture of chloroplast envelope membranes (11), broke 70% of the cauliflower plastids. Freeze-thawing (7) alone is even less efficient: only 20% of cauliflower plastids are broken. However, when intact cauliflower plastids (0.5 mL, 50–80 mg protein) are frozen (for 30 min at −80°C), then thawed to room temperature, treated with 3 mL of hypotonic swelling medium (10 mM tricine-NaOH [pH 7.8], 4 mM MgCl2) and homogenized with a Potter-Elvehjem apparatus having a loose fitting pestle, the envelope membranes of almost 100% of the plastids are ruptured, as judged by the lack of latency of gluconate 6-phosphate dehydrogenase, a stromal enzyme (17).

The broken plastid suspension (3.5 mL, 50–80 mg protein) thus obtained is layered on top of a linear sucrose gradient (total volume, 7.5 mL) made of 0.4 to 1.2 M sucrose, 10 mM tricine-NaOH (pH 7.8), and 4 mM MgCl2 (Fig. 1). At the bottom of the gradient, a 2 mL sucrose (1.2 M) cushion, containing 10 mM tricine-NaOH (pH 7.8) and 4 mM MgCl2, was layered prior to the sucrose gradient (Fig. 2). Centrifugation (SW 40 rotor, Beckman) for 15 h at 71,000g (Rmax), resulted in the separation of three fractions: a supernatant, containing the soluble material, on top of the tube; a dense white starch pellet covered by a loose greenish pellet at the bottom of the tube; and a large yellow band, in the middle of the tube (Fig. 1). For most of the studies, the content of the tube was fractionated in about 20 fractions (650 μL each), using a peristaltic pump (Gilson minipuls) and a fraction collector, whereas the green pellet was resuspended in a minimal volume of 10 mM tricine-NaOH (pH 7.8). In some experiments, the supernatant and the yellow band were removed successively from the top of the tube using a Pasteur pipette. In this case, the yellow fraction was diluted with swelling medium devoid of MgCl2 (final volume, 13 mL) and centrifuged (rotor SW 40, Beckman) for 1 h at 218,000g (Rmax). The pellets obtained were resuspended in a minimal volume of medium containing 0.3 M sucrose, 10 mM tricine-NaOH (pH 7.8) using a Potter-Elvehjem homogenizer.

Figure 2A shows the protein distribution and the sucrose density along the tube after centrifugation. Most of the protein (70–90%) was recovered on top of the gradient; the green pellet represented a significant part of the loaded protein (10–25%)
whereas the yellow band represented only about 2 to 3% of the loaded protein (Table I). The sucrose density at the maximum of the protein peak for the yellow band was 1.122 g/cm³, a value very close to that previously determined for chloroplast envelope vesicles (12). Characterization of the different fractions was done by following the distribution of marker enzymes along the sucrose gradient.

**Distribution of Marker Enzymes Along the Sucrose Gradient.** Since the cauliflower plastids used for the experiment were devoid of contamination by cytosolic enzymes (see above), it was possible to use the distribution of phosphoglucone isomerase—an enzyme from plastid glycolysis (17)—for the characterization of stroma. As expected, phosphoglucone isomerase was exclusively localized in the top part of the gradient (Fig. 2B). MGDG synthase, a marker enzyme for the chloroplast envelope (12), was mostly present (70% of the total activity) in the yellow band (Fig. 2B). Therefore, one can conclude that the yellow band contains envelope membranes from cauliflower plastids. MGDG synthase activity in the pellet represented about 20% of the total activity recovered in the gradient. In fact, careful washing of the pellet followed by centrifugation on sucrose gradients removed most of the MGDG synthase from the pellet (see below, Table II), demonstrating that the green pellet was contaminated by envelope membranes.

In addition, we have verified that none of the plastid subfractons, and especially the envelope membrane fraction, were contaminated by microsomal, mitochondrial, or peroxisomal membranes since they did not exhibit any activities typical of these membranes such as NAD(P)H: Cyt c, succinate: Cyt c oxidoreductase (11). Analyses of envelope lipids (see below, Table II) also demonstrate the very low level of contaminating extraplastidial membranes. Using the procedure described above, the yield of purified envelope membranes was 1 to 2 mg protein (i.e. about 2–3% of the plastid protein), starting from 4 to 5 kg of cauliflower buds. Similar yields were obtained when using a discontinuous sucrose gradient (0.6 and 0.93 M, Fig. 1) to recover the envelope membrane fraction at the 0.6 M/0.93 M interface.

**Modification of the Procedure for the Preparation of Envelope Membranes from Sycamore Amyloplasts.** The differences with the method described above are the following: a) a simple osmotic shock was sufficient for the disruption of the envelope membranes, b) the swelling medium as well as the layers of the sucrose gradient were devoid of MgCl₂, c) protease inhibitors were added in all media used and at all the steps of the purification.² With sycamore amyloplasts, only a starch pellet, devoid of any membrane, was obtained at the bottom of the tube. The

² Addition of protease inhibitors was essential for the preparation of envelope membranes from sycamore cells. In the absence of these inhibitors, the envelope polypeptide pattern was totally altered: for instance, the phosphate translocator is present in much larger amounts in the envelope fraction when protease inhibitors are present at all stages of the experiment.
sycamore amyloplast envelope membranes were also characterized by (a) their density (1.12 g/cm³), (b) the presence of carotenoids (see below), and (c) the presence of MGDG synthase (see below, Table II). From 150 to 200 g of isolated sycamore cells, the yield of purified envelope membranes was only 0.2 to 0.3 mg, i.e. about 20 to 30% of the amyloplast protein (Table I). The membrane to soluble protein ratio is much higher in amyloplasts than in cauliflower plastids (Table I), owing to the large starch grains.

**Absorption Spectrum.** The presence of a yellow band and a green pellet after fractionation of cauliflower bud plastids prompted us to analyze their absorption spectra. The yellow envelope band obviously contains carotenoids, as shown by the typical three-banded absorption spectrum of a lipid extract of the fraction (Fig. 3). The amount of carotenoid was about 3 to 5 µg/mg envelope protein. This is an additional line of evidence supporting the characterization of this fraction as envelope membranes (12). The presence of green membranes, just on top of the starch pellet, at the bottom of the tube was surprising since cauliflower buds are apparently devoid of Chl. Indeed, the absorption spectrum of a lipid extract of the pellet clearly demonstrates the presence of a peak at 664 nm corresponding to Chl (Fig. 3). Thus, it is likely that the pellet consists of fragments of the poorly developed internal membrane system (prethylakoids) of cauliflower bud plastids (see Fig. 1 from Ref. 17). Their limited development within these plastids was responsible for the apparently white color of cauliflower buds. However, the major peak at 450 nm in the absorption spectrum of the green pellet demonstrates that carotenoids are present in larger amounts (compared to Chl) than in normal chloroplast thylakoids (12). Since most of these carotenoids can be removed by a careful washing of the pellet, they reflect, in large part, the contamination of the green pellet by envelope membranes containing carotenoids, as suggested above. Furthermore, the lack of Chl in the envelope fraction (Fig. 2) demonstrates the absence of cross-contamination of envelope membranes by the green internal membranes.

With envelope membranes from sycamore amyloplasts, an absorption spectrum typical of those of cauliflower plastids (Fig. 2) and spinach chloroplasts was obtained (not shown), thus demonstrating the presence of carotenoids. In this case, the carotenoid content of the sycamore envelope membranes was 6 to 8 µg/mg protein; of course, no Chl was detected.

**Immunodot Analyses.** Preliminary experiments by double immunodiffusion using antibodies to polypeptides from chloroplast envelope, stroma, and thylakoids (22) have demonstrated that envelope membranes from cauliflower plastids and sycamore amyloplasts react with antibodies to chloroplast envelope polypeptides, but not with antibodies to stroma or thylakoid polypeptides. To determine unambiguously whether the envelope fraction from nongreen plastids contained both envelope membranes, we analyzed the presence of E30 (a polypeptide corresponding to the phosphate translocator), and E24, inner and outer envelope membrane markers, respectively, by immunoblotting (5). With cauliflower envelope membranes, the immunodot microfiltration assay was performed using a wide range of protein concentrations and rabbit antibodies to E24 and E30 (Fig. 4). A strong reaction was obtained with anti-E30 diluted from 1/100 to 1/1000 at envelope concentrations between 2.46 µg and 64 ng protein (Fig. 4A). A much weaker, but obvious, reaction was obtained between 10 µg and 125 ng envelope protein with anti-E24 diluted from 1/50 to 1/5000 (Fig. 4B). In contrast, no reaction was obtained when preimmune sera were used (not shown). Therefore, we can conclude that the envelope fraction from cauliflower bud plastids contains both outer and inner envelope membranes. Similar results were obtained with envelope membranes from sycamore amyloplasts. Finally, analyses of the green pellet obtained after fractionation of cauliflower plastids with antibodies to E30 and E24 also demonstrate that some envelope membranes indeed contaminated this fraction, as suggested above.

**Polypeptide Composition.** In whole cauliflower plastids and isolated subfractions (stroma, envelope, and pellet), the polypeptides separated with our gel system had Mr values from less than 15,000 to more than 100,000 (Fig. 5). The polypeptide patterns were extremely complex, each fraction analyzed containing more than 100 distinct bands after staining with Coomassie blue. Since addition of protease inhibitors to the different media used for cauliflower bud plastid fractionation had only a limited effect, we can conclude that the complexity of the polypeptide patterns is not due to proteolytic digestion of polypeptides during the purification procedure.

The polypeptide patterns of the stroma and the envelope fraction from cauliflower plastids were strikingly different. For

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**Table 1. Distribution of Protein and Chl in the Various Fractions Obtained after Fractionation of Cauliflower Bud Plastids and Sycamore Amyloplasts**

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Protein</th>
<th>Chl</th>
</tr>
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<tbody>
<tr>
<td>Stroma</td>
<td>50</td>
<td>87</td>
</tr>
<tr>
<td>Envelope</td>
<td>1.5</td>
<td>2.6</td>
</tr>
<tr>
<td>Pellet</td>
<td>6</td>
<td>10.4</td>
</tr>
<tr>
<td>Total</td>
<td>57.5</td>
<td>100</td>
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*tr = traces.

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**Fig. 3.** Absorption spectra of chloroform extracts of envelope membranes (left) and the green membrane pellet (right) from cauliflower bud plastids. Each fraction extracted for lipids corresponded to 0.3 mg protein. Note the absorbance, in the green pellet, around 450 and 480 nm, probably due to contaminating carotenoids from envelope membranes.
instance, the stromal fraction was characterized by a doublet around 90,000 D (Fig. 5, lane 1) which was barely detectable in the envelope fraction (Fig. 5, lane 2). This observation confirms the previous data (Fig. 2B) showing the absence of a stromal enzyme (phosphoglucone isomerase) in the envelope fraction. The $M_r$ of the major polypeptide in the cauliflower envelope fraction was about 28,000. This polypeptide was barely detectable in the stroma fraction, and was present in low amounts in the pellet (Fig. 5, lanes 1–3). Its low abundance in the whole plastid preparation (Fig. 5, lane 4) reflects, as previously shown (Fig. 2), the minor importance (on a protein basis) of the cauliflower envelope membranes within the plastid fraction.

Comparison of cauliflower envelope and pellet polypeptide patterns is interesting (Fig. 5, lanes 2 and 3). As described above, the envelope polypeptides are present in low amounts in the pellet. This situation probably reflects the contamination of the pellet by envelope proteins. However, several stained bands, such as a 31,000-D polypeptide distinct from the phosphate translocator (see below), are present in almost similar proportions in the envelope fraction and in the pellet (Fig. 5, lanes 2 and 3). It is possible that these polypeptides are different and just have a similar electrophoretic mobility in common. However, it cannot be excluded that they are identical. A possible explanation for such a result is provided by the unique structural features of cauliflower bud plastids: the internal membranes are frequently connected to the inner envelope membrane (see Fig. 1 from Ref. 17). Therefore, it is possible that the two membrane systems share some common proteins, such as the 31,000-D polypeptide (Fig. 5, lanes 2 and 3). Work is currently in progress in our laboratory to characterize more clearly the relationship between the inner envelope membrane and the internal membranes from cauliflower plastids.

We have previously shown that envelope membranes from cauliflower bud plastids and sycamore amyloplasts react with an antibody to E30, a polypeptide involved in the phosphate/triose phosphate transport across the inner envelope membrane (15). Western blotting experiments with our antibody to spinach E30 clearly demonstrate that the major 28,000-D polypeptide of the cauliflower envelope fraction and the phosphate translocator from spinach chloroplasts have closely related antigenic sites (Fig. 6). The same experiment made using envelope membranes from sycamore amyloplasts demonstrates that the major 30,000-D polypeptide also reacts with antibody against the spinach...
phosphate translocator (Fig. 7). These observations demonstrate that there are probably only limited differences between the sequence of the phosphate translocator in spinach, cauliflower, and sycamore, since good cross-reactivity of the antibody was observed between all these species, despite the differences in M.

Glycerolipid Composition. A galactolipid:galactolipid galactosyltransferase, localized on the outer envelope membrane of spinach chloroplasts (8), catalyzes the interlipid exchange of galactose between galactolipid molecules and induces the formation of unnatural galactolipids such as tri-GDG and tetra-GDG, and diacylglycerol from MGDG during envelope purification (29). Analyses of the glycerolipid composition of sycamore and cauliflower plastid envelope membranes suggest that this enzyme was also active in nongreen plastids: Table II shows that envelope membranes from both sycamore and cauliflower plastids (which have a very similar glycerolipid composition, see Table II) contain diacylglycerol, tri-GDG, and tetra-GDG. Consequently, in order to obtain a glycerolipid composition which represents the in vivo situation, the galactolipid:galactolipid galactosyltransferase must be destroyed prior to fractionation of cauliflower plastids. This was achieved by thermolysin treatment of intact plastids, as demonstrated for chloroplasts by Dorne et al. (8). Unfortunately, due to the extreme fragility of sycamore amyloplasts containing large starch grains, such a treatment was not possible with these organelles.

The glycerolipid composition of envelope membranes from cauliflower bud plastids, after thermolysin treatment, is given in Table II. The major components are galactolipids (MGDG and DGDG) and PC. As expected, after thermolysin treatment no diacylglycerol was detected. The MGDG to DGDG ratio was 1:15 (but only 0.5 when untreated cauliflower plastids were used). Comparison with envelope membranes from spinach chloroplasts or from pea etioplasts, after thermolysin treatment, demonstrated an almost identical pattern (13). Thus, one can conclude that the glycerolipid content is highly stable among the different plastids. It is also possible to suggest that the high amounts of PC found in envelopes from cauliflower bud plastids and sycamore amyloplasts (Table II) reflects the presence of the outer envelope membrane in our fractions. In pea (7) as well as in spinach (6) chloroplasts, the outer envelope membrane contains much higher amounts of PC than the inner envelope membrane. Furthermore, Dorne et al. (9) have demonstrated that, in chloroplasts, PC was concentrated in the outer leaflet of

Table II. Glycerolipid Composition of Envelope Membranes Isolated from Cauliflower Bud Plastids and Sycamore Cell Amyloplasts

<table>
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<tr>
<th>Envelope Lipids</th>
<th>Thermolysin-treated Plastids</th>
<th>Untreated Plastids</th>
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<tr>
<td>MGDG</td>
<td>31.5</td>
<td>13</td>
</tr>
<tr>
<td>DGDG</td>
<td>27.5</td>
<td>28</td>
</tr>
<tr>
<td>Tri-GDG</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Tetra-GDG</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>SL</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>PC</td>
<td>20</td>
<td>21</td>
</tr>
<tr>
<td>PG</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>PI</td>
<td>4.5</td>
<td>4</td>
</tr>
<tr>
<td>PE</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>DG</td>
<td>0</td>
<td>10.5</td>
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Cauliflower: Untreated and thermolysin-treated plastids from cauliflower buds and untreated amyloplasts from sycamore cells (see "Materials and Methods") were used for these experiments. Due to the fragility of amyloplasts, thermolysin treatment is not possible with these plastids. Envelope membranes were prepared as described under "Materials and Methods." Glycerolipids were extracted and analyzed as described under "Materials and Methods." This composition is from a representative experiment. The values are expressed as weight percent fatty acids.

Sycamore Untreated Plastids

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the outer envelope membrane. Finally, it is now widely accepted
that the presence of PE in chloroplasts reflects contamination by
extraplasmidial membranes (12, 13). Table II confirms the obser-
vation that low levels (less than 1%) of contaminating extraplas-
midial membranes are present in envelope fractions, as indicated
above.

Distribution of Enzymatic Activities Involved in Glycerolipid
Biosynthesis. We have previously characterized more than 20
different enzymatic activities in envelope membranes from spin-
ach chloroplasts (10, 12). To determine whether this was a general
feature in envelope membranes from cauliflower bud plastids
and from sycamore amyloplasts, we analyzed some of the chlo-
roplast enzymes involved in lipid metabolism. Figure 2 dem-
strates that envelope membranes from cauliflower bud plastids
contain the enzyme responsible for MGDG biosynthesis. The
same is true for envelope membranes from sycamore amyloplasts
(Table III).

Furthermore, Table III presents the distribution of two acyl-
transferases involved in the biosynthesis of phosphatidic acid.
The first enzyme, an acyl-CoA(ACP):sn-glycerol 3-phosphate
acyltransferase, involved in the biosynthesis of lyso phosphatidic
acid was recovered together with the soluble fraction (Table III).
This result is in complete agreement with data on chloroplasts
(2, 19), but in contrast with the data of Fishwick and Wright (14)
which showed that in potato tuber amyloplasts, the enzyme was
membrane-bound. One reason for such a discrepancy is not yet
clear, since in amyloplasts from sycamore cells (Table III), we
found the same result as in spinach chloroplasts and cauliflower
bud plastids.

The second enzyme, an acyl-CoA (ACP:monoacyl-sn-glycerol
3-phosphate acyltransferase, was associated with the envelope
membrane fraction (Table III). Again this result was confirmed
with envelope membranes from sycamore amyloplasts (Table
III) and is in good agreement with our observations with chlo-
roplast envelope membranes (19). However, in cauliflower bud
plastids, about 50% of the total activity was recovered with the
green pellet, which was a higher figure than expected from our
data on galactosyltransferase distribution (Fig. 1; Table III). Most
of the activity could be removed by washing the pellet (Table
III), but the presence of this enzyme in the pellet cannot be
totally ruled out (this problem is presently under investigation).

Table III also shows the distribution of two enzymes associated
with acyl-CoA thioesters (acyl-CoA synthetase and acyl-CoA
thioesterase). Again, both enzymes are associated with envelope
membranes of plastids from cauliflower buds and sycamore cells
(Table III), a situation identical to that observed in chloroplasts
(12).

DISCUSSION

Several lines of evidence can be presented for the characteriza-
tion of the membrane fraction isolated from cauliflower bud
plastids as the envelope. First, this membrane fraction has a
density of 1.122 g/cm3, a value almost identical to that of spinach
chloroplast envelope membranes. Second, this membrane is
yellow and contains carotenoids. Third, this membrane fraction
is a major site for lipid synthesis: the site of incorporation of
galactose from UDP-galactose into MGDG, it contains the en-
zeymes of the Kornberg-Pricer pathway together with acyl-CoA
synthetase and thioesterase. Fourth, we have identified immu-
nologically the phosphate translocator, involved in the exchange
of triose phosphate and phosphate between the cytosol and the
plastid stroma (15). Finally, the glycerolipid composition of
nongreen plastid envelope fraction is almost identical to that of
spinach chloroplast envelope. Therefore, this identification is
unambiguous. In addition, both envelope membranes are present
in our membrane fraction. The presence of the inner envelope
membrane is demonstrated by the following observations: the
envelope preparation contains the phosphate translocator and
the MGDG synthase. On the other hand, the presence of the
outer envelope membrane is supported by the immunological
reaction of anti-E24 with the envelope fraction, by the presence
of a galactolipid:galactolipid galactosyltransferase and an acyl-
CoA synthetase, and by the high level of PC in the fractions.
The same observations were used for the characterization of envelope
membranes from sycamore amyloplasts. We have done prelimi-
nary experiments to separate the outer and inner envelope
membranes from nongreen plastids. With cauliflower plastids,
the main problem is breaking the plastids (as discussed above),
whereas with sycamore amyloplasts, yields were a major limita-
tion. However, with cauliflower as well as with sycamore, we
have indeed obtained a light membrane fraction containing acyl-

Table III. Distribution, Within Sycamore Amyloplasts and Cauliflower Bud Plastids, of Enzymic Activities
Involved in Lipid Metabolism

The different fractions were prepared after fractionation of cauliflower bud plastids or sycamore amyloplasts
within a discontinuous sucrose gradient as described in Figure 1. The different enzymic activities were measured
as described under "Materials and Methods." For the cauliflower pellet, in addition to analyses in the crude
pellet (a) directly obtained from the tube, some activities were also determined after two washings of the pellet
(b) to remove most of the envelope contamination. The values are from representative experiments.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Sycamore Stroma</th>
<th>Sycamore Envelope</th>
<th>Cauliflower Stroma</th>
<th>Cauliflower Envelope</th>
<th>Pellet a</th>
<th>Pellet b</th>
</tr>
</thead>
<tbody>
<tr>
<td>acyl-CoA(ACP):sn-glycerol 3-phosphate acyltransferase (nmol lysoPA*) formed/h/mg protein</td>
<td>3.35</td>
<td>0</td>
<td>7</td>
<td>0.35</td>
<td>0.3</td>
<td>ND</td>
</tr>
<tr>
<td>acyl-CoA(ACP):monoacyl-sn-glycerol 3-phosphate acyltransferase (nmol PA formed/h/mg protein)</td>
<td>0</td>
<td>6.8</td>
<td>1</td>
<td>57.8</td>
<td>18.7</td>
<td>7.8</td>
</tr>
<tr>
<td>MGDG synthase (nmol galactose incorporated/h/mg protein)</td>
<td>0</td>
<td>81</td>
<td>20</td>
<td>2300</td>
<td>190</td>
<td>29.2</td>
</tr>
<tr>
<td>acyl-CoA synthetase (umol acyl-CoA formed/h/mg protein)</td>
<td>0.28</td>
<td>18.2</td>
<td>0.27</td>
<td>2.85</td>
<td>0.9</td>
<td>0.15</td>
</tr>
<tr>
<td>acyl-CoA thioesterase (nmol fatty acid released/h/mg protein)</td>
<td>7.7</td>
<td>127.6</td>
<td>7.8</td>
<td>108</td>
<td>61</td>
<td>9</td>
</tr>
</tbody>
</table>

* PA, phosphatidic acid; ND, not detected.
CoA synthetase and a heavy membrane fraction containing the phosphate translocator. Therefore, it is likely that separation of outer from inner envelope membranes is possible. Unfortunately, the yields are presently so low that a serious study cannot yet be achieved.

The envelope membranes prepared from nongreen plastids are very pure: they are devoid of contamination by stromal enzymes (phosphoglucone isomerase, acyl-CoA[ACP]-sn-glycerol 3-phosphate acyltransferase); in addition, in cauliflower envelope membranes, the absorption spectrum demonstrates a negligible level of Chl, and therefore of internal membranes. Furthermore, no enzymic activities characteristic of microsomal, peroxisomal, or mitochondrial membranes and very low levels of PE could be detected in envelope membranes from nongreen plastids.

Finally, the major polypeptide in envelope membranes from starch-containing nongreen plastids is the phosphate translocator (Figs. 6 and 7). This result was not obvious since in pea etioplasts, Soll (28) was unable to identify any polypeptide having the same electrophoretic mobility. However, the polypeptide involved in phosphate transport has $M_r$ values ranging from 28,000, in cauliflower plastids, to 30,000, in sycamore amyloplasts.

Carotenoids are present in envelope membranes from cauliflower bud plastids and sycamore amyloplasts as in all carefully prepared envelope membranes: the absorption spectrum obtained with envelope membranes from these nongreen plastids were identical to those from spinach chloroplasts or potato tuber amyloplasts (12). In addition, Fishwick and Wright (14) have found that the carotenoid content and composition of amyloplast envelope membranes were similar to those of spinach chloroplasts. Therefore, we can conclude that carotenoids are genuine constituents of envelope membranes from chloroplasts as well as from nongreen plastids.

The glycolipid composition of envelope membranes is remarkably stable among the different plastids. Only a few percent difference can be noticed in the glycolipid content of envelope membranes from spinach chloroplasts, pea etioplasts, or cauliflower plastids (12, 13). Therefore, it is clear that envelope membranes contain characteristic polar lipids in a fixed molar ratio that is probably determined genetically. Comparison with the few data available in the literature for etioplasts, chloroplasts, or amyloplasts (1, 12, 23, 24, 26) also supports this conclusion despite the fact that in all these experiments the galactolipid:galactolipid galactosyltransferase was not destroyed prior to the analyses. Finally, the presence of this unique enzyme, on the cytosolic side of all high plant plastids (chloroplasts as well as etioplasts or any starch-containing plastids) analyzed so far, is also remarkable. The physiological significance of the galactolipid:galactolipid galactosyltransferase is still a subject of controversy, but Van Besouw and Wintermans (29) have postulated the major role for this enzyme in DGDG synthesis.

The presence of typical plastid components, such as galactolipids or carotenoids, in chloroplasts as well as in nongreen plastids suggests that envelope membranes from the various plastid types play a major role in their biogenesis (12). The results obtained with purified envelope membranes from cauliflower buds strongly support this hypothesis. In cauliflower and in sycamore, all the enzymes involved in the acylation of sn-glycerol 3-phosphate, the formation of diacylglycerol and the biosynthesis of MGDG are present in, or associated with, envelope membranes. The same is true for amyloplasts, etioplasts, or chromoplasts (1, 14, 23, 24, 26). These results demonstrate that the biosynthetic capabilities of envelope membranes are not restricted to chloroplasts with a well developed thylakoid network, but are also present in plastids whose membranes are almost limited to their envelope membranes. The presence of these enzymes probably reflects the flexibility of envelope membranes surrounding the starch grains.

Consequently, our observations—together with the data available in the literature (for a review, see Douce et al. [12])—demonstrate that there is a high degree of uniformity in the structure, chemical composition, and functions of the plastid envelope membranes among the different plastid types. However, we must keep in mind that differentiation of all plastid types and their developmental transitions are associated with, or dependent on, marked changes in their specific enzymic complement. It is obvious that this is also true for envelope proteins. Characterization of these tissue-specific or plant-specific envelope components that are differentially expressed during plant development is a challenging goal for further studies.

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**LITERATURE CITED**


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