MEMBRANE LIPID METABOLISM IN 
CHLAMYDOMONAS REINHARDTII 137* 
AND Y-1:
I. BIOCHEMICAL LOCALIZATION AND 
CHARACTERIZATION OF ACYLTRANSFERASE 
ACTIVITIES

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SUMMARY

The acyltransferases involved in the synthesis of the chloroplast membrane glycerolipids were analysed biochemically in dark-grown and greening Chlamydomonas reinhardtii y-1 as well as in the synchronous wild-type algae (strain 137+) and wild-type membranes. Using oleoyl-CoA as a substrate, three acyltransferase enzyme activities were detected. Glycerol-3-phosphate (glycerol-3-P) acyltransferase exhibited a pH optimum of 8.0 and was inhibited by addition of N-ethylmaleimide (MalNEt). Lysophosphatidate (PtdLys) acyltransferase exhibited a pH optimum of 7.0 and was not affected by the addition of MalNEt. From preliminary analyses, the activity at pH 5.5 appeared to be associated with dihydroxyacetone phosphate acyltransferase activity.

Both glycerol-3-P and PtdLys acyltransferases were analysed further and found to be present in dark-grown and light-induced y-1 cells as well as in synchronous 137+ cells and their photosynthetic membranes. Both enzyme activities were enriched at least 10-fold in the photosynthetic membranes of 137+ chloroplasts relative to the activities present in the whole cells. This enrichment is indicative of their intrinsic localization in the thylakoids, suggesting that the photosynthetic membranes exhibit a greater degree of autonomy with respect to the synthesis of their membrane lipids than previously reported.

A role for glycerol-3-P and PtdLys acyltransferases in the synthesis of the chloroplast membrane lipids is suggested further by the increases in both enzyme activities coincident with and preceding thylakoid biogenesis following light induction of dark-grown y-1 cells. Increased acyltransferase activity preceded the increase in the chlorophyll content of greening y-1 cells, which is a generally accepted marker for thylakoid synthesis. The increase in the PtdLys acyltransferase activity upon light-induction of the y-1 cells was both more immediate and more dramatic than the increase in glycerol-3-P acyltransferase activity. PtdLys acyltransferase activity was negligible in dark-grown cells and the dramatic increase upon light induction may be important in the subsequent initiation of chloroplast membrane lipid synthesis. On the basis of the localization of acyltransferase enzyme activities to the photosynthetic membranes of 137+ cells and the increase in acyltransferase activity both preceding and occurring in concert with thylakoid synthesis, we propose a direct role for the photosynthetic membranes in the synthesis of their membrane lipid components.

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INTRODUCTION

Elucidation of the processes involved in membrane biogenesis requires analysis of the sites of synthesis and assembly of membrane proteins and lipids. Previous research in this laboratory has contributed to the localization of lipid synthesis during membrane biogenesis in mammalian cells. These studies have dealt primarily with cells actively involved in membrane synthesis, either as a result of development (Benes, Higgins & Barnett, 1973), physiological stress (Levine, Higgins & Barnett, 1972) or drug administration (Higgins & Barnett, 1972). The results of these studies have repeatedly demonstrated that the lipids required for membrane assembly can be formed in situ in several subcellular compartments, particularly at times of rapid membrane synthesis. The present study investigates the role of the chloroplast photosynthetic membranes (thylakoids) in the synthesis of their glycerolipids.

The high percentage of unusual acyl lipids in thylakoids would argue a priori for chloroplast involvement in the synthesis of its membrane lipids. Four-fifths of the polar thylakoid lipid are glycolipids (Wintermans, 1960) and of these, 90% are either monogalactosyl or digalactosyl diglyceride, with sulpholipid and phospholipid contributing much of the remainder (Benson, 1971). Since phosphatidic acid is the common precursor of all these diacylglycerolipids, the enzymes responsible for phosphatidic acid formation seem to be enzymes critical to chloroplast membrane synthesis. Acyltransferases catalyse the transfer of fatty acids from acyl coenzyme A (acyl-CoA) to glycerol 3-phosphate (glycerol-3-P) or dihydroxyacetone phosphate (glycerone-P) to yield the monoacylated forms of these compounds. The resultant lysophosphatidic acid (PtdLys) can then accept a second acyl moiety from acyl-CoA to yield diacylglycerol 3-phosphate, i.e. phosphatidic acid. Acyltransferase activity, together with phosphatidic acid phosphatase, the enzyme that is responsible for diglyceride formation, have been reported to be associated with the envelope of spinach chloroplasts (Joyard & Douce, 1977, 1978, 1979). Similarly, the chloroplast envelope appears to be the site for the incorporation of galactose into galactolipids (Douce, 1974; VanHummel, Hulsebos & Wintermans, 1975; Joyard & Douce, 1976). In all instances, the photosynthetic membranes themselves have been reported to be devoid of lipid-synthesizing activity. However, localization of lipid-synthesizing enzymes to the chloroplast envelope of spinach has been by way of enzymic analysis of membranes purified from isolated chloroplasts; the possible loss of specific membrane activities in such studies, either by detachment or inactivation during the isolation procedures, remains to be assessed. It is the absence of membrane-bound glycerol-3-P acyltransferase activity in the studies on the spinach chloroplasts (Joyard & Douce, 1977) that specifically raises some question about this possibility, based on the reported ease with which microsomal and mitochondrial glycerol-3-P acyltransferase are solubilized (Yamashita & Numa, 1972; Monroy, Chroboczek-Kelker & Pullman, 1973).

These earlier studies, furthermore, have been performed exclusively in systems in which photosynthetic membranes are already assembled, rather than in systems undergoing thylakoid biogenesis. Our studies in mammalian systems have shown repeatedly that during periods of rapid membrane formation, subcellular membranes
prove to be more autonomous than expected on the basis of analysis of homeostatic systems, suggesting a role for these membranes in the synthesis of their lipids. This may represent a real difference in the localization of the lipid-synthesizing enzymes during different stages of development, or may merely reflect the limits of detection of the assay procedures employed, with enzyme activity being present at all stages of development, but measurable only when activity is maximal. Therefore, in the present study, we chose to analyse cells in which active synthesis of the photosynthetic membranes was occurring.

The green alga *Chlamydomonas reinhardti* has been used extensively to study the biogenesis of photosynthetic membranes. Most of the previous work, however, has been concerned primarily with the synthesis and assembly of the membrane polypeptides. These studies have demonstrated convincingly that proteins of both cytoplasmic and chloroplast origin are required for synthesis and assembly of the photosynthetic lamellae (Eytan & Ohad, 1970). The extent to which the chloroplast contributes to the synthesis and assembly of the lipid components of these membranes remains debatable. To date, synthesis of thylakoid lipid in *C. reinhardti* has been studied either by *in situ* radiolabelling and radioautographic studies (Goldberg & Ohad, 1970a, b; Eytan & Ohad, 1972), or by biochemical analysis of membranes isolated during the late stages of greening (dePetrocelli, Siekevitz & Palade, 1970). Neither the radioautographic studies nor radiolabelling studies can ascertain unequivocally the actual site(s) of membrane lipid synthesis. The biochemical analyses in turn are susceptible to the criticisms listed above, i.e. possible loss or inactivation of enzyme activities during membrane isolation. Additionally, there is the possibility that enzymes intrinsic to the thylakoids may be considerably less active late in the greening period when reproducible membrane isolation is possible. Therefore, enzyme activities maximally expressed early in the greening period, when the majority of photosynthetic membrane synthesis is occurring but when membrane isolation is difficult, may not be detected in the membranes isolated during the late stages of greening.

While recognizing the limitations of such biochemical approaches, we have attempted to deal with these issues by analysing two strains of *C. reinhardti*, each of which has properties that are ideally suited to more definitive analyses of the role of the photosynthetic membranes in the synthesis of their membrane lipids. The yellow mutant (strain y-1) synthesizes chloroplasts upon exposure to light. This property enables one to monitor more closely the temporal relationship between the activity of the acyltransferases and the synthesis and assembly of the photosynthetic membranes. Since the thylakoid membranes are difficult, if not impossible, to isolate during the early stages of greening (dePetrocelli *et al.* 1970), analysis of these cells alone can only allow one to assess whether changes in acyltransferase activity can be shown to be related causally to thylakoid membrane synthesis. On the other hand, the chloroplast photosynthetic membranes of the wild-type algae (strain 137⁺) have been routinely isolated in highly purified form (dePetrocelli *et al.* 1970). Additionally, these cells can be synchronized using alternating periods of light and dark, and under these conditions, the 137⁺ cells exhibit fluctuations in chloroplast synthetic activity, which allows for biochemical analysis of the isolated photosynthetic membranes for intrinsic lipid-
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synthesizing activity during periods of maximal synthetic activity. In this study, both dark-grown and light-induced y-i cells as well as synchronous 137+ cells and their photosynthetic membranes were analysed for acyltransferase activity biochemically and subsequently, cytochemically (Michaels, Jelsema & Barnett, 1981), to determine whether the thylakoids have the capacity for the synthesis of their own membrane lipids and the extent to which such in situ enzymes might be contributing to the membrane lipid synthesis occurring during chloroplast biogenesis.

MATERIALS AND METHODS

Treatment of cells

Wild-type (137+) and y-i mutant cells of C. reinhardtii were obtained from stock cultures maintained on 2% agar slants containing the culture media of Sager & Granicke (1953). Using aseptic techniques, flasks containing 100 ml of sterile culture medium were inoculated with light-grown cells from 4-day-old agar slants. The seed cultures were grown 3 days at 25 °C and 7534 lx (white fluorescent light) with continuous aeration and shaking (Ohad, Siekevitz & Palade, 1967). Inocula from these 3-day-old cultures were added to 3 l Fernbach flasks containing 1 - 2 l of sterile culture medium and the cells were grown at room temperature with aeration and shaking. Wild-type cells were grown in an alternating 12-h light/12-h dark cycle for at least 3 days and were harvested from these synchronous cultures 6 h into the light phase when cell density approached 0-4 x 10^6 cells/ml. This time point was chosen due to the high levels of membrane synthesis and chloroplast accumulation at this stage of the light period (Schor, Siekevitz & Palade, 1970). y-i cells were grown in the dark to a maximal density of 1 x 10^6 cells/ml. For induction of chloroplast synthesis, i.e. greening, the dark-grown y-i cells were exposed to light at a specified time in late logarithmic growth phase, and samples of cells were removed and analysed at given time intervals throughout the greening period (Ohad et al. 1967).

Cells were harvested by centrifugation for 15 min in a Sorvall SS-34 rotor operated at 30 000 g (16 000 rev./min) and washed twice with 25 mM-Tris-HCl (pH 7.6), containing 25 mM-KCl, 1 mM-MgCl2 and 3 mM-EDTA (TKME buffer). Cells were resuspended to a density of 0.5 x 10^6 to 1 x 10^6 cells/ml in 25 mM-Tris-HCl (pH 7.6), containing 0.22 M-sucrose. This suspension was homogenized using a chilled French pressure cell operated at 2760 kPa.

Isolation of chloroplast membranes

Chloroplast thylakoid membranes were isolated from the wild-type cells by a modification of the procedure of Bourgignon & Palade (1976). After homogenization of the harvested cells, 10-ml portions of the suspension were layered over discontinuous sucrose density gradients consisting of 10 ml 2 M-sucrose, 10 ml 1.5 M-sucrose and 8 ml 1 M-sucrose, all in TKME buffer. The gradients were centrifuged 1 h at 4 °C in a Beckman Spinco SW27 rotor operated at 100 000 g (25 500 rev./min). The 1.0 to 1.5 M-interface and some of the 1.0 M layer were removed and the suspension was adjusted to 1.5 M-sucrose using solid sucrose so as to maintain as small a volume as possible. A refractometer was used to monitor the sucrose concentration. Approximately 3 ml of this solution was then placed in a SW27 centrifuge tube and 35 ml of a continuous sucrose gradient (1.0 M to 1.5 M-sucrose) was formed above the suspension. The gradient was centrifuged at 100 000 g (25 500 rev./min, Beckman SW27 rotor) for 18 h at 4 °C. The green band was removed with a Pasteur pipette, diluted 1:5 with TKME buffer and centrifuged for 1 h at 100 000 g (25 500 rev./min, Beckman SW27 rotor) to obtain a thylakoid membrane pellet.

Glycerol 3-phosphate and lysophosphatidate acyltransferase assays

Both glycerol-3-P acyltransferase and PtdLys acyltransferase catalyse the transfer of activated fatty acids, fatty acyl-CoA, to their respective substrates. This transfer leads to release of the CoA moiety and formation of PtdLys or phosphatidic acid respectively. The enzyme activities
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were monitored by the amount of radiolabelled fatty acid or radiolabelled lipid precursor incorporated into acylated lipid. The kinetics and pH optima were determined for both enzymes. Initial pH optimum experiments were performed using 50 μM-[14C]oleoyl-CoA, [14C]stearyl-CoA or [14C]palmitoyl-CoA (obtained from New England Nuclear) in the absence or presence of 10 μM-glycerol-3-P, PtdLys or glycerone-P. In these preliminary experiments, all the radiolabelled acyl-CoA moieties were observed to have the same pH maxima, but oleoyl-CoA incorporation exceeded that of the other acyl-CoAs at all pH values, and was therefore employed as the acyl donor in all subsequent assays. To determine the identity of the three acyltransferases, oleoyl-CoA and either [14C]glycerol-3-P (New England Nuclear) or [14C]-PtdLys were employed as the radiolabels in the assay procedure. [14C]PtdLys was prepared from [14C]lysophosphatidylcholine (New England Nuclear) by action of phospholipase A; (Sigma) and separated from the substrate by the two-dimensional thin-layer chromatographic method of Parsons & Patton (1967). The pH optima experiments were all performed at 37 °C in a shaking water-bath and incubation was for 60 min in the presence of 10 μM-glycerol-3-P, 10 μM-glycerone-P or 10 μM-PtdLys using 50 mM-acetate buffer. The acetate buffer retained its buffering capacity in the range from pH 5-0 to 9-0, but Tris-HCl buffer subsequently replaced the acetate buffer since the enzyme activities of interest were in the range from pH 7-0 to 8-0 and there was a slight enhancement of activities using Tris-HCl-buffered media. Both enzyme assays were subsequently optimized for substrate concentrations and reaction time. Protein concentration was measured by the Lowry procedure (Lowry, Rosebrough, Farr & Randall, 1951) using the linear transformation correction of Coakley & James (1978).

For the biochemical assay of glycerol-3-P acyltransferase activity, [14C]glycerol-3-P and oleoyl-CoA were used as precursors. The reaction mixture, after optimization of the reaction conditions, consisted of 0.16 mM-[14C]glycerol-3-P (1 x 10^4 d.p.m./ml), 18 μM-oleoyl-CoA and 50 mM-Tris-HCl (pH 8.0), in 4.5 % dextrose. The reaction mixture was preincubated 5 min at 37 °C prior to addition of the cell or membrane suspensions. After incubation for 90 min at 37 °C in a shaking water-bath, the reaction was stopped by addition of methanol in a ratio of 1:2 (reaction mixture/methanol). Lipids were extracted by the procedure of Bligh & Dyer as modified by Marshall & Kates (1972). The combined, washed chloroform phases were transferred to scintillation vials, evaporated to dryness under nitrogen and counted in a Packard Tri-Carb scintillation spectrometer using 10 ml Biofluor. Sample counts were adjusted for counting efficiency and quench corrected using the automatic external standard method (Rogers & Moran, 1966).

To assess the specificity of the enzyme reaction, 4 ml samples were removed from the reaction mixture at 15, 30, 60 and 90 min into the incubation period followed by two-dimensional thin-layer chromatographic analysis of the lipid-extracted material. The radioactivity was recovered from the thin-layer plates and the radioactivity associated with specific phospholipids was determined by scraping and counting the silica gel in 10 ml Biofluor, following visualization of the lipids using iodine vapours and their identification by co-chromatography with reference lipids.

For biochemical analysis of PtdLys acyltransferase activity, use of [14C]oleoyl-CoA and PtdLys as substrates should favour incorporation of acyl-CoA by way of PtdLys acyltransferase activity. However, to ensure the specificity of the reaction, enzyme activity was monitored in the presence of N-ethylmaleimide. This is a specific inhibitor of glycerol-3-P acyltransferase activity (Lands & Hart, 1965). The final reaction mixture, after optimization of reaction conditions, consisted of 18 μM-[14C]oleoyl-CoA (1 x 10^4 d.p.m./ml), 292 μM-PtdLys, 50 mM-Tris-HCl (pH 7.6), with 4.5 % dextrose and 6.7 mM-N-ethylmaleimide (MalNEt). The reaction mixture was preincubated at 37 °C for 5 min prior to addition of the cells or membranes. After 90 min at 37 °C in a shaking water-bath, the reaction was stopped by addition of methanol in a ratio of 1:2 (reaction mixture/methanol). Lipids were extracted and counted as described above.
RESULTS

Biochemical characterization of acyltransferase activities

Synchronous 137+ C. reinhardtii cells harvested 6 h into the light phase were used as the test system to determine the pH optima for both acyltransferase activities. Preliminary studies showed oleoyl-CoA to be the preferred substrate when radiolabelled oleoyl, palmitoyl or stearoyl-CoAs were incubated with intact 137+ cells at various pH values in either the absence or the presence of exogenous acceptors (data not shown). Oleoyl-CoA was, therefore, used as the substrate in all subsequent assays. Reactions were performed at pH values ranging from 5.0 to 9.0 in the presence or absence of either glycerol-3-P, glycerone-P or PtdLys as the exogenous acyl acceptor and [14C]-oleoyl-CoA as the acyl donor (Fig. 1). Under these conditions, three acyltransferase activities were consistently detected: one with a pH optimum at 8.0, another at pH 7.0 and a third at pH 5.5. Addition of exogenous glycerol-3-P to the reaction mixture enhanced the activities at both pH 8.0 and pH 7.0, while decreasing activity at pH 5.5; the greater increase occurred at pH 8.0. Addition of exogenous glycerone-P enhanced the activities at pH 5.5 and pH 7.0, while decreasing the activity at pH 8.0 (Fig. 1); the greater increase occurred at pH 5.5. Addition of exogenous PtdLys caused an
increase only in the activity at pH 7.0, while both the activities at pH 5.5 and pH 8.0 were depressed. These results indicate a pH optimum of 8.0 for glycerol-3-P acyltransferase and a pH optimum of 7.0 for PtdLys acyltransferase. The activity at pH 5.5 was identified as glycerolone-P acyltransferase.

The specificity of the activities of acyltransferase at pH 7.0 and pH 8.0 was verified subsequently by thin-layer chromatographic analysis of the radiolabelled reaction products obtained by lipid extraction of the reaction mixture at specified times during the time course of the enzyme assays. With [14C]oleoyl-CoA employed as the radiolabelled precursor, the distribution of radioactivity in the glycerolipids after 90 min revealed a predominant labelling of the PtdLys intermediate (68%) when the assay was performed at pH 7.0, whereas when the assay was performed at pH 8.0 the major lipid that was radiolabelled was phosphatidic acid (73%). At the earlier times, an even greater proportion of the radiolabel was associated with these specific lipids at the appropriate pH. The remainder of the radioactivities were distributed among the galactolipids and phospholipids further along the pathway. At no time did the assay mixture at pH 7.0 exhibit greater than 15% labelling of the PtdLys intermediate and this labelling was abolished upon addition of MalNEt to the reaction mixture. The radioactivity associated with PtdLys at pH 7.0 thus appears to relate to activity of a glycerol-3-P and/or glycerone-P acyltransferase isozyme at this pH.
The activity at pH 5.5 has been identified as glycerone-P acyltransferase. The reciprocal inhibition of the activities at pH 5.5 and pH 8.0 upon addition of glycerol-3-P or glycerone-P supports the hypothesis that the activity at pH 5.5 is representative of glycerone-P acyltransferase activity. Glycerol-3-P and glycerone-P acyltransferases would be expected to compete for the radiolabelled acyl-CoA (Fig. 2), and addition of either glycerol-3-P or glycerone-P would favour the appropriate enzyme activity at the expense of the other, as was observed to occur in these studies (Fig. 1). Such a pattern of reciprocal competitive inhibition has also been observed with glycerol-3-P and glycerone-P acyltransferases of rat liver and rat fat cells (Schlossman & Bell, 1976, 1977).

The increased [14C]oleoyl-CoA incorporation at pH 7.0 that occurs upon addition of either glycerol-3-P or glycerone-P (Fig. 1) initially seems inconsistent with the identification of the activity at pH 7.0 as PtdLys acyltransferase. In this assay, addition of glycerol-3-P and glycerone-P should have no effect on [14C]oleoyl-CoA incorporation via PtdLys acyltransferase activity. The increased activity observed at this pH upon addition of glycerol-3-P and glycerone-P thus appears to support the existence of
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pH 7.0 isozymes for both the acyltransferases. An alternative possibility is that the addition of glycerol-3-P and glycerone-P leads to enhanced production of endogenous PtdLys, thereby increasing the level of [14C]oleoyl-CoA incorporated via PtdLys acyltransferase. This alternative, however, also requires the presence of pH 7.0 acyltransferases that would give rise to endogenous PtdLys, and furthermore would not be expected to occur under conditions in which the PtdLys substrate concentration had been previously optimized. It would appear, then, that the acyltransferase activity measured at pH 7.0 is a composite of all three types of acyltransferase activity, with the bulk of the activity being PtdLys acyltransferase activity.

Substitution of [14C]PtdLys and unlabelled oleoyl-CoA in the acyltransferase assay resulted in a peak of activity only at pH 7.0 (Fig. 3), consistent with PtdLys acyltransferase activity at this pH. Substitution of [14C]glycerol-3-P and unlabelled oleoyl-CoA as the reactants in this assay produced a major peak at pH 8.0, consistent with glycerol-3-P acyltransferase activity at this pH. However, a significant amount of [14C]glycerol-3-P incorporation was also observed at pH 7.0 and a minor amount at pH 5.5 (Fig. 3). The [14C]glycerol-3-P incorporation into lipids at pH 5.5 and 7.0 appears to indicate the existence of glycerol-3-P acyltransferase isozymes at these pH values. An alternative explanation is that [14C]glycerol-3-P incorporation into lipids may be occurring by way of CDP-diglyceride:glycerol-3-P acyltransferase activity, an enzyme specifically involved in synthesis of phosphatidyglycerol and cardiolipin.

However, preliminary studies revealed that the extent of radiolabelling in these lipids was not significantly different from the radiolabelling in other glycerolipids at these specific pH values when exogenous CTP was not present. In contrast, upon addition of CTP to the incubation media, there was a fourfold increase in [14C]glycerol-3-P incorporation into phosphatidyglycerol at pH 7.0, but not at pH 8.0 or 5.5. The possible incorporation of [14C]glycerol-3-P into lipids at pH 7.0 by way of CDP-diglyceride:glycerol-3-P phosphatidyltransferase cannot, therefore, be eliminated by these studies. However, the increased incorporation of [14C]oleoyl-CoA at this pH upon addition of exogenous glycerol-3-P, together with the low level of glycerol-3-P phosphatidyltransferase activity in the absence of exogenous CTP, strongly suggest that incorporation of [14C]glycerol-3-P at this pH is due to the presence of a pH 7.0 glycerol-3-P acyltransferase isozyme. The incorporation of [14C]glycerol-3-P into lipids at pH 5.5 similarly cannot be exclusively ascribed to the presence of a pH 5.5 glycerol-3-P acyltransferase isozyme due to the possible interconversion to its oxidized form, glycerone-P, with retention of the radiolabel and subsequent incorporation into lipids by way of glycerone-P acyltransferase activity.

When the amount of [14C]PtdLys incorporated into lipids at pH 7.0 is compared to the amount of [14C]oleoyl-CoA incorporated into lipids on a pmol/10⁶ cell basis (compare Figs. 1 and 3), PtdLys acyltransferase activity is observed to account for only 80% of the total acyltransferase activity detected at this pH. If one assumes that [14C]glycerol-3-P radiolabelling of lipids at pH 7.0 occurs by way of a glycerol-3-P acyltransferase isozyme rather than by CDP-diglyceride:glycerol-3-P phosphatidyltransferase activity, the extent of glycerol-3-P-associated acyltransferase activity on a pmol/10⁶ cell basis would account for about 10-15% of the total acyltransferase
Table 1. Effect of N-ethylmaleimide on acyltransferase activities

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<th>[14C]oleoyl-CoA incorporation</th>
<th>% Control*</th>
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<tr>
<td></td>
<td>pH 5.5</td>
</tr>
<tr>
<td>+ MalNEt</td>
<td>52.4 ± 5.4</td>
</tr>
<tr>
<td>+ glycerol-3-P + MalNEt</td>
<td>48.6 ± 6.7</td>
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<tr>
<td>+ PtdLys + MalNEt</td>
<td>54.9 ± 4.2</td>
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* Control values refer to specific activities obtained by monitoring [14C]oleoyl-CoA incorporation into lipids either without exogenous acceptor, or with glycerol-3-P or PtdLys added to the reaction mixture. See Fig. 1 for control values at each pH.

activity detected at this pH as measured by [14C]oleoyl-CoA incorporation into lipids. This would correlate with the observation that PtdLys acyltransferase at this pH constitutes 80% of the total acyltransferase activity detected, leaving about 5–10% of the observed acyltransferase activity unaccounted for. This remaining acyltransferase activity would most likely be due to the presence of a glycerone-P acyltransferase isozyme. This possibility is strengthened by the observed increase in [14C]oleoyl-CoA incorporation at pH 7.0 upon addition of exogenous glycerone-P (Fig. 1).

On the basis of radiolabelling experiments with either radiolabelled oleoyl-CoA in the presence or absence of exogenous glycerol-3-P or PtdLys acceptor or radiolabelled glycerol-3-P or PtdLys in the presence of exogenous oleoyl-CoA donor, the activity at pH 8.0 thus appears to be exclusively representative of glycerol-3-P acyltransferase and the activity at pH 5.5 appears to be exclusively glycerone-P acyltransferase activity. In contrast, the activity at pH 7.0 appears to be a composite of PtdLys acyltransferase activity together with other acyltransferases that appear to be pH 7.0 isozymes of the glycerol-3-P and glycerone-P acyltransferases, based on the increased incorporation of radiolabelled oleoyl-CoA at this pH upon addition of either glycerol-3-P or glycerone-P. However, the primary activity (80%), was PtdLys acyltransferase.

The results obtained upon addition of N-ethylmaleimide to the assay mixtures further substantiates these interpretations. Both glycerol-3-P and glycerone-P acyltransferases are susceptible to inhibition by MalNEt (Lands & Hart, 1965), although resistant isozymes have been reported (Coleman & Bell, 1980). The maximum inhibition observed upon addition of MalNEt was at pH 8.0, consistent with the identification of this activity as glycerol-3-P acyltransferase. [14C]oleoyl-CoA incorporation at pH 8.0 was depressed about 80–90% upon addition of MalNEt, whether measured in the absence or presence of PtdLys (Table 1). Incorporation of [14C]glycerol-3-P at pH 7.0 and a portion (approx. 10%) of the [14C]oleoyl-CoA incorporated at this pH was also inhibited by treatment with MalNEt (Fig. 3), which strongly supports the presence of a glycerol-3-P acyltransferase isozyme at this pH. The inhibition of [14C]glycerol-3-P incorporation at pH 5.5 upon addition of MalNEt cannot be similarly used to determine whether the glycerol-3-P incorporation at this
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Fig. 4. Glycerol-3-P and PtdLys acyltransferase activities in light-induced y-1 cells in the presence and absence of MalNEt. (▲, △) PtdLys acyltransferase; (●, ○) glycerol-3-P acyltransferase; (---) − MalNEt; (-----) + MalNEt.

pH is occurring by way of a glycerol-3-P acyltransferase isozyme or glycerone-P acyltransferase activity, since both enzymes are MalNEt-sensitive.

The incorporation of radiolabelled oleyl-CoA at pH 7.0 was inhibited only 10–15% upon treatment with MalNEt (Fig. 3), considerably less than at the other two pH values, and MalNEt had no effect on [14C]PtdLys incorporation (Fig. 3). This is in agreement with the absence of any sulphydryl reagent effect on PtdLys acyltransferase activity. Thus, the major activity at pH 7.0, and the only activity at pH 7.0 observed in the presence of MalNEt, was PtdLys acyltransferase. There was an increase in [14C]oleoyl-CoA incorporation at pH 7.0 when MalNEt was added to the reaction mixture in the presence of exogenous glycerone-P or glycerol-3-P (Fig. 1), which cannot be explained by the presence of MalNEt-sensitive glycerol-3-P or glycerone-P acyltransferase. This increase in oleyl-CoA incorporation can perhaps be best explained on the basis of the decreased competition of PtdLys acyltransferase for the acyl-CoA due to the MalNEt inhibition of the glycerol-3-P and glycerone-P acyltransferase isozymes at this pH. This conclusion is supported to some extent by the absence of a similar increase in activity at pH 7.0 upon addition of MalNEt to the reaction mixture containing [14C]oleoyl-CoA (Fig. 2) under conditions in which substrate concentrations are optimal. However, the possibility of the presence of MalNEt-insensitive isozymes at this pH cannot be excluded. Both sets of data thus indicate that the activity at pH 7.0 is specific for PtdLys acyltransferase when MalNEt is present, accounting for 96% of the transferase activity detected. The PtdLys acyltransferase assays were, therefore, all performed in the presence of MalNEt whenever [14C]PtdLys was not used as the reactant.

The pH 8.0 glycerol-3-P acyltransferase activity and the pH 7.0 PtdLys acyl-
transferase activity were both found to have maximum activity between 15 and 30 μM-oleoyl-CoA. At concentrations greater than 50 μM, oleoyl-CoA was inhibitory to both enzymes. The acyltransferases were found to differ with respect to reaction kinetics. There was a gradual increase in glycerol-3-P acyltransferase activity throughout the 2-h incubation period (Fig. 4) as determined by the incorporation of [14C]-glycerol-3-P into lipids at pH 8·0 in the absence of exogenous CTP. This activity was sensitive to MalNEt, further substantiating that this activity represented glycerol-3-P acyltransferase activity. In contrast, PtdLys acyltransferase activity, measured at pH 7·0 by [14C]oleoyl-CoA incorporation into lipids in the presence of MalNEt, exhibited marked activity initially (up to 10 min into the incubation) and then declined. In the absence of MalNEt, [14C]oleoyl-CoA incorporation at this pH reached a second maximum 45 min into the incubation period and a gradual increase in activity was again observed after 60 min (Fig. 4). Since these two latter activities were not measurable in the presence of MalNEt and were not observed upon assay with [14C]PtdLys as the radiolabelled substrate, the latter two peaks were identified as representative of pH 7·0 glycerol-3-P and/or glycerone-P acyltransferase isozymes.

Biochemical analyses of acyltransferase activities: quantitation and localization

Table 2 summarizes the specific activities of glycerol-3-P and PtdLys acyltransferases in dark-grown and light-induced log-phase y-i cells as well as synchronous 137+ wild-type cells and membranes. The wild-type cells were harvested 6 h into the light cycle, when chloroplast synthesis has been reported to be maximal (Schor et al. 1970). Both glycerol-3-P and PtdLys acyltransferase activities were enriched at least tenfold in the thylakoid membrane fraction of 137+ cells, indicative of the presence of these enzymes in the photosynthetic membranes of 137+ chloroplasts. As previously mentioned, it is difficult, if not impossible, to isolate photosynthetic membranes from y-1 cells early in chloroplast biogenesis, but analysis of photosynthetic membranes from y-1 cells 8 h after light induction revealed comparable enrichment of the acyltransferase activities in the y-1 cells. Contamination of the thylakoid membrane fraction by chloroplast envelope is minimal (dePetrocelli et al. 1970; Michaels, Jelsema & Barnett, unpublished) and cannot account for the tenfold enrichment of the glycerol-3-P and PtdLys acyltransferases. The possible adherence of soluble or solubilized enzymes to the isolated membranes in quantities sufficient to give rise to such high levels of activity is also highly unlikely. Therefore, in C. reinhardtii 137+ cells, and in y-1 cells 8 h after light induction, both glycerol-3-P and PtdLys acyltransferases are intrinsic to the photosynthetic membranes.

The increased acyltransferase activity in the y-1 cells soon after light induction suggests a role for these enzymes in the synthesis of the photosynthetic membrane lipids in y-1 cells, since exposure to light induces chloroplast synthesis. However, coincident increases in acyltransferase activity with increased synthesis of the chloroplast membranes does not directly reveal the relationship between the two events. The possibility exists that the light-induced increase in the acyltransferase enzyme activities is merely a reflection of their intrinsic location in the chloroplast membranes and their
Biochemistry of acyltransferase activities

10 r

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig5}
\caption{Light induction of chlorophyll and acyltransferase activities in y-i cells. (▲) PtdLys acyltransferase; (●) glycerol-3-P acyltransferase; (■) chlorophyll.}
\end{figure}

consequent activation as a result of membrane synthesis, rather than being required for membrane synthesis.

In an attempt to resolve this issue the time-course of chloroplast biogenesis and acyltransferase activation was monitored in the y-1 cells following light induction. The chlorophyll content of the y-1 cells increased after 3 h of light (Fig. 5), while both acyltransferase activities were already increased after 1 h of exposure to light. Glycerol-3-P acyltransferase activity steadily increased up to 8 h after light induction (Fig. 4), whereas the activity of PtdLys acyltransferase was maximal after 1 h of light and then declined. Thus, the increase in acyltransferase activities upon light induction preceded the increase in chlorophyll content (Fig. 5) and, therefore, preceded as well as occurring in concert with chloroplast membrane synthesis. The early increase in the acyltransferase activities does not correlate with their activation occurring as a result of membrane synthesis, but rather suggests a direct role for these enzymes in the synthesis of the photosynthetic membrane lipids.

The PtdLys acyltransferase activity in 137+ and y-1 cells was at least half that of glycerol-3-P acyltransferase activity (Table 2). In contrast, the PtdLys acyltransferase activity in dark-grown y-1 cells was less than 5% that of the glycerol-3-P acyltransferase activity. This relates primarily to the low PtdLys acyltransferase activity in the dark-grown y-1 cells. At 6 h PtdLys, acyltransferase activity in the light-induced y-1 cells was approximately 15 times that of the comparable enzyme activity in the dark-grown y-1 cells, whereas glycerol-3-P acyltransferase activity in light-induced y-1 cells
Table 2. Glycerol-3-phosphate and lysophosphatidate acyltransferase activities of y-i and 137+ thylakoid membranes

<table>
<thead>
<tr>
<th>Sample treatment</th>
<th>Glycerol-3-P transferase†</th>
<th>PtdLys transferase‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark-grown y-i cells</td>
<td>0.43 ± 0.09</td>
<td>0.02 ± 0.03</td>
</tr>
<tr>
<td>Light-induced y-i cells</td>
<td>0.77 ± 0.07</td>
<td>0.31 ± 0.10</td>
</tr>
<tr>
<td>137+ cells</td>
<td>0.90 ± 0.08</td>
<td>0.46 ± 0.09</td>
</tr>
<tr>
<td>137+ thylakoid membranes</td>
<td>16.78 ± 2.06</td>
<td>5.29 ± 0.71</td>
</tr>
</tbody>
</table>

Specific activities = nmol/mg protein per h.
† Acyl-CoA:glycerol-3-phosphate acyltransferase; reaction conditions as in Table 1.
‡ Acyl-CoA:lysophosphatidic acid acyltransferase; reaction conditions as in Table 1.

y-i cells were harvested in log phase and either kept in the dark for 6 h or light-induced for 6 h prior to assay. 137+ cells were harvested from synchronous log-phase cultures 6 h into the light phase. 137+ photosynthetic membranes were prepared from 137+ cells harvested 6 h into the light phase following the procedures of Bourginon & Palade (1976), modified as described in Materials and Methods.

was only twice the enzyme activity in dark-grown y-i cells. These results suggest a close correlation between PtdLys acyltransferase activity and light induction. This concept is supported by the almost negligible PtdLys acyltransferase activity in the dark-grown cells and the immediate increase in this enzyme activity upon light induction of y-i cells (Fig. 5).

The higher enzyme activities in the 137+ cells relative to the light-induced y-i cells are somewhat surprising (Table 2). However, this appears to be partially a reflection of the burst of protein synthesis in the light-induced y-i cells, leading to a higher protein content and giving rise to a somewhat low estimate of the light-induced y-i enzyme activities on the basis of specific activity per mg protein. While the 137+ cells and dark-grown y-i cells are fairly similar in their protein content, the 137+ cells have slightly more protein (dePetrocelli et al. 1970). There is, however, a protein content about threefold higher in 6 h light-induced y-i cells relative to dark-grown y-i cells. Similarly, there is a 2.5-fold difference between the protein content of light-induced y-i cells and 137+ cells. As a result, when the light-induced y-i and 137+ cell activities are compared on a per cell basis rather than per mg protein, the glycerol-3-P acyltransferase and PtdLys acyltransferase activities of light-induced y-i cells are, in actuality, 1.5 times the respective 137+ activities. When compared to the enzymic activities in the dark-grown y-i cells, glycerol-3-P acyltransferase activity in the light-induced y-i cells would be five times that of the comparable enzyme activity in the dark-grown y-i cells, while the PtdLys acyltransferase activity in the light-induced y-i cells would be nearly 40 times that of the dark-grown y-i cells. Thus, on a per cell basis, the effect of light treatment on PtdLys acyltransferase activity is even more striking. On the basis of these studies, PtdLys acyltransferase seems to be an enzyme that is activated upon light treatment.
DISCUSSION

Three distinct acyltransferases appeared to be active in *C. reinhardtii* 137+ cells. This observation was based on the use of oleoyl-CoA as substrate, either alone or together with equimolar but suboptimal amounts of glycerol-3-P, glycerone-P and PtdLys added as the exogenous acyl acceptors. The acyltransferase activity having a pH optimum of 8.0 was determined to be glycerol-3-P acyltransferase on the basis of four lines of evidence: (1) its susceptibility to inhibition by MalNεt; (2) increased acylation at pH 8.0 when exogeneous glycerol-3-P was added to the assay mixture; (3) the parallel pH optimum observed when [14C]glycerol-3-P and unlabelled oleoyl-CoA were used as the reactants in the reaction mixture; (4) and the high percentage of radiolabel associated with phosphatidic acid, particularly early in the incubation period, upon separation and identification of the reaction products obtained when either [14C]oleoyl-CoA or [14C]glycerol-3-P was used as the radiolabelled substrate.

The acyltransferase activity observed at pH 7.0 was similarly identified as being primarily representative of PtdLys acyltransferase on the basis of its insensitivity to MalNεt-enhanced acylation at pH 7.0 upon addition of exogeneous PtdLys to the reaction mixture; the parallel pH optimum observed when [14C]PtdLys and unlabelled oleoyl-CoA were used as the reactants in the assay procedure; and the high percentage of radioactivity initially associated with PtdLys upon analysis of the reaction products obtained with either [14C]oleoyl-CoA or [14C]PtdLys as the radiolabelled substrate. However, the presence of isozymes of glycerol-3-P acyltransferase and glycerone-P acyltransferase at pH 7.0 also appears to be indicated by several lines of evidence. When [14C]oleoyl-CoA was used as the radiolabelled substrate in the assay, approximately 80% of the activity was not affected by MalNεt. When [14C]glycerone-P was employed as the radiolabelled substrate, MalNεt had no effect on PtdLys incorporation. In addition, there was increased incorporation of [14C]oleoyl-CoA at this pH upon addition of glycerol-3-P or glycerone-P, whereas these increases were not observed in the presence of MalNεt; secondly, [14C]oleoyl-CoA incorporation was stimulated upon addition of MalNεt in the presence of PtdLys concentrations previously determined to be optimal, which either results from the presence of MalNεt insensitive and glycerol-3-P:glycerone-P acyltransferase isozymes or the decreased competition for the [14C]oleoyl-CoA upon inhibition of MalNεt-sensitive glycerol-3-P and/or glycerone-P acyltransferase isozymes with MalNεt; and lastly, MalNεt-inhibitable glycerol-3-P acyltransferase activity was observed at pH 7.0 when [14C]glycerol-3-P was employed as a substrate. In the presence of MalNεt, however, the activity at pH 7.0 appears to be highly specific for PtdLys acyltransferase, accounting for 96% of the total acyltransferase activity at this pH.

The third acyltransferase, having a pH optimum of 5.5, was identified as glycerone-P acyltransferase on the basis of both the increased acylation at pH 5.5 and the concomitant decrease in acylation at pH 8.0 when glycerone-P was added to the assay containing [14C]oleoyl-CoA, since glycerone-P acyltransferase and the glycerol-3-P acyltransferase compete for the radiolabelled acyl-CoA (Schlossman & Bell, 1976).

Using the synchronous wild-type 137+ cells as the test system, the reaction con-
ditions for the glycerol-3-P and PtdLys acyltransferases were subsequently optimized biochemically with respect to substrate concentration and incubation time. Biochemical analysis of photosynthetic membranes isolated from synchronous 137+ cells harvested 6 h into the light cycle revealed a tenfold enrichment in activities of both acyltransferases in the purified membranes relative to whole cell activities. Since the biochemical assays were performed using specific radiolabelled substrates, i.e. [14C]-glycerol-3-P or [14C]PtdLys, the acyltransferases being measured in the isolated membranes were unquestionably glycerol-3-P and PtdLys acyltransferases, respectively. The different susceptibilities of these enzymes to inhibition with MalNEt substantiated this further. The question, then, was whether these activities were truly endogenous or represented cross-contamination of membrane-bound enzymes or non-specific adherence of cytosolic enzymes. Activation of contaminating microsomal membranes, for example, has been invoked to explain the presence of significant amounts of the phospholipid-synthesizing enzyme, choline phosphotransferase, in mitochondrial fractions of rat liver (Jungalwala & Dawson, 1970). The enzyme is associated primarily with endoplasmic reticulum and Golgi apparatus of rat liver (Jelsema & Morré, 1978), but sequestration of CaE+ by mitochondria is thought to activate the contaminating microsome-related enzymes (Roberts & Bygrave, 1973) leading to a spuriously high choline phosphotransferase activity in mitochondrial fractions.

This paper demonstrates that on the basis of biochemical analysis of purified membranes isolated from 137+ cells, photosynthetic membranes of C. reinhardtii 137+ chloroplasts contain both glycerol-3-P and PtdLys acyltransferase, enzymes that are essential for glycerolipid synthesis. The photosynthetic membranes also were shown to contain phosphatidic acid phosphatase (Jelsema et al. 1977), the enzyme that converts the acylated phosphatidic acid to the diglyceride precursor required for synthesis of the chloroplast-enriched galactolipids and sulpholipids. The finding that the chloroplast contains enzymes of lipid synthesis is in agreement with previous reports on the partial autonomy of the chloroplast with respect to the synthesis of its membrane lipid. However, in contrast to previous reports that cite the chloroplast envelope as the primary or exclusive site of membrane lipid synthesis, we report that the photosynthetic membranes contain acyltransferase enzymes and are, themselves, capable of synthesizing their membrane lipids. Furthermore, a direct and presumably causal correlation between the onset of chloroplast biogenesis and increased chloroplast acyltransferase activity was observed with the inducible y-1 cells. The increase in the photosynthetic membranes as measured by increased chlorophyll content was preceded by increases in the acyltransferase activities of these cells. The major difference upon light induction was observed in PtdLys acyltransferase activity, which increased markedly in the y-1 cells within the first 10 min of light treatment. These findings argue for the presence of the acyltransferase enzymes in the thylakoid membranes and the direct involvement of chloroplast acyltransferases in the synthesis of the thylakoid membranes.

These results are in contrast with the findings of Joyard & Douce (1977), who reported that the acyltransferase activity of intact spinach chloroplast occurred almost exclusively in the chloroplast envelope, and that the chloroplast glycerol-3-P acyl-
transferase was a soluble enzyme. In *C. reinhardtii*, both glycerol-3-*P* and PtdLys acyltransferase were membrane-bound enzymes and were found to be associated with and enriched at least tenfold in isolated chloroplast photosynthetic membranes. The discrepancy between these reports, while it may relate to the phylogenetic differences between higher plants and algae, requires some attempt at resolution. The presence of acyltransferase activity in the envelope of spinach chloroplasts is indisputable. The potential cross-contamination of the spinach chloroplast envelope fraction by thylakoid fragments appears to have been minimal in the studies of Joyard & Douce (1976, 1977), based on both electron micrographs and chlorophyll content. The high envelope activity thus appears to result from intrinsic enzymes. However, the reported absence of acyltransferase activity in the thylakoids and the absence of membrane-bound glycerol-3-*P* acyltransferase in particular is open to question. The possible inactivation or release of thylakoid-bound enzymes during the isolation procedure cannot be dismissed; the absence of membrane-bound glycerol-3-*P* acyltransferase activity in the spinach chloroplast may relate directly to the ease of detachment of the enzyme, as was suggested by Joyard & Douce (1977), on the basis of the ease with which microsomal and mitochondrial glycerol-3-*P* acyltransferase is solubilized (Yamashita & Numa, 1972; Monroy, Rola & Pullman, 1972). The detachment or solubilization of membrane-bound glycerol-3-*P* acyltransferase activity during the isolation of the spinach chloroplast subfractions thus appears to be highly probable. The persistent association of plastoglobuli with spinach thylakoids even after fractionation (Joyard & Douce, 1976), further suggests a lipid-synthesizing capacity in the thylakoids in spinach chloroplasts since lipid-rich plastoglobuli do not appear to be vehicles of lipid transport. Rather, plastoglobuli appear to result from the disproportionate synthesis of membrane lipids relative to membrane proteins (Benson, 1971) and, therefore, tend to retain their association with the membrane of origin, the thylakoids. Thus, localization of lipid-synthetic enzymes to the photosynthetic membranes of chloroplasts is suggested to occur in the higher plants as well.

Synthesis of chloroplast lipids at the site of membrane synthesis obviates the need for massive transport of lipids across the chloroplast stroma. A mechanism for lipid transport becomes a prerequisite if indeed the chloroplast envelope is the exclusive or primary site of chloroplast lipid synthesis. The logistic problems posed when the sites of membrane lipid synthesis are separated from their subsequent sites of utilization in membrane assembly or biogenesis cannot be ignored in view of the water-insolubility of the lipids. Further, the absence of any reports of lipid exchange proteins operative in the chloroplast or any significant amount of association of developing thylakoids with the chloroplast envelope reinforces the hypothesis of *in situ* synthesis of the thylakoid membrane lipids.

If synthesis of the lipid components of chloroplast photosynthetic membranes occurs by such *in situ* activities during chloroplast biogenesis, one would expect high specific acyltransferase activities *in vivo* concomitant with chloroplast membrane synthesis, followed by the rapid decline of this activity. Such changes are characteristic of the acyltransferase enzymes in *C. reinhardtii* y-1, particularly PtdLys acyltransferase activity, and would thus appear indicative of thylakoid lipid synthesis occurring by

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Biochemistry of acyltransferase activities

Despite the conflicting reports giving evidence of localization of the chloroplast-associated lipid-synthetic activities primarily, if not exclusively, to the chloroplast envelope in spinach (Joyard & Douce, 1978, 1979), it would be difficult to argue for the exclusive localization of membrane lipid synthesis in the envelope of higher plants when algal cells give evidence of significant levels of in situ synthesis of the chloroplast thylakoid lipids. These results, therefore, together with the persistent association of plastoglobuli with thylakoids of chloroplasts of higher plants, strongly suggest a reevaluation of the currently accepted theory that the chloroplast envelope is the primary, if not exclusive, site of chloroplast-related membrane lipid synthesis in plant cells. On the basis of both biochemical and cytochemical evidence (Michaels et al. unpublished), we propose a direct role for the photosynthetic membranes in the synthesis of their lipids.

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REFERENCES


Biochemistry of acyltransferase activities


SCHLOSSMAN, D. M. & BELL, R. M. (1976). Triacylglycerol synthesis in isolated fat cells. Evidence that the m-glycerol-3-phosphate and dihydroxyacetone phosphate acyltransferase...
activities are dual catalytic functions of a single microsomal enzyme. J. biol. Chem. 231, 5738–5744.


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