The Role of Chloroplasts and Microsomal Fractions in Polar-Lipid Synthesis from [1-¹⁴C]Acetate by Cell-Free Preparations from Spinach (*Spinacia oleracea*) Leaves

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1. Isolated spinach (*Spinacia oleracea*) chloroplasts were incapable of accumulating polar lipids when incubated with [1-¹⁴C]acetate in a cofactor-free medium. When CoA, ATP and glycerol 3-phosphate were added to incubation media, the accumulated products were non-esterified fatty acids, acyl-CoA and 1,2-diacylglycerol, all intermediates of lipid metabolism. 2. Chloroplast acyl-CoA was used to synthesize phosphatidylcholine only when a microsomal fraction was added back to the incubation medium. 3. The 1,2-diacylglycerol synthesized by isolated chloroplasts was converted almost quantitatively into diacylgalactosylglycerol when exogenous UDP-galactose was available. 4. Stereospecific analyses of the isolated lipids suggested that the diacylglycerol synthesized by isolated chloroplasts may be an important precursor for the synthesis *in vivo* of diacylgalactosylglycerol and phosphatidylglycerol but was unlikely to be a precursor of phosphatidylcholine. 5. A scheme for plant-lipid biosynthesis is presented that integrates the functions of chloroplasts, the cytoplasm and the endoplasmic reticulum.

There is increasing evidence for a considerable interdependence of plant cell organelles in the biosynthesis of glycerolipids. Much of this evidence suggests that plastids are the sites of long-chain-fatty-acid synthesis *de novo* (Weaire & Kekwick, 1975; Vick & Beevers, 1978; Ohlrogge *et al.*, 1979), whereas the endoplasmic reticulum (Chenier, 1965; Sumida & Mudd, 1970; Marshall & Kates, 1972; Devor & Mudd, 1971; Moore *et al.*, 1973; Vick & Beevers, 1977), the mitochondria (Douce & Dupont, 1969; Bahl *et al.*, 1970) and the chloroplast envelope (Douce, 1974; Joyard & Douce, 1977) are the major sites of polar-lipid assembly. However, reports of significant incorporation of [¹⁴C]acetate into polar lipids of isolated plastids are at variance with this concept. Kannangara & Stumpf (1972) found that almost half of the long-chain fatty acids synthesized by isolated spinach (*Spinacia oleracea*) chloroplasts were incorporated into polar glycerolipids, and Panter & Boardman (1973) reported that 30–40% of the [¹⁴C]acetate incorporated into the lipids of plastids isolated from developing pea (*Pisum sativum*) leaves was in polar glycerolipid. By contrast, Roughan *et al.* (1976) and Murphy & Leech (1978) reported that polar lipid accounted for <15% and 2% respectively of the total lipid synthesized from [1-¹⁴C]acetate by isolated spinach

chloroplasts. These inconsistencies probably reflect varying degrees of contamination of the chloroplast preparations by other organelles; the preparations of Murphy & Leech (1978), purified by sucrose-density-gradient centrifugation, were probably the least contaminated by other cell fractions and showed the lowest incorporation of newly synthesized fatty acid into polar lipid.

Chloroplasts purified from maize (*Zea mays*) and spinach leaves were capable of the CoA- plus ATP-dependent incorporation of *sn*-[U-¹⁴C]glycerol 3-phosphate into phosphatidic acid, 1,2-diacylglycerol and diacylgalactosylglycerol (Douce & Guillet-Salmon, 1970) and the separate enzymic steps for these syntheses were subsequently shown to be localized in the chloroplast stromal and envelope fractions (Douce, 1974; Joyard & Douce, 1976; Roughan & Slack, 1977). Intact photosynthetically active chloroplasts, however, did not incorporate [¹⁴C]acetate into diacylgalactosylglycerol but accumulated diacylglycerol when incubated in the presence of [¹⁴C]acetate, *sn*-glycerol 3-phosphate and Triton X-100 (Roughan *et al.*, 1976, 1979a).

In the present paper we show that the addition of other cell fractions is required for the further metabolism of the glycerolipid intermediates ac-

cumulated from [1- 14 C]acetate by illuminated chloroplasts *in vitro*.

Experimental

Spinach (*Spinacia oleracea*) plants [Hybrid 102 (Arthur Yates Ltd., Auckland, New Zealand) or Hybrid 424 (Ferry Morse Seed Co., Mountain View, CA 94040, U.S.A.)] were grown as previously described (Roughan *et al.*, 1979a) and expanding leaves were harvested for chloroplast isolation (Nakatani & Barber, 1977) and for [1- 14 C]acetate feeding via the cut petioles. Isolated chloroplasts were incubated in the basal assay system {(25 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH (pH 7.9)/10 mM-NaHCO₃/2 mM-EDTA/1 mM-MnCl₂/1 mM-MgCl₂/0.5 mM-dithiothreitol/0.3 mM-K₂HPO₄/0.2 mM-[1- 14 C]acetate (Roughan *et al.*, 1979a)}, modified as described in the legends to the Tables. Lipids extracted from incubations were resolved both by unidirectional t.l.c. using layers consisting of 5% (w/w) H₃BO₃ in silica gel with chloroform/acetone (24:1, v/v) as developing solvent, and by two-dimensional t.l.c. (Roughan *et al.*, 1978). Non-esterified fatty acids and diacylglycerol were located on unidirectional chromatograms by I₂ staining of co-chromatographed standards (Roughan *et al.*, 1979a) and the origins were assumed to contain polar lipids. Polar lipids separated by two-dimensional t.l.c. were detected by radioautography and the chromatograms were subsequently stained in I₂ vapour to assist in the identification of the 14 C-labelled compounds. In some experiments, chloroplast polar lipids were co-chromatographed with a lipid extract from developing soya beans (*Glycine max*) that had been frozen and thawed twice before extraction. This procedure increased the amount of phosphatidate in the soya-bean extract (Wilson & Rinne, 1976), which, when chromatographed with the chloroplast extract, provided a suitable spectrum of polar glycerolipids to act as carriers and markers in the analysis. The positional specificity of acyl transfer to *sn*-glycerol 3-phosphate within chloroplasts was measured by treating, with pancreatic lipase (type VI, Sigma Chemical Co., St. Louis, MO, U.S.A.), 14 C-labelled diacylglycerol or 14 C-labelled diacylgalactosylglycerol (Noda & Fujiwara, 1967) isolated from chloroplast incubations. The 14 C-labelled diacylglycerol was diluted, before enzymic digestion, with non-radioactive diacylglycerol prepared from egg-yolk phosphatidylcholine (Gurr & Brawn, 1970). Both the monoacyl derivatives and the non-esterified fatty acids formed in the hydrolysis were purified by t.l.c., transformed into fatty acid methyl esters and analysed by a combination of argentation t.l.c. and g.l.c. The 14 C-labelled diacylglycerol was also converted into phosphatidylphenol (Christie & Moore, 1971), which was treated

with phospholipase A₂ from king-cobra (*Ophiophagus hannah*) venom (Sigma), and the products of the digestion were analysed as above. Detached spinach leaves were fed [1- 14 C]acetate in the light by petiole uptake and lipids were extracted as previously described (Slack & Roughan, 1975). Positional distribution of the 14 C-labelled fatty acids within purified glycerolipids was determined by treatment with pancreatic lipase or with phospholipase A₂ (see above). Washed microsomal fractions were prepared as follows. Six expanding spinach leaves were homogenized in 50 ml of 0.33 M-sorbitol, 0.1 M-Tris/HCl, pH 7.5, 1 mM-MgCl₂, 1 mM-EGTA, 1 mM-EDTA, 10 mM-cysteine hydrochloride and 0.1% (w/v) defatted bovine serum albumin (Slack *et al.*, 1979) and the filtrate was centrifuged at 13 000 g for 10 min. The supernatant was then centrifuged at 100 000 g for 60 min and the resulting pellet resuspended in 16 ml of the Hepes-based 'running' buffer used for measuring photosynthetic O₂ evolution and [1- 14 C]acetate incorporation (see above). After re-centrifuging for 60 min at 100 000 g, the washed 'microsomal' pellet was dispersed in 5 ml of the same buffer. [1- 14 C]Acetate was from The Radiochemical Centre, Amersham, Bucks., U.K., and all other biochemicals were supplied by Sigma.

Results

The combination of both the one- and two-dimensional t.l.c. systems permitted an accurate analysis of the 14 C distribution among the different lipid classes. However, 5–10% of the total lipid 14 C remained at or near the origin in the unidirectional chromatography, and was therefore classed as polar lipid, but migrated with high mobility in both solvents in the two-dimensional system. This uncharacterized lipid may possibly have been monoacylglycerol (Murphy & Leech, 1978).

Lipids synthesized by isolated chloroplasts

When incubated in the basal assay medium, chloroplasts isolated in low-ionic-strength media synthesized very little polar lipid from [1- 14 C]acetate (Table 1). Instead, non-esterified fatty acids plus diacylglycerol consistently accounted for 85–90% of the total 14 C incorporated, although these compounds represented less than 1% of total lipid 14 C when [1- 14 C]acetate was fed to whole leaves (P. G. Roughan, R. Holland & C. R. Slack, unpublished work; see also below). Polar-lipid synthesis was not significantly enhanced when CoA plus ATP were included in incubation media, and the acyl-CoA that accumulated was apparently not utilized for phosphatidic acid synthesis when *sn*-glycerol 3-phosphate was also present during [1- 14 C]acetate incorporation (Table 1). Larger amounts of diacylglycerol and its precursor, phosphatidic acid, were observed in incubations containing added *sn*-gly-

Table 1. *Lipids synthesized from [1-¹⁴C]acetate by isolated intact spinach chloroplasts*

Chloroplasts equivalent to 50µg of chlorophyll were incubated for 15 min at 25°C in 0.25 ml of the basal incubation mixture containing the additions shown. Reactions were stopped by adding 5 ml of chloroform/methanol/acetic acid (335:165:1, by vol.) followed by 0.75 ml of water and shaking. Lipids in the chloroform phase were separated by two-dimensional t.l.c. and were detected by radioautography. Radioactivity recovered in fatty acids of the aqueous-methanol phase after saponification was assumed to indicate thioesters. Additions were: CoA, 0.5 mM; ATP, 2.0 mM; *sn*-glycerol 3-phosphate (G3P), 0.48 mM; Triton X-100, 0.13 mM; UDP-galactose, 0.4 mM; CTP, 2.0 mM. Rates of [1-¹⁴C]acetate incorporation were 1.2–1.6 µmol/h per mg of chlorophyll. Other abbreviations used: Or, chromatographic origin; PI, phosphatidylinositol; PA, phosphatidic acid; PC, phosphatidylcholine; SL, diacylsulphoquinovosylglycerol; DDG, diacyldigalactosylglycerol; PG, phosphatidylglycerol; NFA, non-esterified fatty acids; DGG, diacylgalactosylglycerol; DAG, 1,2-diacylglycerol; THIO, long-chain thioesters. Phosphatidylethanolamine (PE) contained no radioactivity. The asterisk (*) indicates less than 1%.

Additions to basal medium	Percentage of total lipid radioactivity										
	Or	PI	PA	PC	SL	DDG	PG	NFA	DGG	DAG	THIO
None	1	*	1	*	3	*	1	68	*	17	5
CoA, ATP	1	*	1	1	3	*	2	46	*	16	25
CoA, ATP, G3P	3	*	7	1	3	*	3	19	*	33	21
G3P, Triton X-100	6	*	11	*	3	*	1	16	*	48	7
G3P, Triton X-100, CTP	3	*	6	*	4	*	2	16	4	51	5
G3P, Triton X-100, UDP-Gal	4	*	6	*	5	7	1	16	45	8	6

Table 2. *Positional specificity of esterification to *sn*-glycerol 3-phosphate of fatty acids synthesized from [1-¹⁴C]acetate by isolated spinach chloroplasts*

Chloroplasts equivalent to 180µg of chlorophyll were incubated in 1 ml of the standard assay medium containing the additions shown. Incubation was for 20 min at 25°C and reactions were stopped by adding 5 ml of chloroform/methanol (2:1, v/v). 1,2-Diacylglycerols were isolated by t.l.c. and were either treated with pancreatic lipase to yield 2-acylglycerol or were converted into phosphatidylphenol, which was subsequently treated with phospholipase A₂.

Additions to incubation medium	Percentage distribution of radiocarbon within					
	1,2-Diacylglycerol		2-Acylglycerol		1-Acylphosphatidylphenol	
	C _{16:0}	C _{18:1}	C _{16:0}	C _{18:1}	C _{16:0}	C _{18:1}
1 mM- <i>sn</i> -Glycerol 3-phosphate	66	34	99	1	25	75
0.13 mM-Triton X-100	51	49	99	1	3	97
1 mM- <i>sn</i> -Glycerol 3-phosphate + 0.13 mM-Triton X-100	60	40	99	1	16	84

cerol 3-phosphate, particularly in the presence of Triton X-100. Attempts to encourage CDP-diacylglycerol synthesis by adding CTP to reaction media were unsuccessful, but endogenous diacylglycerol was converted in high yield (more than 90%) into diacylgalactosylglycerol in the presence of exogenous UDP-galactose (Table 1). These results compliment those obtained by Douce & Guillot-Salmon (1970), who incubated isolated chloroplasts with *sn*-[¹⁴C]glycerol 3-phosphate. Considering the relatively low incorporation of [1-¹⁴C]acetate into diacylsulphoquinovosylglycerol (sulpholipid) of whole leaves (Roughan, 1970; Bolton & Harwood, 1978), it was unexpected that this should be the most heavily labelled polar lipid synthesized by isolated chloroplasts in basal incubation media.

The diacylglycerol synthesized by isolated chloroplasts contained approximately equal amounts of saturated and monoenic ¹⁴C-labelled fatty acids, depending on the concentration of *sn*-glycerol 3-phosphate added to incubation media (Roughan *et al.*, 1979a). These fatty acids were not randomly distributed, in that the 2-position was found to contain saturated ¹⁴C-labelled fatty acids (more than 95% palmitate) exclusively (Table 2). This was also true for the diacylgalactosylglycerol synthesized from diacylglycerol in the presence of UDP-galactose (results not shown). Hence the chloroplast diacylglycerol synthesized in the presence of Triton X-100 and low concentrations (less than 0.4 mM) of *sn*-glycerol 3-phosphate was 1-oleoyl-2-palmitoyl-*sn*-glycerol.

Effects of supernatant and microsomal fractions

In a second experiment, chloroplasts were incubated under conditions where non-esterified fatty acids, diacylglycerol and acyl-CoA were expected to accumulate in approximately equal amounts and the effect of adding back the 16000 g supernatant of the original homogenate to the incubation media was examined. Although phosphatidylcholine, diacylgalactosylglycerol and phosphatidylglycerol incorporated significant amounts of ^{14}C in these incubations (results not shown), it became apparent that factors in the supernatant could modify the products as well as inhibit the rates of $[1-^{14}\text{C}]$ acetate incorporation into lipids of isolated chloroplasts.

However, it seemed probable that the light-dependent incorporation of $[1-^{14}\text{C}]$ acetate into fatty acids was independent of the subsequent metabolism of the ^{14}C -labelled fatty acids. Hence, chloroplasts were first incubated in the light with $[1-^{14}\text{C}]$ acetate and then subsequently incubated in darkness with further additions to the reaction mixture. These results (Tables 3 and 4) were consistent with those shown in Table 1, but demonstrated the different phases of lipid metabolism, long-chain-fatty-acid synthesis *de novo* and polar-lipid synthesis, more clearly. During the 'dark metabolism' there appeared to be some breakdown of diacylglycerol to non-esterified fatty acids, but this was avoided if the diacylglycerol was converted into diacylgalactosylglycerol. The acyl-

Table 3. Subsequent 'dark metabolism' of lipid intermediates accumulated by chloroplasts from $[1-^{14}\text{C}]$ acetate in the light. Chloroplasts equivalent to 48 μg of chlorophyll were incubated for 20 min at 25°C in 0.25 ml of the basal medium before the lights were turned off to terminate fatty acid synthesis. Homogenizing buffer (0.25 ml) or a 16000 g supernatant fraction from the spinach leaf homogenate (0.25 ml), containing 0.125 μmol of CoA, 0.5 μmol of ATP, 0.06 μmol of glycerol 3-phosphate and 0.1 μmol of UDP-galactose as indicated, was added and the incubation was continued for a further 15 min in the dark. Reactions were stopped and analysed as described in Table 1. Abbreviations are as for Table 1. The asterisk (*) indicates less than 1%.

Additions for dark incubation	Percentage of total lipid radioactivity										
	Or + PI	PA	PC	SL	DDG	PG	PE	NFA	DGG	DAG	THIO
Buffer alone	1	*	1	4	*	1	*	70	*	6	6
+CoA, ATP	2	*	3	4	*	1	*	37	1	9	34
+CoA, ATP, G3P	6	2	3	4	*	2	*	33	*	10	30
+UDP-gal	1	*	1	3	1	*	*	67	12	1	6
16000 g supernatant	1	*	4	3	3	1	*	58	11	3	6
+CoA, ATP	3	2	29	5	3	1	4	24	5	8	7
+CoA, ATP, G3P	3	3	32	4	3	2	3	17	5	10	7
No dark incubation	1	*	1	3	*	1	*	67	*	13	5

Table 4. Dark metabolism by a washed microsomal preparation of lipid intermediates accumulated by isolated chloroplasts in the light

Chloroplasts equivalent to 90 μg of chlorophyll were incubated for 20 min in 0.25 ml of the basal buffer supplemented with either 0.5 mM-CoA (non-esterified fatty acid mode) or 0.13 mM-Triton X-100 plus 0.24 mM-*sn*-glycerol 3-phosphate (1,2-diacylglycerol mode). The lights were extinguished and 0.25 ml either of the basal buffer or of 0.62 mg of microsomal protein in the same buffer, both containing 1 μmol of ATP, 1 μmol of CTP and 0.125 μmol of CoA as indicated. Incubation was continued in the dark for a further 20 min before reactions were stopped and lipids analysed as described in Table 1. Abbreviations are as for Table 1. The asterisk (*) indicates less than 1%.

Additions for dark incubation		Percentage of total lipid radioactivity									
		Or	PA	PC	SL	PG	PE	NFA	DGG	DAG	THIO
(a) Non-esterified-fatty-acid mode											
Buffer only		*	*	*	4	1	*	79	*	10	3
+CoA and ATP		2	*	6	5	2	*	21	*	14	47
Microsomal fraction only		*	*	3	5	1	*	70	*	10	2
+CoA and ATP		2	*	30	6	3	3	6	*	18	29
(b) Diacylglycerol mode											
Buffer only		1	*	2	5	2	*	47	*	30	2
+ATP and CTP		*	2	3	6	2	*	39	*	35	3
Microsomal fraction only		*	*	2	6	2	*	46	1	30	2
+ATP and CTP		*	4	22	7	4	2	14	1	33	3

CoA synthesized from non-esterified fatty acids in the dark was not utilized for polar-lipid synthesis by isolated chloroplasts, even in the presence of *sn*-glycerol 3-phosphate (see also Table 1). This was surprising in view of the reported acyl-CoA-dependent acylation of *sn*-[^{14}C]glycerol 3-phosphate by stroma and envelopes isolated from spinach chloroplasts (Joyard & Douce, 1977). Our results suggested that the newly synthesized acyl-CoA was located strictly outside the chloroplasts, which remained intact during the course of the experiment. The supernatant alone promoted some diacylgalactosylglycerol synthesis, but there was a major transfer of non-esterified fatty acids to phosphatidylcholine, presumably via acyl-CoA to lysophosphatidylcholine, when supernatant plus CoA and ATP were added to the preincubated chloroplasts (Table 3). A further decrease in non-esterified fatty acid in the additional presence of *sn*-glycerol 3-phosphate may indicate some microsomal synthesis of phosphatidic acid and diacylglycerol.

On the assumption that endoplasmic-reticulum fragments would be responsible for the further processing of lipid intermediates accumulated by isolated chloroplasts, the latter were charged with non-esterified fatty acids or diacylglycerol before incubation in the dark with a washed microsomal fraction from spinach leaf (Table 4). Non-esterified fatty acids were efficiently incorporated into acyl-CoA and phosphatidylcholine, but attempts to encourage further metabolism of the accumulated diacylglycerol via diacylglycerol kinase and CTP-phosphatidate cytidyltransferase were unsuccessful, even when a concentrated microsomal fraction washed free of potential inhibitors was used. A significant conversion of non-esterified fatty acids to phosphatidylcholine in the absence of added CoA (Table 4) may indicate that bound CoA was added along with the microsomal fraction (Baker & Lynen, 1971).

Distribution of ^{14}C -labelled fatty acids within lipids of whole leaves

To determine how these findings might relate to acetate metabolism *in vivo*, spinach leaves were fed [$1\text{-}^{14}\text{C}$]acetate via the cut petioles for 30 min before the lipids were extracted and analysed. The most heavily labelled lipids at this time were phosphatidylcholine, diacylgalactosylglycerol and phosphatidylglycerol, which accounted for 45, 20 and 15% respectively, of total lipid ^{14}C . Saturated fatty acids of diacylgalactosylglycerol were located exclusively at the 2-position (Table 5), whereas the 1-position was enriched in unsaturated fatty acids. This distribution was very similar to that found in diacylglycerol and diacylgalactosylglycerol synthesized by isolated chloroplasts. Similarly, the saturated ^{14}C -labelled fatty acids of phosphatidylglycerol were concentrated at the 2-position, suggesting that the chloroplast diacylglycerol could be a significant acyl source for phosphatidylglycerol synthesis *in vivo*. On the other hand, the saturated ^{14}C -labelled fatty acids of phosphatidylcholine were located exclusively at the 1-position, and there was a more even distribution of unsaturated ^{14}C -labelled fatty acids between the two positions.

Discussion

Kinetic studies of [$1\text{-}^{14}\text{C}$]acetate or $^{14}\text{CO}_2$ incorporation into the fatty acids of lipids in whole leaves have shown the earliest labelled products to reside in phosphatidylcholine, phosphatidylglycerol and diacylgalactosylglycerol (Roughan, 1970, 1975; Slack & Roughan, 1975; Williams *et al.*, 1976; D. J. Murphy & R. M. Leech, personal communication). A high content of ^{14}C -labelled oleate distinguished phosphatidylcholine, whereas phosphatidylglycerol was relatively enriched in ^{14}C -labelled palmitate.

Table 5. *Positional distribution within some glycerolipids of spinach leaves of ^{14}C -labelled fatty acids synthesized from [$1\text{-}^{14}\text{C}$]acetate*

Expanding spinach leaves were fed [$1\text{-}^{14}\text{C}$]acetate (3.4 mM, 58 mCi/mmol) by petiole uptake for 30 min. Lipids were extracted and purified as previously described (Slack & Roughan, 1975). Isolated glycerolipids were treated with phospholipase A_2 or with pancreatic lipase and the monoacyl compounds re-isolated for determination of fatty acid ^{14}C by argentation t.l.c. of the derived methyl esters.

Glycerolipid	Position	Percentage of total fatty-acyl ^{14}C in positions 1 and 2 of purified lipid			
		Saturated	Monoenoic	Dienoic	Trienoic
Phosphatidylcholine	1	11.2	33.5	5.5	0
	2	0	40.1	11.2	0
Diacylgalactosylglycerol	1	(-3.8)	17.6	20.9	15.7
	2	37.0	5.2	7.8	0
Phosphatidylglycerol	1	14.8	18.6	16.6	2
	2	40.2	3.0	2.4	2

Isolated chloroplasts, on the other hand, efficiently synthesized long-chain fatty acids but were apparently capable of very little polar-lipid synthesis *per se*, even when endogenous carbon pools were maintained by rates of CO₂ fixation of 75–100 µmol/h per mg of chlorophyll (Roughan *et al.*, 1979a). Instead, when incubated with [1-¹⁴C]acetate, the chloroplasts accumulated intermediates of lipid metabolism that showed certain similarities to the products synthesized *in vivo*. Thus the accumulated non-esterified fatty acids (about 90% oleate) could be the predominant acyl source for phosphatidylcholine synthesis, and the accumulated diacylglycerol (more than 50% palmitate) could be the main, if indirect, precursor for phosphatidylglycerol synthesis. Indeed, at least one aspect of [1-¹⁴C]acetate metabolism *in vivo*, the synthesis of ¹⁴C-labelled oleate and its transfer to microsomal phosphatidylcholine (Slack & Roughan, 1975), could be reconstituted in a cell-free system by mixing washed chloroplasts, soluble cytoplasmic constituents (CoA and ATP) and a washed microsomal preparation. Such a system, however, could not have affected a net synthesis of phosphatidylcholine, and the observed labelling must have been due to acylation of endogenous lysophosphatidylcholine.

Attempts to stimulate polar-lipid synthesis from the diacylglycerol accumulated by isolated chloroplasts were only partially successful. On the one hand, this diacylglycerol was efficiently utilized for diacylgalactosylglycerol synthesis when UDP-galactose was supplied exogenously, and yet it was not utilized at all for the synthesis of phosphatidic acid, CDP-diacylglycerol or phosphatidylglycerol when exogenous ATP, CTP and *sn*-glycerol 3-phosphate were supplied along with the microsomal fraction. Even so, it seems likely that this chloroplast diacylglycerol would be an important precursor of phosphatidylglycerol synthesis for the following reasons. (1) The 2-position of diacylglycerol synthesized by isolated chloroplasts contained almost exclusively ¹⁴C-labelled palmitate, and during the early stages of labelling with [1-¹⁴C]acetate, 80% of the total ¹⁴C at the 2-position of phosphatidylglycerol from whole spinach leaves was as ¹⁴C-labelled palmitate. (2) *trans*-Hexadec-3-enoate is found exclusively at the 2-position of phosphatidylglycerol from green leaves and algae (Haverkate & van Deenen, 1967), and is considered to be synthesized from palmitate only after incorporation of the latter into phosphatidylglycerol (Bartels *et al.*, 1967; Roughan, 1975; Slack & Roughan, 1975). (3) All of the enzymes required for converting diacylglycerol into phosphatidylglycerol have been described in plant materials (Bradbeer & Stumpf, 1960; Douce *et al.*, 1968; Douce & Dupont, 1969; Marshall & Kates, 1975), and all appear to be exclusive of chloroplasts, which is consistent with

our finding that isolated chloroplasts accumulated diacylglycerol rather than polar lipids. (4) Variations in the relative amounts of non-esterified fatty acids and diacylglycerols synthesized by chloroplasts would account for the variations in the relative amounts of [1-¹⁴C]acetate incorporated into phosphatidylcholine and phosphatidylglycerol respectively of leaves on pumpkin (*Cucurbita pepo*) plants of differing physiological status (Roughan, 1975).

Our recent finding (Roughan *et al.*, 1979b) that the fatty acids of the diacylglycerol synthesized by isolated chloroplasts were desaturated after conversion of the diacylglycerol into diacylgalactosylglycerol suggests that some of this glycolipid synthesized by spinach leaves is derived from a diacylglycerol of the type synthesized by isolated chloroplasts. This would also explain the presence of hexadecatrienoate exclusively at the 2-position of diacylgalactosylglycerol of spinach leaf (Siebertz & Heinz, 1977). However, although chloroplast diacylglycerol may be utilized for the synthesis of diacylgalactosylglycerol in the short term, as indicated in the present study, it seems more likely that 80% of diacylgalactosylglycerol synthesis occurs via more unsaturated diacylglycerol, possibly generated from microsomal phosphatidylcholine (Slack *et al.*, 1976, 1977; Williams *et al.*, 1976).

The only polar lipid synthesized by isolated chloroplasts was phosphatidic acid, and its existence was transitory. Chloroplast phosphatidic acid was probably synthesized by the direct transfer of acyl groups from acyl-primed acyl carrier protein to *sn*-glycerol 3-phosphate, the first acylation being that of palmitate at position-2, which probably occurred in the stroma (Joyard & Douce, 1977; Bertrams & Heinz, 1976). The second acylation, that by oleate from oleoyl-primed acyl carrier protein, would have taken place in the chloroplast envelope (Joyard & Douce, 1977) and may have been stimulated by low concentrations of Triton X-100 (Roughan *et al.*, 1979a). Although Joyard & Douce (1977) and Bertrams & Heinz (1976) used acyl-CoA for the acylation of *sn*-[¹⁴C]glycerol 3-phosphate in their experiments and apparently ruled out an intermediary role for acyl carrier protein; acyl-primed acyl carrier protein was in fact not tested. A direct transfer of palmitate from acyl carrier protein to *sn*-glycerol 3-phosphate appears to be the most plausible interpretation of the effect of added *sn*-glycerol 3-phosphate on the ratio of oleate/palmitate synthesized from [1-¹⁴C]acetate by isolated chloroplasts (Roughan *et al.*, 1979a) and the direct transfer of acyl groups from [¹⁴C]-acyl-primed acyl carrier protein to *sn*-glycerol 3-phosphate in extracts of autotrophic *Euglena gracilis* cells was reported by Renkonen & Bloch (1969). In that study, diacylgalactosylglycerol was

Scheme 1. A model of plant lipid metabolism showing the proposed interrelationships between chloroplasts and cytoplasm. Bold arrows indicate the predominant pathway of fatty acid metabolism in isolated chloroplasts. Abbreviations used; ACP, acyl carrier protein; others as given in Table 1. The notation '(C_{18:3})DGG', for example, serves to indicate that the C_{18:3} moiety is a part of the diacylgalactosylglycerol molecule.

the main ^{14}C -labelled product, particularly when UDP-galactose was added to incubation media, and this suggests that the plastid envelope was the site of diacylglycerol synthesis in those experiments. A chain-length specificity for the acylation of *sn*-glycerol 3-phosphate as observed in the present study may explain the relatively large amounts of monoacyl compounds synthesized from *sn*- ^{14}C glycerol 3-phosphate by chloroplast preparations in other work (Joyard & Douce, 1977; Bertrams & Heinz, 1976).

A summary of the probable sequence of events from [$1\text{-}^{14}\text{C}$]acetate to polar lipids in developing plant tissues is shown in Scheme 1. The scheme is based on studies of long-chain-fatty-acid synthesis and metabolism by isolated spinach chloroplasts (Stumpf *et al.*, 1967; Jaworski *et al.*, 1974; Jacobson *et al.*, 1974; Shine *et al.*, 1976; Roughan & Slack, 1977; Joyard & Douce, 1977; Roughan *et al.*, 1979a,b) and on reports concerning the mechanisms and subcellular localization of polar-lipid assembly in plant cells (Douce, 1974; Kates & Marshall, 1975; Vick & Beevers, 1977; Lord *et al.*, 1972; Vijay & Stumpf, 1971; Dubacq *et al.*, 1976; Slack *et al.*, 1976, 1977, 1979; Styme & Appelqvist, 1978). It applies equally to developing oilseeds, except that diacylgalactosylglycerol synthesis is replaced by triacylglycerol accumulation (Slack *et al.*, 1978). The scheme predicts that the key intermediate in polar-lipid synthesis, phosphatidic acid, should be

synthesized by acylation of *sn*-glycerol 3-phosphate either with acyl-primed acyl carrier protein in the chloroplast or with acyl-CoA in the endoplasmic reticulum, and this seems to differentiate a 'prokaryotic' pathway, resulting in the synthesis of phosphatidylglycerol and perhaps sulpholipid, from a 'eukaryotic' pathway, which is responsible for the synthesis of phosphatidylcholine, phosphatidylethanolamine and indirectly, diacylgalactosylglycerol. One major difficulty resulting from this concept is that the chloroplast envelope contains two pools of diacylglycerol, one synthesized *in situ* and one derived from microsomal phosphatidylcholine, which have to be distinguished for different syntheses.

There is a similarity between this integrated model for lipid metabolism and the acyl-carrier protein and CoA routes of acyl-group metabolism proposed by Shine *et al.* (1976).

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