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Galactosyltransferases Involved in Galactolipid Biosynthesis Are Located in the Outer Membrane of Pea Chloroplast Envelopes[1]

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ABSTRACT

The galactosylation steps in the biosynthesis of galactolipids involve two different enzymes; a UDP-Gal:diacylglycerol galactosyltransferase and a galactolipid:galactolipid galactosyltransferase. Previous localization studies have shown that in spinach these enzymes are located in the chloroplast envelope. Our results with peas (*Pisum sativum* var Laxton's Progress No. 9) confirm these results and extend the localization by providing evidence that the galactosyltransferases are in the outer membrane of the envelope. The specific activity of UDP-Gal:diacylglycerol galactosyltransferase in outer membrane preparations was 6 to 10 times greater than that exhibited by inner membrane preparations. In addition, using quantitative sodium dodecyl sulfate-polyacrylamide gel electrophoresis, it was possible to show that the UDP-Gal:diacylglycerol galactosyltransferase activity associated with inner membrane preparations could be accounted for by outer membrane contamination. It is concluded from these results that this enzyme is located predominantly, if not exclusively, in the outer membrane of the envelope. An analysis of the galactolipid products synthesized by the highly purified outer membrane showed that the galactolipid:galactolipid galactosyltransferase is also present, suggesting that this enzyme is also an outer membrane enzyme. The implication of these results is that the final assembly of galactolipids is carried out on the outer membrane of the chloroplast envelope.

Galactolipids, the major lipids of chloroplast membranes, consist of a diacylglycerol moiety to which one (MGDG[2]) or two (DGDG) galactosyl residues (4, 6) are attached. The synthesis of galactolipids is a major cellular process which involves the assembly of compounds deriving from the cell cytoplasm (*e.g.* UDP-Gal) with compounds which are made in the chloroplast (*e.g.* fatty acids) (3, 6). The enzymic steps of this process have not been definitively established, but a tentative scheme has been proposed based upon enzyme activities associated with isolated spinach chloroplast envelopes (6). In this scheme, diacylglycerol is formed from fatty acids and glycerol-3-P by a series of enzymes which includes an acyl CoA synthetase, two fatty acyl transferases, and a phosphatidic acid phosphatase. MGDG and DGDG are then formed from diacylglycerol by at least two galactosyltransferases. The first enzyme, a UDP-Gal: diacylglycerol galactosyltransferase, forms MGDG by transferring a galactosyl residue from UDP-Gal to diacylglycerol (6). This enzyme is the more thoroughly characterized of the galactosyltransferases. Evidence has been presented that it is located exclusively in the chloroplast envelope (6). For this reason, it is thought that final assembly of all galactolipids occurs in the envelope membranes.

The mechanism of DGDG formation is as yet unresolved. Two different enzymes have been described which are capable of forming DGDG. One enzyme, a UDP-Gal:MGDG galactosyltransferase, transfers a galactosyl residue from UDP-Gal to MGDG (11). This enzyme is found in the soluble fraction of ruptured plant cells and has been proposed to be cytoplasmically located (11). A second enzyme, located in the chloroplast envelope, forms DGDG by galactolipid-galactolipid galactosyl transfer in which the galactosyl residue from one MGDG is transferred to another, forming DGDG and diacylglycerol (12).

In order to fully understand the mechanisms involved in the synthesis of galactolipids, it is necessary to determine in which of the two membranes of the envelope each of the biosynthetic enzymes is located. Until recently, answering these questions was not feasible due to the fact that the two membranes of the envelope had not been separated. However, we have recently developed a method which subfractionates the envelope into two membrane fractions which have been tentatively identified as the inner and outer envelope membranes (4). The purpose of the present study is to determine in which of these envelope membrane subfractions the envelope-bound galactosyltransferases are located.

MATERIALS AND METHODS

Hepes, Tricine, Percoll, and UDP-Gal were obtained from Sigma. UDP-[^{3}H]galactose (10.4 Ci/mmol) and UDP-[^{14}C]galactose (305 mCi/mmol) were purchased from Amersham. All other chemicals were reagent grade.

Purification and Subfractionation of Chloroplast Envelope Membranes. Intact chloroplasts were purified from homogenates of 2- to 3-week old pea seedlings (*Pisum sativum* var Laxton's Progress No. 9) by differential centrifugation followed by Percoll density gradient centrifugation as previously described (4).

Chloroplast envelope membranes were purified and subfractionated by the procedure described previously (4). Briefly, purified intact chloroplasts were suspended in 0.6 M sucrose and incubated on ice for 10 min. The chloroplasts were then ruptured by a freeze-thaw cycle and subsequently fractionated by flotation centrifugation. During this centrifugation, envelope membranes rise to a 0.3/1.2 M sucrose interface, whereas thylakoid membranes and the soluble chloroplast extract remain in the lower region of the gradient. The envelope membranes obtained by this flotation centrifugation were previously called freeze-thaw/flotation membranes (4), but here they will be referred to as unfractionated envelope membranes. Thylakoid membranes were prepared by diluting the lower region of the flotation gradient to approximately 0.3 M sucrose followed by sedimenting at 90,000g_{max} for 1 h. The supernatant of this centrifugation contains the soluble chloroplast components. The unfractionated envelope membranes were subfractionated into inner and outer membranes by sedimentation

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[2] Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PAGE, polyacrylamide gel electrophoresis; TGDG, trigalactosyldiacylglycerol; TeGDG, tetragalactosyldiacylglycerol.

through a 27- to 29-ml linear 0.6 to 1.2 M sucrose gradient at 113,000g_{max} for ~14 h at 4°C in a SW27 rotor.

Envelope membranes were recovered from sucrose gradients by dilution followed by pelleting at 90,000g_{max} for 1 h. All solutions used for preparing envelope membranes by this procedure were buffered with 10 mM Tricine/NaOH (pH 7.5) containing 2 mM EDTA. All dilutions for recovery of envelope and thylakoid membranes were made with 10 mM Tricine/NaOH (pH 7.5).

Assays. Chlorophyll was determined by the method of Arnon (2). Protein was estimated by the modified Lowry procedure described by Markwell *et al.* (10) using BSA as a standard.

The diacylglycerol content of membrane samples was determined by first extracting the polar lipids as described (7) followed by separating the diacylglycerol from other polar lipids by TLC using a chloroform:methanol:H_2O (65:25:4, v/v/v) solvent system (7). The lipids were detected by I_2 staining, identified by comparison of R_F with that of authentic standards, and quantitated by GLC analysis of the fatty acid methyl esters derived from the lipids as described by Allen and Good (1).

Galactosyltransferase assays were carried out in a total of 0.4 ml of 0.1 M Tricine/NaOH (pH 7.5) containing 4 mM $MgCl_2$ at 25°C. Each assay contained 250,000 to 300,000 cpm of UDP-[^{3}H] galactose and sufficient unlabeled UDP-Gal to achieve the desired final concentration. Reactions were initiated by the addition of membranes. Reactions were terminated and galactolipids extracted as described by Douce (7). The amount of galactolipid formed was determined by liquid scintillation counting of extracted lipid fractions. Lipid preparations from Chl-containing samples were first chromatographed on Kiesegel G plates in diethyl ether to separate pigments from galactolipids (3). In this latter case, the level of radioactivity in the recovered galactolipids was corrected for the 18% loss which occurred during this procedure.

For analysis of the specific galactolipids formed during galactosyl transfer reactions, membranes were incubated with UDP-[^{14}C]Gal (40 μM). After reactions were terminated, the extracted, radiolabeled galactolipids were separated by TLC and visualized by autoradiography as described (7). Radioactivity in individual lipids was quantitated by scraping the silica from the TLC plates followed by liquid scintillation counting.

Buffers used for determining the pH optimum of galactosyltransferase were 0.1 M solutions of: acetate/NaOH (pH 4.5–6.0), Mes/NaOH (pH 5.5–7.5), and Tricine/NaOH (pH 7–9). At a given pH, different buffers gave no significant difference in the rate of galactolipid incorporation.

Unless otherwise stated, the UDP-Gal concentration for galactosyl transferase assays was 250 μM (approximately 8 times the K_m). Specific activities were measured by assaying samples for varying lengths of time (0–8 min) and determining initial rates from plots of galactolipid product *versus* time. Rates of galactose incorporation determined in this manner increase linearly with increasing amounts of membrane. For other galactosyltransferase assays, reactions were carried out for a fixed period of time (<10 min), and activity was computed as the average nmol of galactosyl residues incorporated into galactolipids per min during the course of the reaction.

SDS-PAGE. Discontinuous PAGE in the presence of SDS was performed in 0.8-mm thick gels according to Laemmli (8). The separating gel (10 cm long) was formed from a 7.5 to 15% linear acrylamide gradient accompanied by a 5 to 17.5% linear sucrose gradient. The stacking gel (1–2 cm long) was made from a 4% acrylamide solution. Pelleted membrane samples were dissolved in sample buffer at room temperature and then subjected to electrophoresis at a constant current of 12 mamp for 5 h. After electrophoresis, gels were stained by shaking in stain solution, 0.1% Coomassie Blue R250 in methanol: H_2O:acetic acid (5:5:1, v/v/v) for at least 2 h. Gels were destained with 7.5% methanol, 7.5% acetic acid in water, and then dried between cellophane. Densitometry was carried out on a Joyce Loebl model MK IIIC microdensitometer equipped with either a 1.7 D or 0.8 D gray wedge and a 0.5 D neutral density filter. Scans were made through the center of sample lanes. Integration of densitometer scans was achieved by cutting out the traces and weighing them on an analytical balance.

RESULTS AND DISCUSSION

Preliminary Characterization of Galactosyltransferase from Pea Chloroplast Membranes. Although much is known about the galactosyltransferases of spinach chloroplast envelopes (6, 12, 13), the pea enzymes have not yet been characterized. To ensure that pea galactosyltransferases were assayed near optimal conditions, several characteristics of galactose incorporation from UDP-Gal into galactolipids were determined. The source of galactosyltransferase as well as diacylglycerol, the galactosyl acceptor, was unfractionated envelope membranes which are recovered after the first centrifugation in the purification procedure. Under the assay conditions described in "Materials and Methods," unfractionated envelope membranes synthesized predominantly MGDG (see below). Accordingly, the following characterization studies apply to the UDP-Gal:diacylglycerol galactosyltransferase.

The pea galactosyltransferase has properties similar to the spinach enzyme. Galactose incorporation using pea envelope membranes exhibited a broad pH optimum from pH 7 to 9 and was stimulated by Mg^{2+}. Addition of 1 to 4 mM $MgCl_2$ to the reaction mixture resulted in a 60% stimulation of activity over reactions lacking added $MgCl_2$. Increases above 4 mM resulted in little if any additional increase in enzyme activity. When an excess of EDTA was added, the stimulatory effect of $MgCl_2$ was abolished.

The kinetics of pea galactosyltransferase are depicted in the Eadie-Hofstee plot (Fig. 1). The apparent K_m for UDP-Gal for three determinations was 36 ± 8 μM. The V_{max} values per mg of membrane protein varied considerably among preparations; values as low as 4 nmol/min·mg and as high as 50 nmol/min·mg have been obtained. These values compare with the spinach enzyme which exhibits a K_m of 30 to 45 μM and V_{max} of ~45 nmol/min·mg envelope protein (12). Van Besouw and Wintermans (12) report that in spinach envelopes there is a second active site with a much lower affinity for UDP-Gal (K_m ~300–1,000 μM). Our data with pea envelope membranes give no indication of a second active site.

It has been reported that spinach galactosyltransferase is stimulated by albumin, presumably due to the ability of albumin to bind fatty acids which inhibit the enzyme (13). No stimulating effect of albumin was observed with the pea enzyme. Several pea chloroplast envelope preparations have been tested and up to 20 mg/ml BSA have been used without effect. The pea galactosyltransferase showed good stability to storage, with loss of less than 20% of the original enzyme activity when kept at 4°C or −20°C for up to 4 d.

Based on these results, the assay conditions described in "Materials and Methods" were chosen for routinely assaying galactosyltransferase. Fractionated sucrose gradients and membrane subfractions were assayed either fresh or after storage at −20°C for less than 4 d.

Localization of Galactosyltransferase. It has been previously reported that in spinach leaves, the chloroplast envelope is the sole membrane site of incorporation of galactose from UDP-Gal into galactolipids (6). This conclusion was based upon the fact that isolated envelope membranes possess a much higher specific activity of galactosyltransferase than other cellular membrane fractions. Our results with pea tissue are consistent with this conclusion. First, an accounting of galactosyltransferase activity and chlorophyll content of various membrane preparations obtained during the purification of intact pea chloroplasts indicates that

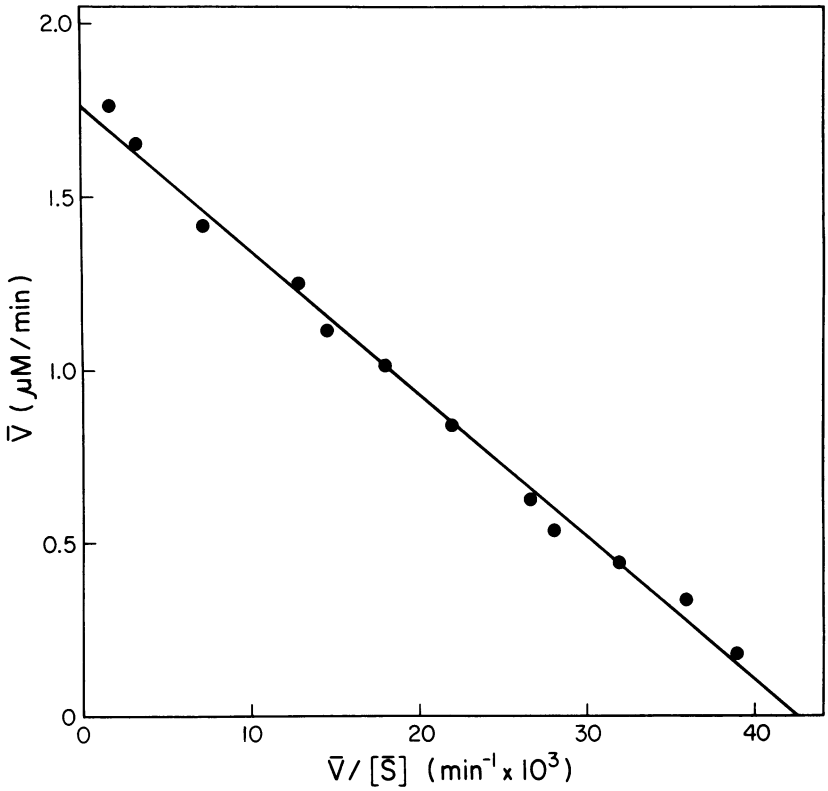

FIG. 1. An Eadie-Hofstee plot of the UDP-Gal dependence of galactosyltransferase. Each assay mixture contained 60 μg of envelope membrane protein, 300,000 cpm UDP-[^{3}H]Gal, and varying amounts of unlabeled UDP-Gal. Assays were conducted for 5 min as described in "Materials and Methods." Average substrate concentrations, [$\bar{S}$], and average reaction velocities, $\bar{V}$, during the course of the reaction were calculated as described (9). The K_m and V_{max} values obtained from the above analysis were 41 μM UDP-Gal and 12 nmol/min·mg protein, respectively.

Table I. *Distribution of Galactosyltransferase Activity in Chloroplast Membranes*

Chloroplasts were ruptured by freezing and thawing as described in "Materials and Methods." An aliquot of the unfractionated, broken chloroplasts was frozen while the remainder was fractionated into envelopes, thylakoids, and soluble fractions by flotation centrifugation. Envelope, thylakoid, and unfractionated chloroplast membranes were recovered by sedimentation at 90,000g_{max} for 60 min, were resuspended in 0.1 M Tricine/NaOH, 4 mM $MgCl_2$ (pH 7.5), and were assayed for galactosyltransferase specific activity. The activity shown below derives from 10 mg Chl of intact chloroplasts. Activity in the soluble fraction of chloroplasts was below detection limits of 2.6 nmol/min.

	Galactosyltransferase	
	Total activity	Specific activity
	nmol Gal/min	*nmol Gal/min·mg protein*
Chloroplast membranes (unfractionated)	29.3	1.42
Envelope membranes	22.6	48.8
Thylakoid membranes	14.8	0.62

chloroplasts can account for all of the membrane-bound galactosyltransferase of cell homogenates (data not shown). Second, the distribution of galactosyltransferase in fractionated chloroplasts indicates that the envelope is the location of galactosyltransferase within the chloroplast (Table I). Chloroplasts, ruptured by freezing and thawing, were fractionated by flotation-centrifugation into thylakoids, soluble fraction, and envelopes (see "Materials and Methods"). The highest galactosyltransferase specific activity was found in the envelope fraction, approximately 80-fold greater than that of the thylakoid fraction. Galactosyltransferase generally could not be detected in the chloroplast soluble fraction. Although a significant percentage of the total galactosyltransferase activity was associated with the thylakoid fraction, this activity most probably results from envelope contamination of the thylakoids (6).

The localization of galactosyltransferase to one membrane of the envelope was accomplished by subfractionating the envelope into its component membranes and assaying the separated membrane fractions for enzymic activity. The subfractionation of envelopes is accomplished by sedimenting unfractionated envelopes through a linear-density sucrose gradient. An optical density profile of the sucrose gradient is shown in Figure 2. Two bands of membranes are seen; one near the top of the gradient and a second near the bottom. We have tentatively identified the membrane material in the upper band (pool 4) as the outer envelope membrane (4). The lower band of membranes is somewhat heterogeneous. The peak fractions (Fig. 2, pool 1) contain predominantly putative inner membrane, whereas the trailing edge of the peak (pool 2) consists of a mixture of inner and outer membranes. Evidence for the conclusions concerning the purity of the lower band comes from electron microscopic data and polypeptide compositions of the membranes (see Ref. 4 and below).

When the gradient was assayed for galactosyltransferase, two peaks of activity were seen (Fig. 2). One centered over the outer membrane band. The second peak of activity centered over the trailing edge of the lower band. This is the region of the gradient which contains a mixture of inner and outer membranes. At least eight separate preparations have been assayed for galactosyltransferase with essentially the same results.

The most straightforward interpretation of this profile is that the galactosyltransferase is located in the outer envelope membrane and is present in inner membrane-containing subfractions

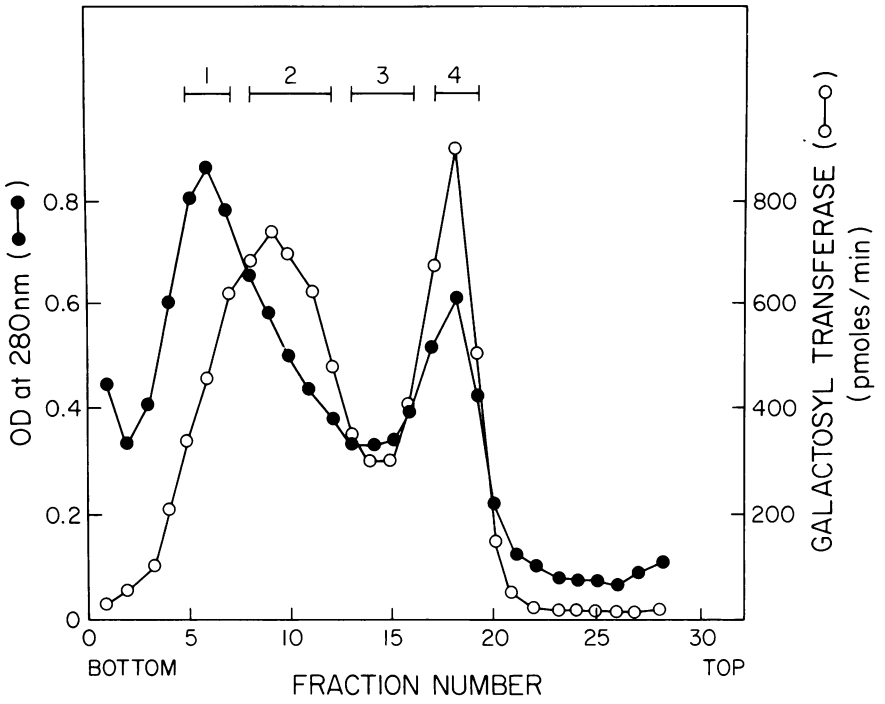

FIG. 2. Galactosyltransferase activity of subfractionated chloroplast envelope membranes. Unfractionated envelope membranes (12 ml, 1.5 mg protein) obtained from 30 mg Chl of intact chloroplasts were sedimented through a 27-ml, 0.6 to 1.2 M linear sucrose gradient. Fractions (1.25 ml) were assayed for turbidity at 280 nm and for galactosyltransferase activity. Galactosyltransferase activities were conducted for 10 min as described in "Materials and Methods" with the exception that the UDP-Gal concentration was 40 μM. Activities are displayed as the enzyme activity per ml in each fraction. For subsequent analysis of the membranes, pools 1 to 4 were made as shown.

Table II. *Galactosyltransferase Activities of Envelope Membrane Subfractions*

Unfractionated envelope membranes, obtained from 10 mg Chl of intact chloroplasts, were subfractionated by density gradient centrifugation as described in Figure 2 and "Materials and Methods." Envelope membranes from different regions of the gradient were pooled as shown in Figure 2 and recovered by centrifugation. Galactosyltransferase specific activities were determined.

Membrane Preparation	Protein	Galactosyltransferase	
		Total activity	Specific activity
	mg	*nmol Gal/min*	*nmol Gal/min·mg protein*
Unfractionated envelopes	5.2	72.9	13.9
Pool 1 (inner membrane)	1.32	8.8	6.7
Pool 2	1.25	20.5	16.2
Pool 3	0.41	10.2	24.9
Pool 4 (outer membrane)	0.4	15.9	40.4

due to outer membrane contamination. If this is true, then the level of galactosyltransferase in the various subfractions should reflect the amount of outer membrane in those subfractions. To test this prediction, the percentage of outer membrane protein which would be expected in the various subfractions was computed from their respective galactosyltransferase specific activities. These values were then compared with values obtained independently by quantitative SDS-PAGE. Table II lists the specific activities of the membrane subfractions from a representative preparation. The highest specific activity was associated with the outer envelope membrane (40.4 nmol/min·mg protein), whereas

Table III. *Estimation of the Percent of Outer Membrane Protein in Envelope Subfractions*

Envelope membrane fractions were obtained as described in "Materials and Methods" and Figure 2. The percent of outer membrane protein in the subfractions was computed first from the galactosyltransferase specific activities of each subfraction. Two assumptions were made for these calculations: that pool 4 is pure outer membrane, and that galactosyltransferase is exclusively an outer membrane enzyme. The percent outer membrane was also estimated by quantitative SDS-PAGE densitometry using the 75 kD polypeptide as a marker for the outer membrane (see Fig. 4 and the text). The displayed values are the mean ± SD obtained from analysis of envelope subfractions from three separate preparations. E_u are unfractionated envelope membranes; pools 1 through 4 are as shown in Figure 2.

Method of Estimation	Outer Membrane Protein				
	E_u	Pool 1 (inner)	Pool 2	Pool 3	Pool 4 (outer)
			%		
Prediction from galactosyltransferase activity	27 ± 7	13 ± 3	38 ± 2	55 ± 12	100
Integration of SDS-PAGE profiles	28 ± 6	9 ± 2	31 ± 10	77 ± 5	100

the lowest specific activity was found in the predominantly inner membrane preparation (6.7 nmol/min·mg). Although the absolute values for specific activities varied among preparations, the relative specific activities of the various subfractions within each preparation were consistent. The inner membrane always had the lowest specific activity and the outer membrane the highest (6–10 times that of the inner). Based on the assumption that galactosyltransferase is located exclusively in the outer envelope membrane,

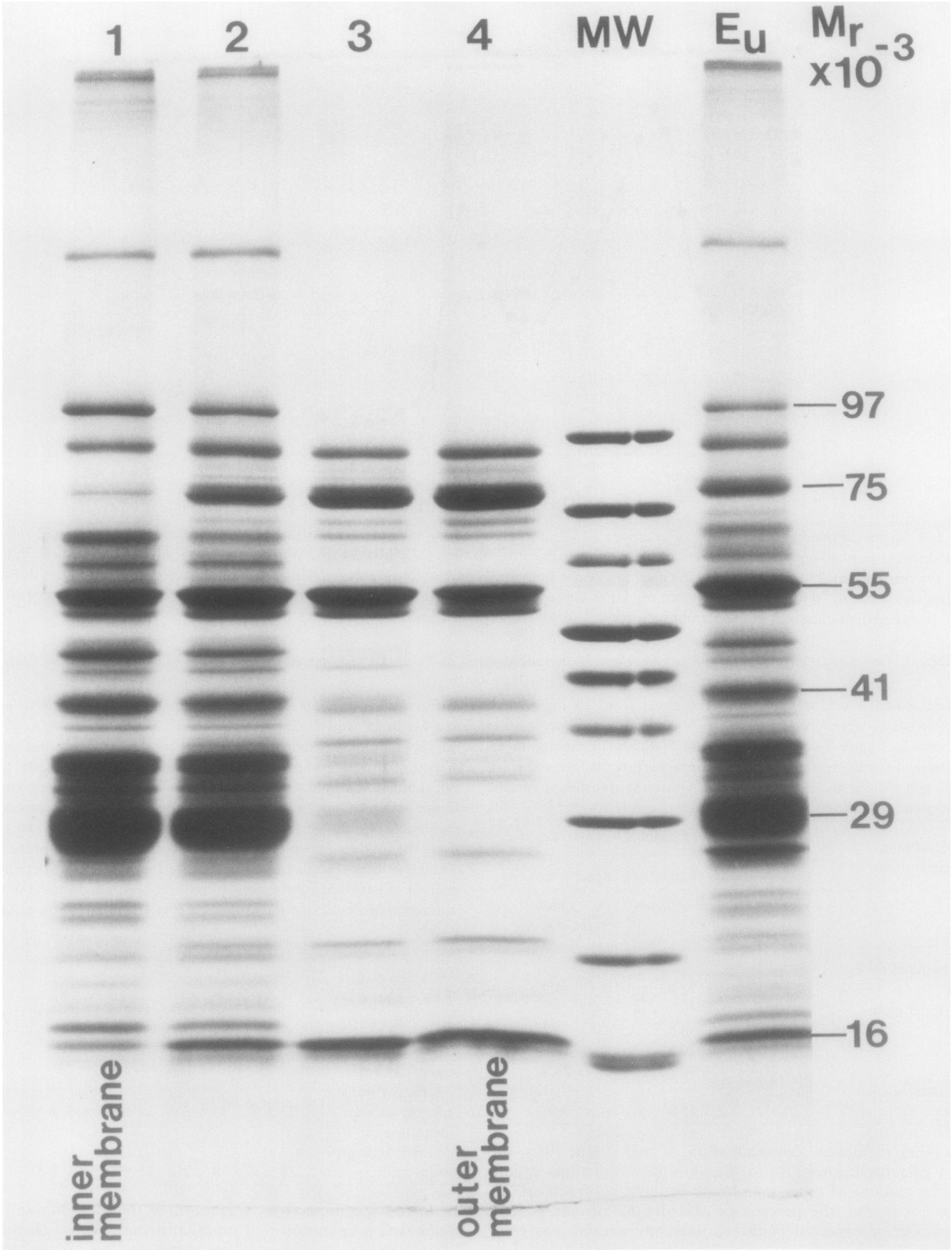

FIG. 3. SDS-polyacrylamide gel electrophoretogram of envelope membrane fractions. Envelope membranes subfractions were prepared and electrophoresis conducted as described in "Materials and Methods." Lanes 1 to 4 are envelope pools 1 to 4, respectively, as shown in Figure 2; E_u are unfractionated envelopes; MW, mol wt markers: phosphorylase b (94,000), BSA (68,000), catalase (57,000), fumarase (49,000), aldolase (40,000), malate dehydrogenase (34,000), carbonic anhydrase (29,000) soybean trypsin inhibitor (21,500), and hemoglobin (14,800). Mol wt of envelope polypeptides were determined using a standard curve of R_f *versus* the logarithm of mol wt for the marker proteins.

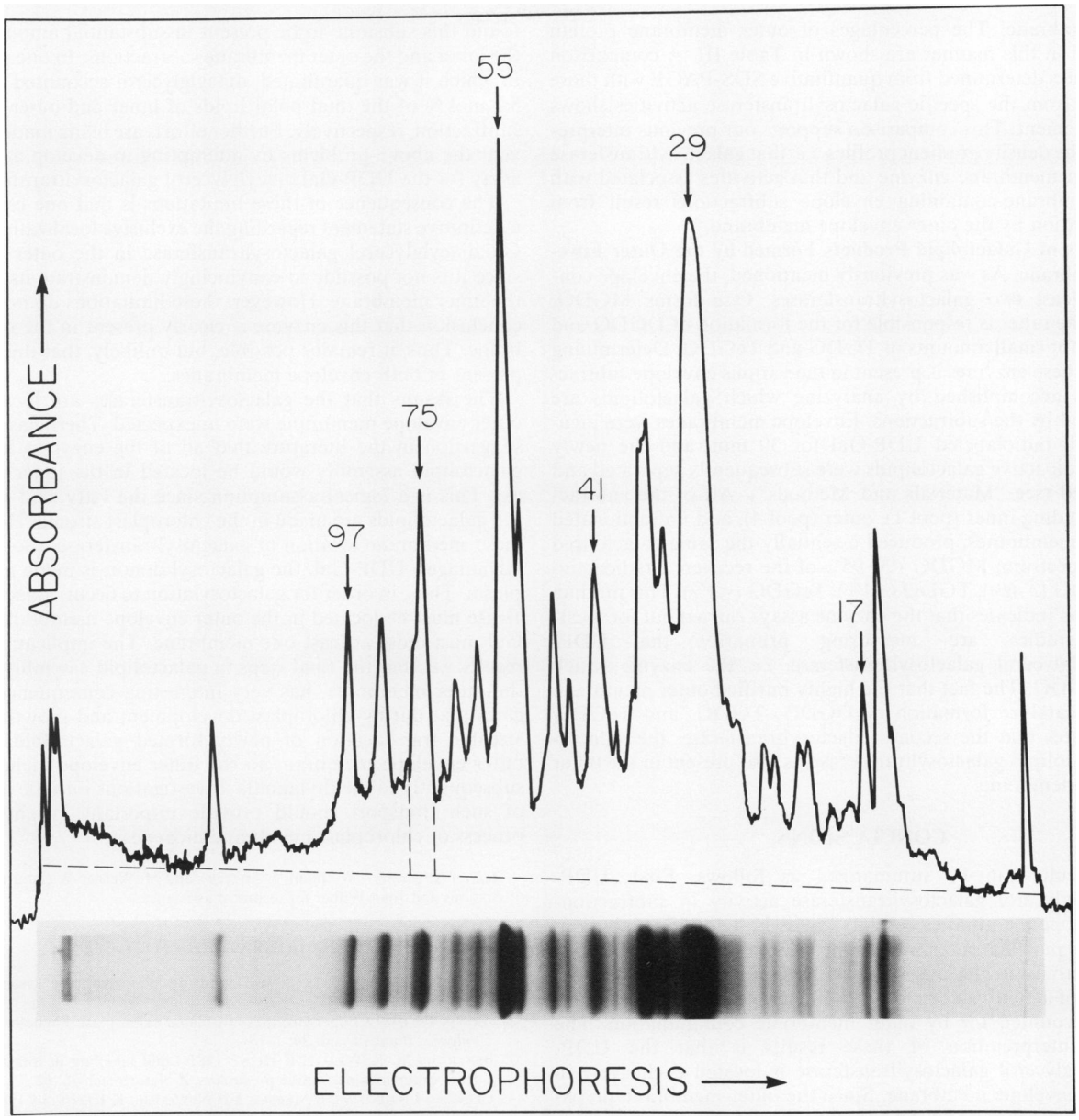

FIG. 4. Quantitation of envelope polypeptides by densitometric analysis of SDS-PAGE profiles. Densitometry of Coomassie-stained gels was performed as described "Materials and Methods." Scans were integrated and the resulting areas taken as relative values for the quantity of protein. The total protein in a gel lane is the area between the scan trace and the baseline. The baseline (dashed line) was arbitrarily chosen as a straight line drawn between a point on the trace near the top of the gel which was devoid of Coomassie staining and a similar point near the bottom of the gel. The amount of individual polypeptides is the area between the scan trace, the baseline, and lines drawn from the base of each side of the peak, perpendicular to the baseline, *i.e.* see the perimeter drawn for the 75 kD polypeptide above. The above scan is of lane 2 of Figure 3.

the percent of outer membrane present in each of the membrane subfractions was calculated from their galactosyltransferase specific activities (Table III). Such an analysis indicates that the purest inner membrane preparation (pool 1) is contaminated approximately 13% with outer membrane protein.

The amount of outer membrane protein in the various subfractions was then estimated by analysis of SDS-polyacrylamide gels of the subfractions. A representative gel profile is shown in Figure 3. A visual examination of the polypeptide patterns yields a qualitative assessment of cross-contamination. The 29 kD polypeptide, which is the dominant polypeptide of the inner envelope membrane (Fig. 3, lane 1), is absent from the outer membrane preparation (Fig. 3, lane 4), indicating that the outer membrane is free from inner membrane contamination. On the other hand, the 75 kD polypeptide, the major outer membrane polypeptide (Fig. 3, lane 4), is present in a small amount in the purest inner membrane subfraction (Fig. 3, lane 1) and to a greater extent in the trailing edge of the inner membrane peak (Fig. 3, lane 2) demonstrating that these membrane subfractions are contaminated by the outer envelope membrane.

Quantitation of this contamination was accomplished by subjecting the Coomassie-stained SDS-PAGE profiles of the membrane subfractions to densitometry. The densitometric scans were integrated and the resulting areas were used as a measure of the amount of protein (Fig. 4). The dominant 75 kD polypeptide was used as a marker with which to measure the amount of outer membrane in other subfractions. For example, in the gel profile shown in Figure 3, the 75 kD polypeptide represented 12.7% of the total protein of the outer membrane (pool 4). In the inner membrane subfraction (pool 1), the 75 kD polypeptide accounted for 1.4% of the total protein. This indicates that (1.4 ÷ 0.127)% or 11% of the total protein in this subfraction originates from the

outer membrane. The percentages of outer membrane protein calculated in this manner are shown in Table III. A comparison of the values determined from quantitative SDS-PAGE with those predicted from the specific galactosyltransferase activities shows good agreement. This comparison supports our previous interpretation of the density gradient profiles, *i.e.* that galactosyltransferase is an outer membrane enzyme and that activities associated with inner membrane-containing envelope subfractions result from contamination by the outer envelope membrane.

Analysis of Galactolipid Products Formed by the Outer Envelope Membrane. As was previously mentioned, the envelope contains at least two galactosyltransferases. One forms MGDG, whereas the other is responsible for the formation of DGDG and probably for small amounts of TGDG and TeGDG. Determining which of these enzymes is present in the various envelope subfractions was accomplished by analyzing which galactolipids are synthesized by the subfractions. Envelope membranes were incubated with radiolabeled UDP-Gal for 30 min, and the newly formed radioactive galactolipids were subsequently separated and quantitated (see "Materials and Methods"). All of the subfractions, including inner (pool 1), outer (pool 4), and unfractionated envelope membranes, produced essentially the same galactolipid product spectrum: MGDG (90–95% of the recovered radioactivity), DGDG (3–6%), TGDG (~1%), TeGDG (<1%). This product distribution indicates that the enzyme assays carried out for localization studies are measuring primarily the UDP-Gal:diacylglycerol galactosyltransferase, *i.e.* the enzyme which forms MGDG. The fact that the highly purified outer membrane will also catalyze formation of DGDG, TGDG, and TeGDG demonstrates that the second galactosyltransferase, the galactolipid:galactolipid galactosyltransferase, is also present in the outer envelope membrane.

CONCLUSIONS

Our results can be summarized as follows. First, UDP-Gal:diacylglycerol galactosyltransferase activity in subfractionated envelope membranes exhibits the highest specific activity in the highly purified outer membrane subfraction and the lowest specific activity in the purest inner membrane fraction. Second, the levels of activity associated with inner membrane subfractions can be accounted for by outer membrane contamination. The simplest interpretation of these results is that the UDP-Gal:diaclyglycerol galactosyltransferase is located exclusively in the outer envelope membrane. Since the outer membrane preparations also synthesize DGDG, TGDG, and TeGDG, we suggest that the galactolipid:galactolipid galactosyltransferase is also located in the outer envelope membrane. This conclusion is consistent with recent results of Dorne *et al.* (5) and our laboratory (in preparation) in which protease treatment of chloroplasts was used to show that the galactolipid:galactolipid galactosyltransferase is an outer membrane enzyme. Thus, the picture which emerges is that both galactosylation steps in the formation of galactolipids occur on the outer envelope membrane.

However, certain limitations of our approach require that a cautionary note be added to the above interpretations. The limitations are those of many studies which rely upon assays of membrane-bound enzymes. For example, access of UDP-Gal to the inside of sealed membrane vesicles may limit detection of the enzyme. In addition, the assays rely upon the presence of an endogenous galactosyl acceptor, diacylglycerol. Under the described assay conditions, UDP-Gal:diacylglycerol galactosyltransferase would be undetected in a membrane fraction which lacks diacylglycerol. In an effort to eliminate this objection, we have measured the diacylglycerol content of subfractions and have found this substrate to be present in substantial amounts both in the inner and the outer membrane subfractions. In one preparation in which it was quantitated, diacylglycerol accounted for 5.6 and 5.2 mol % of the total polar lipids of inner and outer membrane subfraction, respectively. Further efforts are being made to circumvent the above problems by attempting to develop a solubilized assay for the UDP-Gal:diacylglycerol galactosyltransferase.

The consequence of these limitations is that one cannot make a definitive statement regarding the exclusive localization of UDP-Gal:diacylglycerol galactosyltransferase in the outer membrane since it is not possible to convincingly demonstrate its absence in the inner membrane. However, these limitations do not affect the conclusion that this enzyme is clearly present in the outer membrane. Thus, it remains possible, but unlikely, that this enzyme is present in both envelope membranes.

The results that the galactosyltransferases are located in the outer envelope membrane were unexpected. There has been some suggestion in the literature that all of the enzymes involved in galactolipid assembly would be located in the inner membrane (6). This is a logical assumption since the fatty acid moieties of the galactolipids are made in the chloroplast stroma. However, an outer membrane location of galactosyltransferase also has logical advantages. UDP-Gal, the galactosyl donor, is made in the cytoplasm. Thus, in order for galactosylation to occur, galactosyltransferase must be located in the outer envelope membrane or UDP-Gal must cross at least one membrane. The implication of these results, *i.e.* that the final steps in galactolipid assembly occurs on the outer membrane, has very interesting consequences. It indicates that during chloroplast development and growth there is a sizeable translocation of newly formed galactolipids from the outer envelope membrane to the inner envelope membrane and subsequently to the thylakoids. Investigations into the mechanism of such transport should provide important insights into the process of chloroplast membrane biogenesis.

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