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Final Step of Phosphatidic Acid Synthesis in Pea Chloroplasts Occurs in the Inner Envelope Membrane[1]

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ABSTRACT

The second enzyme of phosphatidic acid synthesis from glycerol-3-phosphate, 1-acylglycerophospate acyltransferase, was localized to the inner envelope membrane of pea chloroplasts. The activity of this enzyme was measured by both a coupled enzyme assay and a direct enzyme assay. Using the coupled enzyme assay, phosphatidic acid phosphatase was also localized to the inner envelope membrane, although this enzyme has very low activity in pea chloroplasts. The addition of UDP-galactose to unfractionated pea chloroplast envelope preparations did not result in significant conversion of newly synthesized diacylglycerol to monogalactosyldiacylglycerol. Thus, the envelope synthesized phosphatidic acid may not be involved in galactolipid synthesis in pea chloroplasts.

The synthesis of at least some plastidial glycerolipids occurs in the plastid via PA[3]. *De novo* synthesis of PA can occur upon the addition of glycerol 3-P to intact chloroplasts which are actively synthesizing fatty acids (27, 29); this PA can then be dephosphorylated to form DG. When UDP-Gal is added to spinach chloroplasts, newly synthesized DG is rapidly converted to MGDG (28). In addition, indirect evidence suggests that plastid PG and some SL may also be derived from plastidial-synthesized PA; the precursor-product relationship between these lipids is suggested by the similarities in the patterns of fatty acid pairing and distribution observed in these lipids (16, 30).

To understand more completely the mechanism of PA synthesis and its subsequent distribution into glycerolipids, the localization of its biosynthetic enzymes has been investigated. The assembly of PA appears to occur both in the stroma and in the envelope membranes. The first enzyme involved, glycerophosphate acyltransferase (acyl-ACP: 1-*sn*-glycerol-3-phosphate *O*-acyltransferase), is a soluble plastid activity in both spinach and pea chloroplasts, and is thus probably located in the stroma (4, 5). The second enzyme, 1-acylglycerophosphate acyltransferase (acyl-ACP: 1-acyl-*sn*-glycerol-3-phosphate *O*-acyltransferase), appears to be firmly bound to the envelope membranes in spinach chloroplasts (19). In addition, the enzyme which converts PA into DG, phosphatidate phosphatase (L-α-phosphatidate phosphohydrolase), also appears to be an envelope-bound enzyme (19, 20). Because the envelope preparations used in these earlier localization studies could not be further subfractionated into the inner and outer membranes which together make up the chloroplast envelope (12), the localization of PA synthesis within the envelope system could not be determined.

However, procedures developed in our laboratory allow us to subfractionate pea chloroplast envelopes into two membrane fractions (9). The lighter fraction consists of highly purified outer envelope membranes, while the heavier fraction is greatly enriched in inner envelope membranes (9–11). Thus, we were able to investigate the localization of PA synthesis within pea chloroplast envelope membranes. We first employed an *in vitro* labeling system similar to that used for spinach chloroplasts, in which envelope membranes were incubated with CoA, ATP, [^{14}C]glycerol 3-P and stromal components (19). In subsequent experiments, PA synthesis was directly assayed by adding [^{14}C] palmitoyl-ACP and 1-oleoyl-glycerol 3-P(LPA) to envelope membrane fractions (14).

We report here that 1-acylglycerophosphate acyltransferase, in addition to phosphatidate phosphatase, appeared to be inner envelope membrane activities in pea chloroplasts. However, the addition of UDP-Gal to the *in vitro* labeling system in which DG synthesis occurred did not result in significant MGDG synthesis. Thus, the PA and DG synthesized in pea chloroplast envelopes may not be involved in galactolipid biosynthesis.

MATERIALS AND METHODS

Materials. L-[U-^{14}C]Glycerol 3-P (disodium salt, 144 mCi/mmol) and [1-^{14}C]palmitic acid (53 μCi/μmol) were purchased from New England Nuclear; the labeled glycerol 3-P was diluted to the indicated specific activity with DL-glycerol 3-P (disodium salt, Sigma Chemical Co). Acyl carrier protein (ACP) was purified to ~90% homogeneity from *Escherichia coli* as described by Rock and Cronan (26). Acyl-ACP synthetase was purified from *E. coli* by a modification of the procedure of Rock and Cronan (24) as described previously (22). ATP (disodium salt from equine muscle), CoA (disodium salt from yeast), oleic acid (sodium salt), CDP-choline (sodium salt, from yeast), UDP-galactose (sodium salt), and oleoyl-CoA and palmitoyl-CoA (both as free acids), were purchased from Sigma Chemical Co. Lipid standards were purchased from Serdary, Supelco, or Sigma Chemical Co. All other chemicals were reagent grade. TLC plates (silica gel 60 without fluorescent indicator on glass) were purchased from E. Merck.

Purification and Fractionation of Chloroplasts. Intact chloroplasts were obtained from homogenates of 12- to 16-d old pea seedlings (*Pisum sativum* var Laxton's Progress No. 9) by differential centrifugation followed by Percoll density gradient centrifugation as described (9). Chloroplasts were broken by freeze-

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[3] Abbreviations: PA, phosphatidic acid; ACP, acyl carrier protein; DG, diacylglycerol; glycerol 3-P, glycerol-3-phosphate; LPA, lysophosphatidic acid; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; SL, sulpholipid.

thaw lysis in hypertonic media; envelopes were isolated from broken chloroplasts by flotation centrifugation and then subfractionated by linear density centrifugation (9). The envelope membranes were then used either immediately or within 1 d (after storage at −20°C) for lipid synthesizing assays. Any variations in this procedure are noted in the text and in the figure legends and tables.

Chloroplast stromal extracts were prepared from isolated intact chloroplasts which were lysed by suspension in 10 mM Tricine-NaOH, pH 7.6 (~0.8 mg Chl/ml). This suspension was then centrifuged at 88,000 g_{avg} for 2 h. The straw-colored supernatant, which contained the soluble components of the chloroplasts, was lyophilized to dryness and resuspended in H_2O to ~10 to 15 mg protein/ml. The extracts were then divided into 0.5-ml aliquots and stored frozen (−20°C); they were subsequently used in lipid synthesizing assays within 1 month. The soluble glycerophosphate acyltransferase activity from spinach chloroplasts, when prepared and stored in a similar fashion, was reported to retain most if not all of its activity for at least 1 month (19); the activity of the pea chloroplast enzyme appeared to be as stable.

Lipid Synthesis from [^{14}C]Glycerol 3-P by Pea Chloroplast Envelope Membranes. The procedure for assaying lipid synthesis from [^{14}C]glycerol 3-P was only slightly modified from that described for spinach chloroplasts by Joyard and Douce (19). The lipid synthetic activities were assayed in a coupled enzyme assay, in which envelope membranes were added to CoA, ATP, [^{14}C]glycerol 3-P and soluble chloroplast proteins. Thus, acyl-CoA was synthesized by an envelope membrane enzyme from the added ATP and CoA and endogenous free fatty acids. This acyl-CoA was then utilized by a stromal enzyme to synthesize LPA from glycerol 3-P. The LPA was subsequently metabolized to PA and DG by the envelope membranes.

The reaction mixture contained: Tricine-NaOH, 10 mM, pH 7.6; ATP, 5 mM; [^{14}C]glycerol 3-P (0.6 µCi/µmol), 1 mM; $MgCl_2$, 5 mM; and known amounts of chloroplast stromal extracts and chloroplast envelope membranes in a total reaction volume of 500 µl. Reactions were initiated by the addition of envelope membranes, and incubated at 22 to 24°C for indicated times. The reactions were terminated by extraction of the membrane lipids into chloroform (13). The polar lipids were then separated by two-dimensional TLC (13). Lipid spots were visualized by reversible staining with I_2 vapor, and identified by reactivity with specific stains (1, 23), by R_F values, and by comparison with standards. Radioactive lipids were first located by autoradiography, and then quantitated by scraping and counting the silica which contained them.

Lipid synthesis is expressed as nmol DL-[^{14}C]glycerol 3-P incorporated per mg envelope protein. Total lipid synthesis was determined by counting an aliquot of the chloroform lipid extract; individual lipid synthesis was determined by separating the lipids by TLC and counting the radioactive lipids located by autoradiography. These values were corrected for losses (usually ~20%) which occurred during lipid separation by TLC.

PA Synthesis from [^{14}C]Palmitoyl-ACP and LPA by Pea Chloroplast Envelope Membranes. The procedure for assaying PA synthesis from [^{14}C]palmitoyl-ACP and LPA was that described by Frentzen *et al.* (14). The reaction mixture contained: MOPS-KOH, 250 mM, pH 7.4; LPA, 25 or 50 µM; [^{14}C]palmitoyl-ACP (53 µCi/µmol), 5 µM (3–8 µM); BSA, 30 µg; and 10 to 30 µg envelope protein in a total volume of 80 µl. Reactions were initiated by the addition of envelope membranes, and incubated at 22 to 24°C for up to 10 min. The reactions were terminated by extraction of the membrane lipids into chloroform (5, 14), and a portion of the chloroform layer counted to determine total lipid synthesis. Enzyme activities represent initial rates as determined from plots of product appearance *versus* time. Labeled lipid products were analyzed as described above.

Preparation of [^{14}C]Palmitoyl ACP. [^{14}C]Palmitoyl ACP was prepared by minor modification of the procedure of Rock and Garwin (25). The reaction mixture contained *E. coli* ACP, 75 µM; Tris-HCl, 0.1 M, pH 8; ATP, 5 mM; $MgCl_2$, 10 mM; $LiCl_2$, 0.4 mM; Triton X-100, 2%; DTT, 2 mM; [1-^{14}C]palmitic acid, 188 µM, 15 µCi; and acyl-ACP synthetase, 0.2 units; in a final volume of 1.5 ml. The reaction was allowed to proceed 20 h at 37°C. The mixture was diluted 10-fold with 10 mM Mes, pH 6.1 (buffer 1), and applied to a 1.0-ml DE-52 (Whatman) ion exchange column. The column was washed with several ml of 20 mM Mes, pH 6.1 (buffer 2), followed by 2 to 3 ml of 80% 2-propanol in buffer 2. [^{14}C]Palmitoyl-ACP and unreacted ACP-SH were eluted with 0.6 M NaCl in buffer 2. Fractions containing [^{14}C]palmitoyl-ACP were pooled and applied to a 5-ml column of octyl-sepharose CL-4B (Sigma). The column was washed with 6 ml of 0.6 M NaCl in buffer 2 to remove ACP-SH; the [^{14}C] palmitoyl-ACP was then eluted with 30% 2-propanol in buffer 1, evaporated to dryness under reduced pressure, and dissolved in H_2O to ~80 µM. This solution was then stored frozen at −20°C.

Protein was determined by the Bradford procedure (8) using BSA as the standard. Chl was determined by the method of Arnon (3).

RESULTS

Initial Characterization of Glycerolipid Synthesis from Gylcerol 3-P in Pea Chloroplast Envelopes. Unfractionated pea chloroplast envelope preparations synthesized both PA and DG in the presence of CoA, ATP, glycerol 3-P, and chloroplast stromal extracts (Fig. 1). The initial rates of lipid synthesis were the same when envelopes were obtained from chloroplasts broken either by slow freezing and thawing in hypertonic buffer (Fig. 1A) or by hypotonic lysis (Fig. 1B). The decrease in total lipid synthesis after 40 min by envelopes obtained from frozen and thawed chloroplasts was not observed in subsequent experiments. Thus, the enzymes were not adversely affected by the slow freezing and thawing used to break the chloroplasts. PA was rapidly synthesized from LPA, while DG synthesis from PA occurred much more slowly.

This assay of 1-acylglycerophosphate acyltransferase and phosphatidate phosphatase activities depends upon the synthesis of acyl-CoA by an envelope membrane synthetase from endogenous fatty acids in the presence of exogenous CoA and ATP. However, the origins, quantities, and identities of the endogenous fatty acids were unknown. Efforts to circumvent this difficulty by the addition of either oleic acid (1 mM) in Triton X-100 (0.5%), or oleoyl-CoA (1 mM) instead of CoA and ATP, resulted in decreased glycerolipid synthesis in pea chloroplast envelopes (to 55% or to 10% of control levels, respectively). In addition, the distribution of labeled products were quite different. LPA was the major product after addition of oleic acid with detergent (88.5%), while both LPA and PA were predominant after addition of oleoyl-CoA (47.1% and 38.5%, respectively). These changes may have been due to the detergent effects of either Triton X-100 or oleoyl-CoA, or to the presence of only one acyl substrate, oleoyl-CoA, for glycerolipid synthesis.

The rates of DG synthesis we observed with pea chloroplast envelopes (Fig. 1) occurred at one-tenth to one-half the rates reported previously for spinach chloroplast envelopes (19). Thus, we attempted to increase DG synthesis from glycerol 3-P in pea chloroplast envelopes. Because the optimum activity of spinach chloroplast envelope phosphatidate phosphatase occurs at pH 9 (20), the pH of the reaction mixture to which pea chloroplast envelopes were added was increased to 9 after an initial 30 min incubation at pH 7.6. After a 30-min incubation at pH 9, the pH was then decreased to 8 and the reaction continued for yet another 30 min incubation (Fig. 2). Increasing the pH to 9

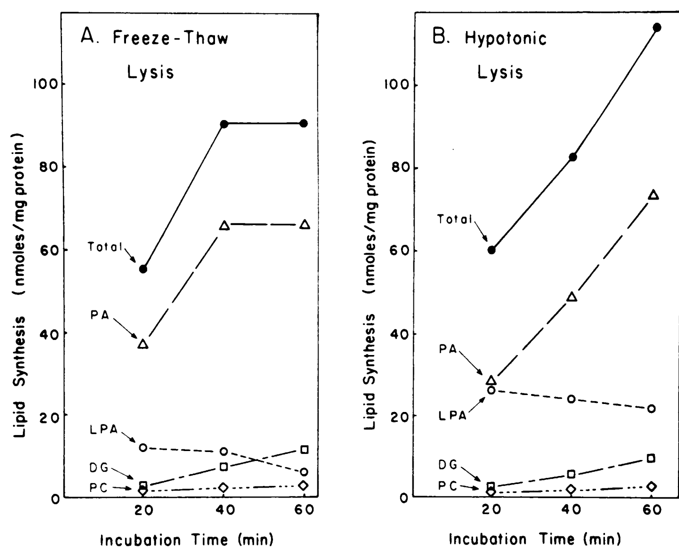

FIG. 1. Lipid synthesis from [^{14}C]glycerol 3-P by pea chloroplast envelope membranes. Envelope fractions were prepared from either: A, chloroplasts broken by slow freezing and thawing while suspended in hyperosmotic medium as described in "Materials and Methods" (Freeze-Thaw Lysis); or B, chloroplasts broken by suspension in hypoosmotic medium (Hypotonic Lysis) as follows. Isolated intact chloroplasts (25 mg Chl) were resuspended in 15 ml 10 mM Tricine-NaOH, pH 7.6 + 2 mM EDTA; after 5 min, sucrose was added to a final concentration of 0.3 M, and the lysed chloroplast suspension layered over two discontinuous sucrose density gradients (11 ml 0.6 M, 8 ml 0.98 M, 7 ml 1.2 M, and 3 ml 1.5 M). The gradients were centrifuged for 80 min, and the envelope membranes collected from the 0.6 M/0.98 M and 0.98 M/1.2 M interfaces as described in "Materials and Methods." Lipid synthesis from [^{14}C]glycerol 3-P was conducted as described in "Materials and Methods," except that [$MgCl_2$] was 1 mM and [glycerol 3-P] was 2 mM, resulting in a specific radioactivity of 0.3 μCi/μmol. Envelope protein in A (0.52 mg) and 0.39 mg of envelope membrane protein in B were added to each sample with 3.0 mg chloroplast soluble proteins. Lipids synthesis is expressed as nmol DL-[^{14}C]glycerol 3-P incorporated per mg envelope protein; MG synthesis (3.6 nmol/mg·h in A and 5.9 nmol/mg·h in B) is not indicated.

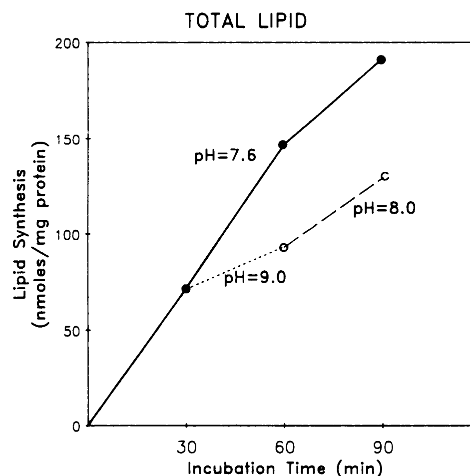

FIG. 2. Effects of changing the reaction mixture pH on total lipid synthesis from [^{14}C]glycerol 3-P by pea chloroplast envelopes. Envelope membranes were prepared and assayed for lipids synthesizing activity from [^{14}C]glycerol 3-P as described in "Materials and Methods." Envelope membrane proteins (0.28 mg) were added to each sample which contained 1.44 mg soluble chloroplast proteins. The reaction mixture pH was increased to 9 by the addition of NaOH, and decreased to 8 by the addition of HCl, at the indicated time. Individual samples were carried through the incubation protocol and terminated at each point. Lipid synthesis is expressed as nmol DL-[^{14}C]glycerol 3-P incorporated per mg envelope protein. (●), Synthesis under control (pH 7.6) conditions; (o), synthesis under changing (pH 9, then pH 8) conditons.

inhibited total lipid synthesis from glycerol 3-P by 70% (Fig. 2); this inhibition was partly reversed by decreasing the pH to 8. When the amounts of radioactivity incorporated into each glycerolipid were examined, the patterns were similar to that observed for total lipid synthesis; the synthesis of each lipid was decreased at pH 9, and only partly, if at all, restored at pH 8. The decrease at pH 9 may have been due to an inhibition of glycerol 3-P acyltransferase activity, as the amount of labeled LPA actually decreased during the high pH incubation. The major observation is that DG synthesis decreased rather than increased at pH 9. One reason may be that $MgCl_2$, which inhibits the spinach chloroplast phosphatidate phosphatase (19), was present in this reaction mixture. However, even lower levels of DG synthesis were observed in the direct assay of PA synthesis (see following), and these assays did not contain $MgCl_2$.

Newly synthesized DG in spinach chloroplast envelopes can be rapidly converted to MGDG upon the addition of UDP-Gal (19). To determine if similar MGDG synthesis can occur in pea chloroplast envelopes, UDP-Gal (final concentration, 1 mM) was included in the reaction mixture to which envelope membranes were added. Only a very small amount of MGDG was synthesized (0.59 nmol synthesized/mg protein·h); this value was about twice that of control reactions which lacked UDP-Gal, and represented only 1.1% of the total radioactivity incorporated into glycerolipids. When attempts were made to increase DG synthesis by increasing the reaction mixture pH to 9, the effects of this pH change on subsequent MGDG synthesis were examined by adding UDP-Gal to the reaction mixture after decreasing the pH to 8. MGDG synthesis increased from 0.11 nmol/mg protein in the absence of UDP-Gal to 1.83 nmol/mg protein when UDP-Gal was added; a concomitant decrease was observed in the amount of DG synthesized. However, these levels are very low when compared to those in spinach chloroplast envelopes, which can synthesize up to 90 nmol MGDG/mg protein·h (19).

Pea chloroplast envelope membranes were also able to synthesize PC from [^{14}C]glycerol 3-P at a low constant rate (Fig. 1). As much as 4 to 5% of the total label incorporated into glycerolipids could be found in PC (Fig. 1 and data not shown). The addition of CDP-choline did not increase the amount of PC synthesized (data not shown).

Localization of 1-Acylglycerophosphate Acyltransferase and Phosphatidate Phosphatase Activities within Pea Chloroplast Envelopes by Lipid Synthesis from Glycerol 3-P. Unfractionated pea chloroplast envelope preparations were subfractionated into two membrane fractions by centrifugation through a linear density sucrose gradient (0.6–1.2 M sucrose) (Fig. 3). The lighter fraction (ρ = 1.08 g/cm^3) consists of highly purified outer envelope membranes, while the heavier fraction (ρ = 1.13 g/cm^3) is enriched in inner envelope membranes (9–11). The indicated fractions were pooled, and the membranes collected by dilution and centrifugation. Each membrane fraction was then assayed for the presence of monoacylglycerol 3-P acyltransferase and phosphatidate phosphatase activities by the coupled enzyme assay described in "Materials and Methods."

The most highly purified inner envelope membrane preparation (pool 1) actively synthesized both PA and DG, while the outer envelope membrane fractions (pools 3 and 4) did so only very slowly (Fig. 4). Thus, it appeared that synthesis of both PA and DG occurred in the inner envelope membrane of pea chloroplasts. A similar localization of phosphatidate phosphatase activity was reported for spinach chloroplasts (7), in which the

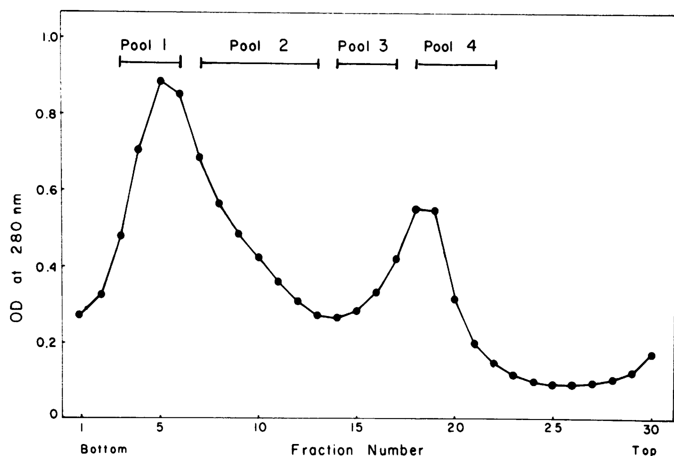

FIG. 3. Fractionation of chloroplast envelope membranes by linear density gradient centrifugation. Unfractionated membranes isolated from 16.8 mg Chl of intact pea chloroplasts were sedimented through a 0.6 to 1.2 M sucrose linear density gradient (9). Gradient fractions were 1.25 ml. For analysis of the membranes, pools 1 to 4 were made as shown, and the membranes recovered by dilution and centrifugation.

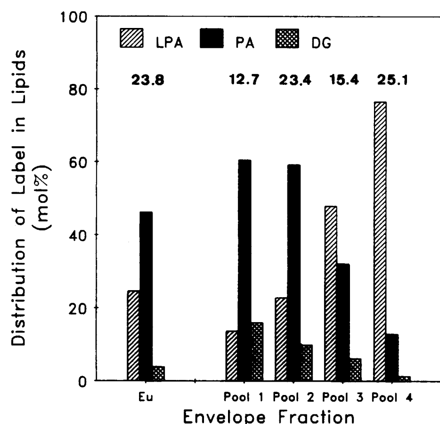

FIG. 4. Lipid synthesis from [14C]glycerol 3-P by various envelope membrane subfractions. Pea chloroplast envelope membranes were prepared and subfractionated as described in Cline *et al.* (9) and Figure 3; lipid synthesis from [14C]glycerol 3-P was assayed as described in "Materials and Methods." Incubations were initiated by adding the following amounts of envelope membrane protein to reaction mixtures which contained 1.44 mg soluble chloroplast proteins: E_u, 0.29 mg; pool 1, 0.27 mg; pool 2, 0.21 mg; pool 3, 0.10 mg; and pool 4, 0.10 mg. Incubations were terminated at 20 min. Total lipid synthesis, indicated above each bar graph, is expressed as nmol DL-[14C]glycerol 3-P incorporated per mg envelope protein. E_u = unfractionated envelope membrane preparations.

same type of coupled enzyme assay was used.

Interpretation of the results in Figure 4 is limited due to several constraints imposed by the nature of the coupled enzyme assay used. First, synthesis of acyl-CoA, which is used as an acyl donor by the acyltransferases, is carried out by an outer envelope membrane enzyme (2, 6). Because inner envelope membrane preparations (pool 1) are contaminated by outer envelope membranes, inner membrane preparations carry out the synthesis of acyl-CoA, albeit at a much lower rate than outer envelope membrane fractions (2). Since each membrane fraction was incubated with the same amount of chloroplast stromal extract and thus the same amount of glycerophosphate acyltransferase activity, the slower rates of acyl-CoA synthesis in the inner envelope membrane fraction probably account for the lower rate of total lipid synthesis observed in this fraction. Second, as described earlier, the assay system depends upon endogenous envelope membrane fatty acids for acyl-CoA synthesis, and the quantity and identity of these fatty acids in each membrane are unknown. Third, PA synthesis is required in order to observe DG synthesis. Thus, it would be difficult to measure accurately the amount of phosphatase activity in a membrane which lacked 1-acylglycerophosphate acyltransferase.

It is interesting to note that low levels of both PA and DG synthesis can be observed in the outer envelope membrane fractions (Fig. 4, pools 3 and 4). Although these fractions are highly purified, some contamination by inner envelope membrane does occur, as indicated by the presence of an inner envelope membrane activity, acyl-CoA thioesterase, in the outer envelope membrane fractions (2). Additionally, the small amounts of lipid synthesizing activities derived from the contaminating inner envelope membrane may be enhanced by the high levels of acyl-CoA synthesized by the outer envelope membrane. Such enhancement may account for the levels of PA and DG synthesis observed in the outer envelope membrane preparation.

Initial Characterization of PA Synthesis from [14C]Palmitoyl-ACP and Exogenous LPA in Pea Chloroplast Envelopes. Because of the problems inherent in the coupled enzyme assay, a more direct measurement of PA synthesis was accomplished by adding the immediate substrates for PA synthesis, LPA, and acyl-ACP to the envelope membranes (14). In this procedure, isolated pea chloroplast envelope membranes were incubated in the presence of exogenous 1-oleoyl-glycerol 3-P (LPA) and [14C]palmitoyl-ACP at pH 7.6 ("Materials and Methods"). Radioactivity was incorporated into only one lipid product, which was analyzed by two-dimensional TLC and determined to be PA (not shown). No lipid products were seen in the absence of added LPA. Thus, the assay was specific for the acyl-ACP 1-acylglycerophosphate acyltransferase activity in the envelope membranes.

The enzyme was 3 times more active at pH 7.6 than at pH 6 (not shown). Although the apparent activity was greater at pH 9, as indicated by chloroform-soluble radioactivity, product analysis indicated that only one-half of this material was PA, while the remainder was free fatty acid. Thus, the net formation of PA occurred at about the same rate at pH 9 as it did at pH 7.6. It is interesting to note that at pH 9, newly synthesized PA was not converted to DG within 6 min. In other experiments, longer incubation periods (up to 42 min) at pH 7.6 resulted in only 3.9% of the incorporated label appearing in DG, even though 50% of the [14C]palmitoyl-ACP was utilized for PA synthesis within the first 2 min. Slightly less labeled DG was observed (2.6%) if the pH was increased to 9 after an initial 2 min at pH 7.6, and the incubation continued for 40 min. The total amount of incorporated label (86–88%) was the same in both cases, and occurred predominantly in PA. These results were similar to the low level of DG formation observed in pea chloroplast envelopes by Frentzen *et al.* (14), although they reported that DG formation is greater at the higher pH.

The transferase activity did not appear to require $MgCl_2$, and was in fact slightly inhibited by the presence of these ions (to 80% of control activity at 2-5 mM $MgCl_2$). The enzyme was stable to storage at either 4 or −20°C; activity decreased to ~70% of control levels after 1 d at 4°C, while at −20°C activity was maintained for up to 2 weeks (~98% of control levels). Finally, although the enzyme was inhibited by higher concentrations of Triton X-100 (to 36% control activity at 0.1% Triton X-100, w/v), it was unaffected by the detergent at 0.01%. This eliminated any potential difficulties with substrate access problems in envelope vesicles.

The addition of [14C]palmitoyl-CoA instead of [14C]palmitoyl-ACP, but at the same concentration (6 μM), resulted in only very low levels of PA synthesis, 0.200 nmol PA/mg·min, which was 7% the activity seen with palmitoyl-ACP. The greatest proportion of the chloroform-soluble lipid products (~93.5%) migrated with

free fatty acids. Thus, palmitoyl-CoA was not a good substrate for the acyltransferase, in agreement with the work of Frentzen *et al.* (14). In addition, the envelope membranes possess an active acyl-CoA thioesterase (2), which competed successfully with the acyltransferase for the acyl-CoA substrate.

Localization of 1-Acylglycerophosphate Acyltransferase Activity within Pea Chloroplasts by PA Synthesis from LPA. Pea chloroplasts were fractionated by a slight modification of the procedure described in "Materials and Methods." Broken chloroplasts were directly fractionated into stromal components, envelopes, and thylakoids by a single sedimentation centrifugation step (Table I); 1-acylglycerophosphate acyltransferase activity was then assayed in each fraction. The stromal and thylakoid fractions were assayed directly from the gradient, while the envelope membrane fraction was washed first by dilution, sedimention, and resuspension, as described. The specific activity was greatest in the envelope membranes (Table I), which leads to the conclusion that the enzyme was located in this fraction.

Table I. *Localization of 1-Acylglycerophosphate Acyltransferase Activity within Pea Chloroplasts*

Isolated intact chloroplasts were subfractionated by a slight modification of the procedure described in "Materials and Methods." The broken chloroplast suspension was diluted to 0.3 M sucrose (6 mg Chl/5 ml) and then layered over the following sucrose step gradient: 3 ml 1.3 M; 1 ml 1.2 M; 4 ml 1.1 M; 4 ml 0.5 M. The gradient was centrifuged for 90 min at 116,000g_{avg}. The stromal components remained within the 0.3 M sucrose sample layer; the envelope membranes sedimented to the 0.5/1.1 M interface; and the thylakoid membranes moved to the 1.3 M layer. Unbroken chloroplasts formed a pellet at the bottom of the gradient, and represented ~30% of the initial chloroplast sample. The stromal components, thylakoid membranes, and envelope membranes were collected separately with a Pasteur pipet; the first two fractions were directly assayed for 1-acylglycerophosphate acyltransferase activity (direct assay of PA synthesis), while the envelope membrane fraction was first concentrated by dilution, sedimentation, and resuspension.

Chloroplast Fraction	Protein Distribution	Enzyme Activity	
		Specific activity	Activity distribution
	% total	*nmol/mg protein·min*	*% total*
Envelopes	0.97	3.20	36.6
Thylakoid	28.3	0.184	61.4
Stroma	70.8	0.0024	2.0

Envelope membranes from pea chloroplasts were isolated and subfractionated as described in "Materials and Methods." The appropriate fractions were pooled and the membranes collected by the procedure described in the text and in the legend to Figure 3. 1-Acylglycerophosphate acyltransferase activity was then assayed in each membrane fraction. The specific activity was greatest in pool 1, indicating that the transferase was located in the inner envelope membrane (Table II). Nearly identical results were obtained in the presence of Triton X-100 (data not shown). The distribution of total activity is similar to that observed for another inner envelope membrane enzyme, acyl-CoA thioesterase (2).

More than one-half of the total 1-acylglycerophosphate acyltransferase activity within pea chloroplasts was associated with the thylakoid fraction (Table I). This is similar to the distribution observed for another inner envelope membrane enzyme, acyl-CoA thioesterase (2), and contrasts with the distribution of two outer envelope membrane enzymes, acyl-CoA synthetase (2) and galactosyltransferase (10), in which the greater proportion of total chloroplast activity is associated with the envelope fraction. These results suggest either that the inner envelopes preferentially contaminate the thylakoid fraction, or that separate activities for both the acyltransferase and the acyl-CoA thioesterase exist in the thylakoid and envelope membranes. Although the greater specific activity in the envelope fraction supports the former hypothesis, it is not yet possible to distinguish between these two possibilities.

DISCUSSION

Pea chloroplast envelopes possessed 1-acylglycerophosphate acyltransferase activity, and at much lower levels, phosphatidate phosphatase activity, These enzymes were assayed by two methods. In the first, both enzymes were measured in the presence of [^{14}C]glycerol 3-P, soluble chloroplast proteins, and necessary

Table II. *Localization of 1-Acylglycerophosphate-Acyltransferase Activity within Pea Chloroplast Envelope Membranes*

Envelope membranes were isolated, fractionated, and pooled as described in "Materials and Methods" and Figure 3; 1-acylglycerophosphate acyltransferase was assayed in each membrane pool as described in "Materials and Methods" by the direct assay of PA synthesis. Experiment I represents a single set of enzyme activity measurements, while experiment II represents the average of two sets of enzyme measurements.

	Membrane Preparation				
	E_u (Unfractionated envelopes)	Pool 1 (Inner envelope)	Pool 2 (Mixed inner and outer envelopes)	Pool 3 (Outer envelope)	Pool 4 (Outer envelope)
Experiment I					
Specific activity (nmol/mg·min)	7.29	10.39	9.58	0.615	0.049
Activity distribution (% total)		40.4	58.1	1.4	0.10
Protein distribution (% total)		27.2	42.4	15.6	14.8
Experiment II					
Specific activity (nmol/mg·min)	3.07	6.99	4.52	0.308	0.027
Activity distribution (% total)		46.6	52.3	1.0	0.10
Protein distribution (% total)		24.5	48.8	14.5	12.2

cofactors, which resulted in the appearance of labeled PA and DG (Figs. 1 and 4). The levels of both activities varied between preparations by as much as 3-fold. The rate of DG synthesis, though generally proportional to the rate of PA synthesis, was always much lower. The highest rates of PA synthesis which we observed in pea chloroplast envelopes were comparable to those observed in spinach chloroplast envelopes (19). However, we observed that DG synthesis occurred consistently at much lower rates than those reported for spinach (19). This low level of phosphatase activity in isolated pea chloroplast envelopes confirms the conclusions derived from several studies of lipid synthesis in intact chloroplasts (15, 17).

In the second assay, 1-acylglycerophosphate acyltransferase activity was directly measured by adding [^{14}C]palmitoyl-ACP and 1-oleoyl-glycerol 3-P (LPA) to envelope membranes. This activity was localized to the inner envelope membranes of pea chloroplasts (Tables I and II); these observations confirmed the localization deduced from the more indirect coupled enzyme assay (Fig. 4). The synthesis of DG from PA occurred at much lower levels in the direct assay system ("Results") than in the coupled enzyme assay (Figs. 1 and 4). This difference may be due to the potentially larger number of molecular species of PA synthesized in the coupled enzyme assay, some of which may be better substrates for the phosphatidate phosphatase.

Although Joyard and Douce (19) report that spinach chloroplast envelopes rapidly convert newly synthesized DG to MGDG upon the addition of UDP-Gal, we observed that pea chloroplast envelopes did so only very slowly ("Results"). This difference is very dramatic; the extent of galactosylation of newly synthesized DG in pea chloroplast envelopes was about one-thirtieth that seen in spinach chloroplast envelopes. These differences observed in isolated envelopes confirm results obtained from investigations of lipid synthesis in intact chloroplasts. Thus, during *de novo* glycerolipid synthesis from newly synthesized fatty acids and endogenous glycerol 3-P in isolated, intact chloroplasts, Heinz and Roughan observed that the addition of UDP-Gal results in a rapid disappearance of DG and a rapid appearance of MGDG in spinach chloroplasts, but in only a very slow appearance of MGDG in pea chloroplasts (17); the difference between spinach and peas in the rate of appearance of MGDG is about 20-fold.

The differences observed in the rates of *de novo* glycerolipid synthesis between pea and spinach chloroplasts may be due to the different metabolic requirements of each plant. The very low levels of galactosylation of newly synthesized DG, as well as the low levels of DG synthesis from newly synthesized PA, in pea chloroplast envelopes suggest that this lipid is not incorporated into pea chloroplast galactolipids. This further supports the hypothesis that, in peas, the DG backbone of the galactolipids may originate in the cytoplasm (28). However, the fact that PA was actively synthesized by the envelopes implies that it could be incorporated into other pea chloroplast lipids, *e.g.* PG.

The localization of 1-acylglycerophosphate acyltransferase to the inner envelope membrane of pea chloroplasts is topologically sensible for at least two reasons. The first is that LPA synthesized by the stromal enzyme glycerophosphate acyltransferase (4, 5) is probably inserted into the nearest membrane, which is the inner envelope membrane, where it can then act as a substrate for 1-acylglycerophosphate acyltransferase. The second reason is that an inner envelope membrane location for 1-acylglycerophosphate acyltransferase allows the enzyme access to its physiological acyl donor, acyl-ACP, which is probably found exclusively in the stroma (21). Finally, the localization of PA and DG synthesis in the inner envelope membrane suggests that, if PA is also a precursor to other plastid synthesized lipids (*e.g.* PG), then the enzymes catalyzing the synthesis of these other lipids may also be localized to the inner envelope membrane.

The synthesis of PC from [^{14}C]glycerol 3-P by pea chloroplast envelopes (Fig. 1 and data not shown) was unexpected, as spinach chloroplast envelopes are unable to synthesize PC from added CDP-choline (18). During their investigation of PG synthesis by isolated intact spinach chloroplasts, Sparace and Mudd (30) noted that up to 2% of the [^{14}C]glycerol 3-P incorporated into spinach chloroplast lipids migrated with PC. In both pea chloroplast envelopes and intact spinach chloroplasts, CDP-choline either did not result in increased PC synthesis (data not shown) or was not metabolized (30). This is in good agreement with the results obtained with spinach chloroplast envelopes. Thus, PC synthesis did not appear to occur via CDP-choline:1,2-diacylglycerol cholinephosphotransferase activity.

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LITERATURE CITED

1. ALLEN CF, P GOOD 1971 Acylipids in photosynthetic systems. Methods Enzymol 23: 523–547
2. ANDREWS J, K KEEGSTRA 1983 Acyl-CoA synthetase is located in the outer membrane and acyl-CoA thioesterase in the inner membrane of pea chloroplast envelopes. Plant Physiol 72: 735–740
3. ARNON DI 1949 Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1–15
4. BERTRAMS M, E HEINZ 1976 Experiments on enzymatic acylation of *sn*-glycerol-3-phosphate enzyme preparations from pea and spinach leaves. Planta 132: 161–168
5. BERTRAMS M, E HEINZ 1981 Positional specificity and fatty acid selectivity of purified *sn*-glycerol-3-phosphate acyltransferase from chloroplasts. Plant Physiol 68: 653–657
6. BLOCK MA, A-J DORNE, J JOYARD, R DOUCE 1983 The acyl-CoA synthetase and acyl-CoA-thioesterase are located on the outer and inner membrane of the chloroplast envelope, respectively. FEBS Lett 153: 377–381
7. BLOCK MA, A-J DORNE, J JOYARD, R DOUCE 1983 The phosphatidic acid phosphatase of the chloroplast envelope is located on the inner envelope membrane. FEBS Lett 164: 111–115
8. BRADFORD M 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal Biochem 72: 248–254
9. CLINE K, J ANDREWS, B MERSEY, EH NEWCOMB, K KEEGSTRA 1981 Separation and characterization of inner and outer envelope membranes of pea chloroplasts. Proc Natl Acad Sci USA 78: 3595–3599
10. CLINE K, K KEEGSTRA 1983 Galactosyltransferases involved in galactolipid biosynthesis are located in the outer membrane of pea chloroplast envelopes. Plant Physiol 71: 366–372
11. CLINE K, K KEEGSTRA, A STAEHLIN 1985 Freeze-fracture electron microscopic analysis of ultrarapidly frozen envelope membranes on intact chloroplasts and after purification. Protoplasma. 125: 111–123
12. DOUCE R, J JOYARD 1979 Structure and function of the plastid envelope. Adv Bot Res 7: 1–116
13. DOUCE R, J JOYARD 1980 Chloroplast envelope lipids: Detection and biosynthesis. Methods Enzymol 69: 290–301
14. FRENTZEN M, E HEINZ, T MCKEON, PK STUMPF 1983 Specificities and selectivities of glycerol-3-phosphate acyltransferase and monoacylglycerol-3-phosphate acyltransferase from pea and spinach chloroplasts. Eur J Biochem 129: 629–636
15. GARDINER SE, PG ROUGHAN 1983 Relationship between fatty-acyl composition of diacylgalactosylglycerol and turnover of chloroplast phosphatidate. Biochem J 210: 949–952
16. HAAS R, HP SIEBERTZ, K WRAGE, E HEINZ 1980 Localization of sulpholipid labelling within cells and chloroplasts. Planta 148: 238–244
17. HEINZ E, PG ROUGHAN 1983 Similarities and differences in lipid metabolism of chloroplasts isolated from 18:3 and 16:3 plants. Plant Physiol 72: 273–279
18. JOYARD J, R DOUCE 1976 L'enveloppe des chloroplastes este-elle capable de synthetiser la phosphatidylcholine? CR Hebd Sceances Acad Sci Ser D 282: 1515–1518
19. JOYARD J, R DOUCE 1977 Site of synthesis of phosphatidic acid and diacylglycerol in spinach chloroplasts. Biochim Biophys Acta 486: 273–285
20. JOYARD J, R DOUCE 1979 Characterization of phosphatidate phosphohydrolase activity associated with chloroplast envelope membranes. FEBS Lett 102: 147–150
21. OHLROGGE JB, DN KUHN, PK STUMPF 1979 Subcellular localization of acyl carrier protein in leaf protoplasts of *Spinacia oleracea*. Proc Natl Acad Sci USA 76: 1194–1198
22. OHLROGGE JB, T-M KUO 1984 Control of lipid synthesis during soybean seed development. Enzymic and immunochemical assay of acyl carrier protein.

Plant Physiol 74: 622–625

23. RENKONEN O, A LUUKKONEN 1976 Thin-layer chromatography of phospholipids and glycolipids. *In* G Marinetti, ed, Lipid Chromatographic Analysis. Marcell Decker, New York, pp 1–50
24. ROCK CO, JE CRONAN JR 1979 Solubilization, purification, and salt activation of acyl-acyl carrier protein synthetase from *Escherichia coli*. J Biol Chem 254: 7116–7122
25. ROCK CO, JL GARWIN 1979 Preparative enzymatic synthesis and hydrophobic chromatography of acyl-acyl carrier protein. J Biol Chem 254: 7123–7128
26. ROCK CO, JE CRONAN JR 1980 Improved purification of acyl-carrier protein. Anal Biochem 102: 362–364
27. ROUGHAN PG, R HOLLAND, CR SLACK 1979 On the control of long-chain fatty acid synthesis in isolated *Spinacia oleracea* chloroplasts. Biochem J 184: 193–202
28. ROUGHAN PG, R HOLLAND, CR SLACK 1980 The role of chloroplasts and microsomal fractions in polar-lipid synthesis from [1-^{14}C] acetate by cell-free preparations from spinach (*Spinacia oleracea*) leaves. Biochem J 188: 17–24
29. ROUGHAN PG, CR SLACK, R HOLLAND 1976 High rates of [1-^{14}C] acetate incorporation into the lipid of isolated spinach chloroplasts. Biochem J 158: 593–601
30. SPARACE SA, JB MUDD 1982 Phosphatidylglycerol synthesis in spinach chloroplasts: Characterization of the newly synthesized molecule. Plant Physiol 70: 1260–1264