Plant Physiol. (1997) 113: 549–557

Characterization of the Glycerolipid Composition and Biosynthetic Capacity of Pea Root Plastids[1]

Lingru Xue, Letitia M. McCune, Kathryn F. Kleppinger-Sparace, Michael J. Brown, M. Keith Pomeroy, and Salvatore A. Sparace*

Plant Science Department, McGill University, Macdonald Campus, 21111 Lakeshore Road, Ste. Anne-de-Bellevue, Québec, Canada H9X 3V9 (L.X., L.M.M., K.F.K.-S., M.J.B., S.A.S.); and Plant Research Center, Research Branch, Agriculture and Agrifood Canada, Ottawa, Ontario, Canada K1A OC6 (M.K.P.)

The glycerolipid composition of pea (*Pisum sativum* L.) root plastids and their capacity to synthesize glycerolipids from [UL-^{14}C]glycerol-3-phosphate were determined. Pea root plastids primarily consist of monogalactosyldiacylglycerol, triacylglycerol, phosphatidylcholine, digalactosyldiacylglycerol, and diacylglycerol. Maximum rates of total glycerolipid biosynthesis were obtained in the presence of 2.4 mM glycerol-3-phosphate, 15 mM $KHCO_3$, 0.2 mM sodium-acetate, 0.5 mM each of NADH and NADPH, 0.05 mM coenzyme A, 2 mM $MgCl_2$, 1 mM ATP, 0.1 M Bis-Tris propane (pH 7.5), and 0.31 M sorbitol. Glycerolipid biosynthesis was completely dependent on exogenously supplied ATP, coenzyme A, and a divalent cation, whereas the remaining cofactors improved their activity from 1.3- to 2.4-fold. Radioactivity from glycerol-3-phosphate was recovered predominantly in phosphatidic acid, phosphatidylglycerol, diacylglycerol, and triacylglycerol with lesser amounts in phosphatidylcholine and monoacylglycerol. The proportions of the various radiolabeled lipids that accumulated were dependent on the pH and the concentration of ATP and glycerol-3-phosphate. The data presented indicate that pea root plastids can synthesize almost all of their component glycerolipids and that glycerolipid biosynthesis is tightly coupled to de novo fatty acid biosynthesis. pH and the availability of ATP may have important roles in the regulation of lipid biosynthesis at the levels of phosphatidic acid phosphatase and in the reactions that are involved in phosphatidylglycerol and triacylglycerol biosynthesis.

Plastids play a central role in plant lipid metabolism. As the only subcellular site for de novo fatty acid biosynthesis, plastids provide the entire plant cell with fatty acids for the synthesis of membrane and storage lipids (Stumpf, 1984; Ohlrogge et al., 1993). Plastids are also actively engaged in their own membrane glycerolipid biosynthesis. Most of the information about the glycerolipid biosynthetic capacities of plastids is derived from studies that have been conducted with photosynthetically active chloroplasts (Roughan and Slack, 1982). Chloroplasts contain and synthesize approximately 75% of the total leaf lipid and are thus the most important sites of glycerolipid biosynthesis in leaves (Harwood, 1980; Roughan and Slack, 1982). In contrast, little information is available about lipid metabolism in nonphotosynthetic plastids. The available information suggests that nonphotosynthetic plastids may be similar to chloroplasts in terms of their autonomy for fatty acid and glycerolipid biosynthesis and the types of lipids comprising their membranes. However, the actual lipid composition of each plastid varies from one type to another, depending on the physiology or function of the tissues from which the plastids are isolated as well as differences in the regulation of lipid metabolism in these plastids (Sparace and Kleppinger-Sparace, 1993).

A few studies have described glycerolipid biosynthesis in nonphotosynthetic plastids from such tissues as cauliflower buds and sycamore suspension cells (Journet and Douce, 1985; Alban et al., 1989a, 1989b) and daffodil flower petals (Kleinig and Liedvogel, 1978), however, none have fully defined the cofactor requirements or the regulatory effects that these cofactors have on glycerolipid biosynthesis in these plastids. Recently, pea (*Pisum sativum* L.) root plastids were used as a system for the study of plastidic glycolytic carbon flow and the energy production that is required for fatty acid and glycerolipid biosynthesis in a nonphotosynthetic plastid (Kleppinger-Sparace et al., 1992; Qi et al., 1994, 1995). The latter studies suggest that glycolytic carbon metabolism, in addition to providing carbon and energy, may modulate glycerolipid biosynthesis in pea root plastids. To more fully understand glycerolipid biosynthesis in nonphotosynthetic plastids and the biochemical factors regulating this activity, we have determined the glycerolipid composition of

[1] This research was supported by grants to S.A.S. from the Natural Sciences and Engineering Research Council of Canada.

* Corresponding author; e-mail sparace@agradm.lan.mcgill.ca; fax 1–514–398–7897.

Abbreviations: ASG, acylated sterol glycoside; BTP, 1,3-bis-[Tris(hydroxymethyl)-methylamino]-propane; CDP-DAG, cytidine diphosphodiacylglycerol; DAG, diacylglycerol; CDPcholine: 1,2-diacylglycerol choline phosphotransferase; DGDG, digalactosyldiacylglycerol; FFA, free fatty acids; G3P, glycerol-3-phosphate; MAG, monoacylglycerol; MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGP, phosphatidylglycerol phosphate; PI, phosphatidylinositol; PS, phosphatidylSer; SE, sterol ester; SQDG, sulfoquinovosyldiacylglycerol; TAG, triacylglycerol. Numbers separated by colons (e.g. 14:0–22:1) correspond to fatty acid carbon number:number of double bonds in that fatty acid.

pea root plastids, defined the optimum cofactor requirements for glycerolipid biosynthesis from G3P, and determined the effects of each cofactor on the amounts of each lipid synthesized.

MATERIALS AND METHODS

Plant Material and Isolation of Plastids

Plastids were isolated from germinating pea (*Pisum sativum* L. cv Improved Laxton's Progress) roots as previously described (Kleppinger-Sparace et al., 1992; Trimming and Emes, 1993). Briefly, the cell-free root homogenate was centrifuged at 500*g* for 8 min to yield the crude plastid fraction. These plastids were resuspended in 0.5 mL of homogenization buffer and purified by centrifugation at 4000*g* through a 10% (v/v) Percoll purifying gradient (Pharmacia). Plastids pelleted through the Percoll gradient were washed and finally resuspended in 1.0 mM BTP (pH 7.5) containing 0.33 M sorbitol to yield a final plastid protein concentration of approximately 1 mg/mL.

Marker Enzymes

Marker enzymes that were used for assessing the purity of subcellular fractions during plastid purification were de novo fatty acid synthesis for the plastids (Stahl and Sparace, 1991), fumarase for the mitochondria (Racker, 1950), and both NADPH-Cyt *c* reductase (Lord et al., 1973) and cholinephosphotransferase (Lord et al., 1973; Moore, 1987) for the ER. Marker enzymes for other subcellular organelles, such as the plasma membrane, tonoplast, and Golgi bodies, were not performed because these organelles are not commonly associated with fatty acid and glycerolipid biosynthesis.

In Vitro Conditions for Glycerolipid Biosynthesis

The assay conditions used in this investigation were adapted from those used in an earlier investigation characterizing de novo fatty acid biosynthesis in pea root plastids (Stahl and Sparace, 1991). Each reaction component was individually optimized for glycerolipid biosynthesis from ^{14}C-G3P. For experiments reported here, glycerolipid biosynthesis was measured in 0.5 mL of a reaction mixture containing 0.31 M sorbitol, 0.25 M BTP (pH 7.5), 0.2 mM sodium-acetate, 15 mM $KHCO_3$, 0.05 mM CoA, 0.5 mM NADH, 0.5 mM NADPH, 2 mM ATP, 2 mM $MgCl_2$, and 0.16 mM [UL-^{14}C]G3P (15 μCi/μmol). Reactions were initiated by the addition of 40 μL of plastids (corresponding to approximately 40 μg of protein), incubated for 1 h at 25 C, and terminated by the addition of chloroform:methanol: acetic acid (1:2:0.1, v/v) followed by the extraction of the lipids (Mudd and DeZacks, 1981). Under these conditions, glycerolipid biosynthesis was essentially linear with respect to plastid protein concentration (up to 200 μg mL^{-1}) and incubation time (up to 6 h).

Glycerolipid and Fatty Acid Analyses

Because standard plastid isolations yield only about 200 μg of plastid lipid, the analyses of endogenous (unlabeled) lipids and fatty acids were performed on pooled lipid extracts, each containing approximately 1 to 2 mg of lipid derived from three to five standard plastid isolations. Individual lipids were separated by TLC and their fatty acids were quantified by GC as described by Pomeroy and Mudd (1993), except that TLC silica gel bands containing unresolved PE and SQDG were eluted and extracted (Bligh and Dyer, 1959) and the two lipids were resolved by further TLC using chloroform:methanol:water (65:25:4, v/v) as the developing solvent. For the analysis of radioactive lipid extracts, TLC plates were first prerun in acetone:acetic acid:water (100:2:1, v/v). Lipid components were then separated by one-dimensional TLC (Sparace and Mudd, 1982). TLC plates were first developed with the above prerun solvent followed by either chloroform:methanol:acetone: acetic acid:water (50:10:20:15:5, v/v) or chloroform: methanol:ammonium hydroxide:water (65:35:2:2, v/v). The identities of all glycerolipids and fatty acids were confirmed by co-chromatography with known standards. All other materials and methods for lipid analysis and the quantification of radioactivity and protein were as previously described (Sparace et al., 1988; Qi et al., 1994). All experiments were performed at least twice. The data shown are averages of duplicate analyses within representative experiments.

RESULTS AND DISCUSSION

Plastid Purity

Plastids represent one of three principal sites of glycerolipid biosynthesis in plant cells. In addition to plastids, the ER and mitochondria also serve as important sites of lipid biosynthesis (Moore, 1982; Roughan and Slack, 1982), especially in nonphotosynthetic tissues (Sparace and Kleppinger-Sparace, 1993). In our earlier studies that emphasized fatty acid biosynthesis (Kleppinger-Sparace et al., 1992; Qi et al., 1994), preliminary analyses showed that the products of de novo fatty acid biosynthesis in pea root plastids were recovered in several glycerolipid products and intermediates. These included TAG and PC, which are not typically associated with or synthesized in large amounts by plastids. Thus, before any detailed investigations of the glycerolipid composition or biosynthetic capacities of pea root plastids could be undertaken, it was essential to eliminate the possibility that other organelles involved in glycerolipid biosynthesis might be contaminating our plastid preparations.

Plastid purity was verified by following the activities of relatively typical markers throughout the subcellular fractionation and purification of the plastids. These were NADPH:Cyt *c* reductase and cholinephosphotransferase for the ER, fumarase for the mitochondria, and de novo fatty acid biosynthesis for the plastids (Quail, 1979). All marker enzyme activities were readily detectable in the crude, cell-

Table I. *Recovery of total activities of marker enzymes among subcellular fractions isolated from pea roots*

The crude root homogenate was centrifuged at 500*g* for 8 min to yield the crude plastid-enriched fraction. Following resuspension, this fraction was layered onto a 10% Percoll gradient and then centrifuged at 4000*g* for 5 min to yield the 4000*g* Percoll supernatant, the 4000*g* Percoll, and the 4000*g* Percoll-purified plastids.

Subcellular Fraction	Fatty Acid Synthesis	Fumarase	NADPH: Cyt *c* Reductase	Choline-Phospho-Transferase
		nmol min^{-1}		
Crude homogenate	110	2120	711	9.28
		% of crude homogenate		
500*g* Supernatant	37.2	54.4	88.9	88.5
500*g* Pellet (crude plastids)	45.5	16.7	10.8	0.98
4000*g* Percoll pellet (purified plastids)	53.3	0	0	0.035

free homogenate (Table I). The initial 500*g* centrifugation yielded roughly one-half of the total activity for fatty acid synthesis in the crude plastid-enriched fraction (500*g* pellet). These plastids contained approximately 17 and 11% of the total activities for fumarase and NADPH:Cyt *c* reductase, respectively, but only 1% of the cholinephosphotransferase activity. When the crude plastid fraction was applied to 10% Percoll and centrifuged at 4000*g*, no measurable fumarase or NADPH:Cyt *c* reductase and an apparent 0.03% of the cholinephosphotransferase co-sedimented with the purified plastids. These observations indicate that the plastids used in this investigation are free from mitochondrial contamination, but may contain trace amounts of ER. However, when plastid-derived lipid extracts for the cholinephosphotransferase assay were subjected to TLC, radioactivity was only recovered at the sample origin with none detected in PC (data not shown). Thus, the small amount of "apparent" cholinephosphotransferase associated with the purified plastids likely represents a carryover of trace amounts of ^{14}C-CDPcholine with the lipid phase during extraction. Overall, our observations indicate that the plastids used in this investigation are free from measurable mitochondrial and ER contamination. In related studies (Qi, 1995; Qi et al., 1995) we have also confirmed that these plastids are free from cytosolic and mitochondrial contamination using additional marker enzymes.

Acyl Lipid Composition of Pea Root Plastids

To understand more completely the lipid biosynthetic capacity of pea root plastids, it is important to know their lipid composition. Since the lipid compositions of root plastids have not been previously determined, we performed a relatively detailed analysis of pea root plastids. As expected, pea root plastids are composed of a variety of acyl lipids that are commonly found in plastids (Table II). However, the amounts of each lipid vary markedly from those reported for other nonphotosynthetic plastids and chloroplasts. Glycolipids and phospholipids represented about one-half of the total plastid lipid. Among the glycolipids, MGDG and DGDG predominated with approximately 15 and 10% of the total plastid lipid, respectively, whereas SQDG represented less than 1% of the total lipid. This is considerably less than the 50 to 80% galactolipid and 5 to 7% sulfolipid found in other plastids (Harwood, 1980; Sparace and Kleppinger-Sparace, 1993). Among the phospholipids, PC, PG, and PE were the major components, comprising about 11, 4, and 2% of the total lipid, respectively. PA, PI, PS, and lyso-PC were all relatively minor components (less than 2%), whereas lyso-PA represented approximately 3% of the total lipid. Neutral lipids were unusually prevalent in pea root plastids. Together, they represented almost 40% of the total acyl lipid with approximately 10 to 13% each of TAG, DAG, and FFA. Finally, pea root plastid lipids were comprised of approximately 4 and 7% of ASG and SE, respectively.

In terms of the fatty acid compositions of each lipid, the more typical plant fatty acids (palmitic, stearic, oleic, linoleic, and linolenic acids) represented 70 to 99% of the total fatty acids of all glycerolipids (Table III). Both galactolipids were highly enriched (64–68%) in linolenic acid with very small amounts of C_{16} fatty acids. The latter observation is consistent with the fact that pea is an "18:3 plant" that relies heavily on the "eukaryotic pathway" for galactolipid assembly (Heinz and Roughan, 1983; Browse and Somerville, 1991). In contrast, the phospholipids and SQDG generally contained 20 to 40% of the C_{16} fatty acids with correspondingly less linolenic acid. Neutral lipids were intermediate in that oleic and linoleic acids represented 50 to 60% of the total fatty acids. The monoacylated lipid derivatives (including SE, lyso-lipids, and MAG) were rel-

Table II. *Acyl lipid composition of pea root plastids*

Data shown represent the average of results from two individually analyzed pooled plastid lipid extracts containing 1.2 and 1.5 mg of lipid derived from five standard plastid isolations.

Lipid	Mol%
Glycolipids	
MGDG	15.3
DGDG	9.8
SQDG	0.7
Phospholipids	
PC	11.3
PG	3.9
PE	2.2
PA	1.7
PI	0.4
PS	0.3
Lyso-PA	3.0
Lyso-PC	1.0
Neutral Lipids	
TAG	13.3
DAG	9.8
MAG	4.8
FFA	10.7
Sterol Derivatives	
SE	7.2
ASG	4.6

Table III. *Fatty acid compositions of pea root plastid acyl lipids*

Lipid	14:0	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	Others[a]
							fatty acid mass %							
Glycolipids														
MGDG	+[b]	2.4	+	+	+	0.9	2.9	24.2	68.2	+	+	+	+	1.4
DGDG	+	5.0	+	+	+	3.7	5.0	20.8	64.1	+	+			1.4
SQDG	2.7	16.5	7.8	7.7	2.9	9.4	12.4	21.0	8.3	3.6	4.7	+		3.0
Phospholipids														
PC	+	13.2	+	+	+	4.3	7.1	60.6	12.7	+	+		+	2.1
PG	1.2	38.9	3.4	0.8	1.2	5.6	7.8	26.2	12.8	+	+	+	++	2.1
PE	1.4	18.8	7.7	2.5	3.9	4.9	11.6	33.6	6.8	2.1	2.2	+	+	4.5
PA	0.8	14.0	4.2	2.1	+	8.1	25.4	36.5	2.7	1.3	2.0	0.9	1.0	1.0
PI	5.0	28.4	6.2	5.6	2.3	22.1	12.4	5.5	2.1	0.8	1.9	1.0		6.7
PS	2.9	19.8	5.5	6.0	1.9	14.8	17.1	11.5	10.9	3.3	1.6	+		4.7
Lyso-PA	5.3	17.5	7.5	6.0	1.4	14.8	12.8	11.0	8.8	5.2	6.6	0.8		2.3
Lyso-PC	4.4	15.4	9.2	7.2	2.9	10.5	14.5	17.1	6.7	+	2.8	1.1		8.2
Neutral lipids														
TAG	0.8	7.1	1.3	+	+	3.6	34.7	34.6	7.6	1.5	2.5	1.2	4.3	0.8
DAG	2.3	14.7	1.2	+	+	5.7	33.2	23.6	4.7	2.4	2.9	2.5	5.9	0.9
MAG	3.8	14.0	3.1	1.4	+	8.0	17.3	16.9	13.7	4.0	2.9	12.5	1.8	0.6
FFA	2.5	14.7	3.7	1.2	.9	9.9	22.9	29.5	2.2	2.5	2.2	2.9	4.4	0.5
Sterol derivatives														
SE	7.0	16.0	11.3	3.7	3.2	6.4	19.5	17.9	2.2	3.1	2.1	4.6	2.8	0.2
ASG	1.7	19.2	3.5	2.1	+	7.7	15.8	23.3	20.1	1.9	1.8	1.2	+	1.7

[a] "Others" correspond to those fatty acids occurring in trace amounts and the a^7 isomers of oleic and linoleic acids. [b] +, Corresponds to trace amounts with values < 0.8%. These values are not included in percentage calculations.

atively unique because they all contained almost equal amounts of the typical fatty acids (approximately 15–20% each). Finally, all lipids contained low and variable amounts (1–7%) of myristic acid, unsaturated C_{16} fatty acids, and very long chain-saturated and monounsaturated C_{20} and C_{22} fatty acids. The occurrence of such very long chain fatty acids in glycerolipids is somewhat unusual because these fatty acids are typically associated with the cuticular lipids of plants (Kolattakudy, 1980). However, these fatty acids have been reported to occur in the glycerolipids of some plants (Kuiper and Stuiver, 1972), although they are generally much less abundant and thus are frequently not detected or reported (Hitchcock and Nichols, 1971).

pH and Cofactor Requirements for Glycerolipid Biosynthesis

The rate of total glycerolipid biosynthesis from G3P was greatly dependent on the pH of the incubation medium (Fig. 1A). Activity was greatest at pH 7.5 in either Tricine or BTP buffers, with the latter giving almost 30% greater activity at the optimum pH (Fig. 1A). This is in contrast to the relatively broad optimum of pH 8 to 8.5, typically reported for fatty acid synthesis, including pea root plastids (Stahl and Sparace, 1991) and spinach chloroplasts (Nakamura and Yamada, 1975). This difference most likely reflects the different pH optima of key enzymes that are involved in the utilization of acetate versus G3P for lipid biosynthesis in plastids. In this regard, acetyl-CoA carboxylase from pea root plastids and maize leaf is optimal at pH 8.0 and 8.4, respectively (Nikolau and Hawke, 1984; Qi et al., 1996), whereas the *sn*-G3P acyltransferases from both pea and spinach chloroplasts are optimal at pH 7.5 (Bertrams and Heinz, 1981).

Glycerolipid biosynthesis was also completely dependent on a supply of G3P, ATP, Mg^{2+}, and CoA (Fig. 1, B–F). The effects of increasing concentrations of G3P and ATP were saturated at 2.4 and 1 mM, respectively (Fig. 1, B and C). Similar effects of ATP were observed when equimolar amounts of ATP + Mg^{2+} were varied (data not shown). The responses to Mg^{2+} and CoA were both almost (> 90%) maximal at the lowest concentrations tested (0.5 mM and 2.0 μM, respectively) (Fig. 1, D and E). Higher concentrations of these cofactors gave small improvements in activity but became slightly inhibitory at the highest concentrations tested. Mn^{2+} could only partly substitute for Mg^{2+} and generally gave approximately 30% of the Mg^{2+}-dependent activity at equivalent concentrations (data not shown). Reduced nucleotides (NADH and NADPH) were not absolutely essential for activity, but greatly improved total lipid synthesis (Fig. 1F). Individually, NADH and NADPH increased activity by up to 50%. However, the greatest stimulation (>2-fold) was obtained in the presence of equimolar concentrations of both nucleotides.

The effects of acetate and bicarbonate on glycerolipid biosynthesis from G3P were also determined. The addition or omission of the optimum concentration of acetate required for de novo fatty acid biosynthesis (200 μM; Stahl and Sparace, 1991) had no effect on the rate of total glycerolipid biosynthesis, whereas the addition of up to 25 mM $KHCO_3$ improved activity by about 15% (data not shown). Similarly, G3P is not essential for fatty acid or glycerolipid

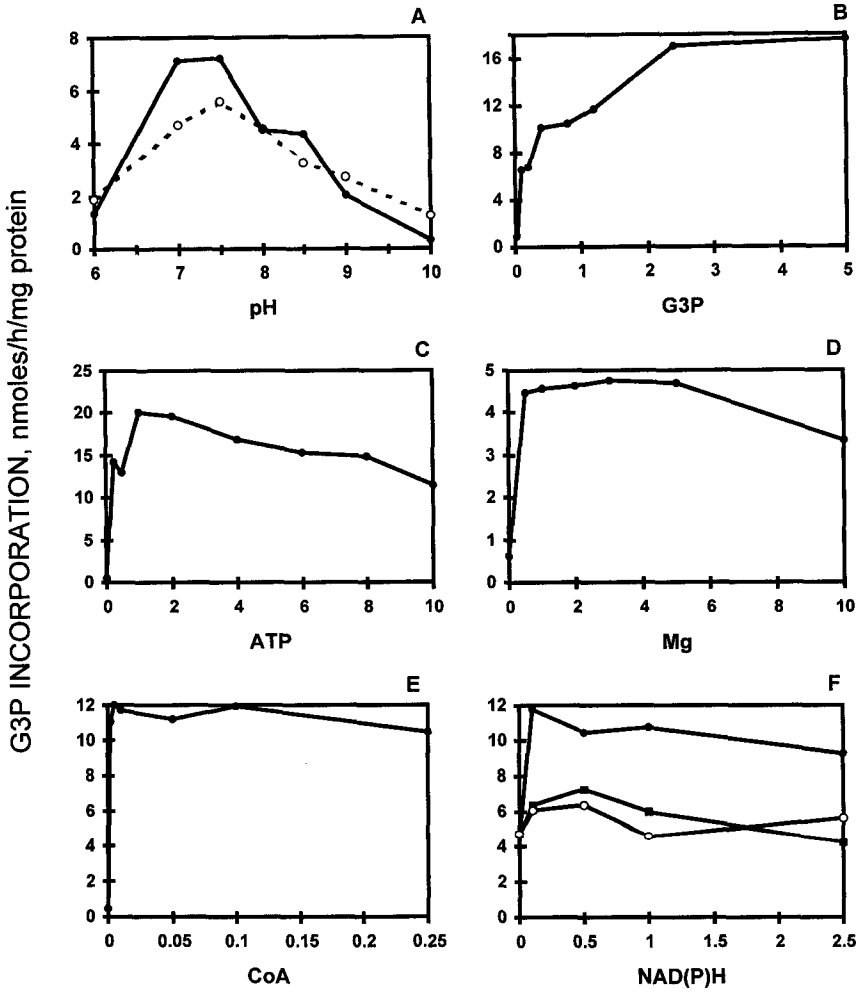

Figure 1. The effects of pH and cofactor concentrations on the total rate of glycerolipid biosynthesis from G3P by pea root plastids. A–F, Experiments performed with different plastid preparations. A, Filled circles with solid lines represent activity in BTP buffer, and open circles with dashed lines represent activity in Tricine buffer. F, Filled squares represent NADH alone; open circles represent NADPH alone; filled circles represent equimolar NADH plus NADPH. All concentrations are mM.

biosynthesis from acetate (Stahl and Sparace, 1991). These observations suggest that the internal pools of carbon precursors for both fatty acid and glycerolipid biosynthesis in pea root plastids are largely adequate for these processes at the time of plastid isolation.

The cofactor and energy requirements shown here for glycerolipid biosynthesis from G3P are in good agreement with our previous work that emphasized fatty acid biosynthesis from acetate (Stahl and Sparace, 1991; Kleppinger-Sparace et al., 1992). With only minor differences in optimum concentrations, both processes have essentially absolute requirements for ATP, CoA, and a divalent cation. Similarly, although not essential, equimolar amounts of NADH and NADPH give a 2-fold stimulation of each process. These similarities indicate that de novo fatty acid biosynthesis and glycerolipid assembly are tightly coupled processes in pea root plastids, and that glycerolipid biosynthesis can only occur when fatty acid biosynthesis is active. This is supported by the fact that 80% or more of the acetate-labeled fatty acids is recovered in various glycerolipids of these plastids (Stahl, 1990; Kleppinger-Sparace et al., 1992). Thus, it is likely that the requirements for ATP, CoA, and reduced nucleotides demonstrated here for glycerolipid biosynthesis from G3P reflect their requirements at key steps in the process of de novo fatty acid biosynthesis from acetate (i.e. acetyl-CoA synthase, acetyl-CoA carboxylase, and acyl-ACP reductases; Ohlrogge et al., 1993).

Effects of pH and Cofactor Concentration on the Distribution of Radioactivity among Labeled Glycerolipids

Radioactivity from G3P was recovered primarily in PA, PG, DAG, TAG, and PC, with lesser amounts recovered in lyso-PA and MAG (Tables IV–VI). This is in agreement with our earlier studies using acetate as a precursor (Kleppinger-Sparace et al., 1992; Qi et al., 1994, 1995). No radioactivity was detected in SQDG, PI, PS, or PE. The latter observations are expected, because these glycerolipids represent a very small fraction (less than 1% each) of pea root plastid lipids (Table II) or are not typically synthesized by plastids. However, in subsequent studies using high specific radioactivity acetate (McCune, 1995), small amounts of SQDG biosynthesis have been detected. Similarly, galactolipid biosynthesis was not detected, because

Table IV. *The effects of pH on distribution of radioactivity among glycerolipids synthesized by pea root plastids*

Samples analyzed were from the experiment illustrated in Figure 1A.

pH	Product Distribution[a]						
	Origin+Lyso-PA	PA	PC	PG	MAG	DAG	TAG
				%			
6.0	9	46	10	7	7	9	12
7.0	5	22	13	22	3	17	18
7.5	4	20	13	23	3	17	18
8.0	4	30	14	10	3	24	15
8.5	2	28	17	8	2	25	18
9.0	4	26	19	7	2	27	15
10.0	11	25	13	18	2	21	10

[a] No apparent differences were observed between product distributions obtained with BTP or Tricine buffers. Data shown represent averages of the combined results from both buffers.

UDP-Gal, which is absolutely essential for galactolipid synthesis (Joyard et al., 1993), was not supplied in this investigation. However, when provided with UDP-Gal, pea root plastids will also synthesize galactolipids (McCune, 1995). Our observations with pea root plastids are in general agreement with related studies of glycerolipid biosynthesis in other nonphotosynthetic plastids and chloroplasts (Sparace and Kleppinger-Sparace, 1993). However, the biosynthesis of TAG and PC remain somewhat of a concern. As mentioned earlier, the biosynthesis of these lipids is typically associated primarily with the ER (Roughan and Slack, 1982; Browse and Somerville, 1991). However, our marker enzyme data presented here (Table I) and elsewhere (Qi, 1995; Qi et al., 1995) indicate that our plastids are free from ER, mitochondrial, and cytosolic contamination. Thus, we believe that the observed TAG biosynthesis is a normal function of pea root plastids. Time-course studies suggest that this biosynthesis proceeds via the classic Kennedy pathway (Kennedy, 1961; data not shown). In support of our observations low amounts of TAG or DAG (1–2%) have been reported for daffodil chromoplasts (Kleinig and Liedvogel, 1978) or amyloplast envelopes (Fishwick and Wright, 1980). Furthermore, TAG appears to be relatively common in whole root tissues, including pea roots (S.A. Sparace, unpublished observations).

The biosynthesis of DAG and TAG by pea root plastids suggests another important anomaly for these plastids. As mentioned earlier, pea is an "18:3 plant" in which galactolipid assembly normally relies on the "eukaryotic" or extraplastidic pathway of lipid metabolism for DAG moieties. This is because PA phosphatase in the "prokaryotic" or plastidic pathway does not have sufficient activity to generate the large amounts of DAG typically required for galactolipid synthesis (Heinz and Roughan, 1983; Joyard et al., 1993). Our observations indicate that PA phosphatase is more active in pea root plastids than in plastids of other 18:3 plants.

The biosynthesis of PC by isolated plastids also remains an enigma. This lipid becomes variously labeled during lipid biosynthesis by a variety of plastids, particularly nonphotosynthetic plastids with pea root plastids among the highest (Sparace and Kleppinger-Sparace, 1993). At present, however, there seems to be no satisfactory explanation for the mechanism of PC biosynthesis in plastids. Again, we considered the possibility of ER contamination in our plastid preparations. In addition to the marker enzyme analyses described earlier, we also tested the effects of exogenously supplied unlabeled CDPcholine in acetate labeling experiments and found that the amount of radiolabeled PC that was synthesized was not affected (data not

Table V. *The effects of G3P concentration on the distribution of radioactivity among glycerolipids synthesized by pea root plastids*

Samples analyzed were from the experiment illustrated in Figure 1B.

G3P	Product Distribution						
	Origin+Lyso-PA	PA	PC	PG	MAG	DAG	TAG
mM[a]				%			
0.02	3	27	11	28	1	20	10
0.1	2	18	11	32	1	22	14
0.2	2	24	9	25	1	20	18
0.4	2	29	8	21	1	21	19
0.8	3	27	9	19	2	21	19
1.2	4	32	9	20	2	20	14
2.4	6	27	9	20	2	22	15
5.0	6	30	9	23	2	17	12

[a] Carrier-free radiolabeled G3P.

Table VI. *The effects of ATP concentration on the distribution of radioactivity among glycerolipids synthesized by pea root plastids*
Samples analyzed were from the experiment illustrated in Figure 1C.

ATP	Product Distribution						
	Origin+Lyso-PA	PA	PC	PG	MAG	DAG	TAG
mM				%			
0.25	1	49	16	6	3	8	17
0.5	3	49	11	12	3	7	16
1.0	1	50	14	11	2	6	15
2.0	2	39	18	12	2	9	18
4.0	1	24	22	10	3	15	25
6.0	1	18	25	9	3	17	27
8.0	1	18	27	9	3	14	28
10.0	4	17	24	9	3	13	30

shown). Therefore, we are left with the possibility that some headgroup exchange or transfer may occur in pea root plastids. This is not completely without precedent, as this, in part, represents the mechanism of DGDG synthesis by the galactolipid:galactolipid galactosyltransferase of chloroplasts (Joyard et al., 1993).

The proportion of various labeled glycerolipids that accumulated was also dependent on the conditions of the reaction mixture. The greatest effects were observed with the pH and the concentrations of G3P and ATP. Among the other cofactors tested, changing the concentrations of divalent cations (Mg^{2+} and Mn^{2+}), CoA, HCO_3^-, and reduced nucleotides all had no apparent effect on the proportions of the various glycerolipids that were synthesized (data not shown). In terms of pH, the amounts of PA, PG, and DAG showed the greatest and most consistent changes (Table IV), with no apparent difference between BTP and Tricine buffers (data not shown). As the reaction pH was shifted from 7.0 to 9.0, the proportion of PA increased from about 20 to 30% at pH 8.0 and then decreased slightly. Similarly, PG decreased from roughly 22 to about 7%, whereas the proportion of DAG increased from 17 to 27%. Glycerolipid distributions at extreme pHs (6.0 and 10.0), due largely to low sample radioactivity, did not completely fit this pattern. Overall, these observations support the idea that PA phosphatase and the enzymes involved in PG synthesis are affected the most by changes in the pH. In this regard, PA phosphatase from spinach chloroplasts is optimal at pH 9.0 with almost no activity at pH 7.0 or less (Joyard and Douce, 1979). In comparison, CDP-DAG synthesis (via CTP:phosphatidate cytidlyl transferase) and PG synthesis (via CDP-DAG:*sn*-G3P phosphatidyltransferase and PGP phosphatase) in several other systems (Douce, 1968; Bahl et al., 1970; Moore, 1974; Kleppinger-Sparace and Moore, 1985), including pea chloroplasts (Andrews and Mudd, 1984), all have slightly acidic to neutral pH optima. Thus, one possible interpretation of the data shown in Table IV is that at relatively neutral pHs (7.0 and 7.5), PG synthesis and accumulation are maximal, causing the greatest depletion of PA. At higher pHs (8.0–9.0) PG synthesis is inhibited (by approximately 60%), leading to an accumulation of PA and DAG, the latter via PA phosphatase.

The concentration of G3P also affected the distribution of radioactivity among glycerolipids (Table V). The greatest and most consistent effects were observed in the amounts of PG and TAG, with somewhat variable changes in the amounts of PA. Over the range of G3P concentrations that were tested, the proportion of label in PG gradually decreased from about 30% to approximately 20%, whereas the amount of label in TAG showed a corresponding increase up to 0.8 mM G3P and then a decrease at higher concentrations. PA showed a variable increase up to about 30% of the total radioactivity. These observations suggest that at low concentrations of G3P, PA produced via acylation of G3P is preferentially used for PG synthesis from PA via CDP-DAG and PGP. At higher concentrations of G3P, more PA becomes available, which can then be used for the synthesis of other lipids, including TAG.

Finally, the concentration of ATP in the reaction mixture also had marked effects on the distribution of radioactivity among the various glycerolipids synthesized (Table VI). At concentrations of 1 mM or less, PA was the major lipid that accumulated, representing approximately 50% of the total radioactivity. At progressively higher ATP concentrations, the level of radioactivity in PA gradually declined to about 18%. This was accompanied by a decrease in the amount of PG with corresponding increases in DAG, TAG, and PC. Together, these observations suggest that the activity of PA phosphatase may be increased at higher concentrations of ATP, thereby diverting PA moieties away from PG biosynthesis. The resulting DAG may then be utilized for TAG and PC synthesis.

CONCLUSIONS

The work presented here indicates that pea root plastids, in comparison with other plastids, have several similarities and differences in terms of their glycerolipid composition and biosynthetic capacities. Whereas they contain essentially the same types of glycerolipids, they are reduced in glycolipids and enriched in neutral lipids, particularly TAG. Like other plastids, they can synthesize almost all of their constituent glycerolipids, including DAG and TAG. PA phosphatase is a key enzyme in pea

root plastid lipid metabolism that is regulated in a manner somewhat different from the enzymes of plastids from other 18:3 plants. The physiological role or significance of TAG biosynthesis in pea root plastids remains poorly understood. As pointed out by Qi et al. (1994), TAG biosynthesis in these plastids may represent a sink for excess lipid carbon, particularly in the presence of abundant ATP or high pH, or under conditions that promote the utilization of glycolytic intermediates for lipid biosynthesis (Qi et al., 1995). Overall, the differences reported here for pea root plastids in comparison with other plastids likely reflect the possibility that pea root plastids are developmentally more distant from chloroplasts than are other plastids that have been studied.

Received August 5, 1996; accepted November 3, 1996.

LITERATURE CITED

Alban C, Dorne A-J, Joyard J, Douce R (1989a) [^{14}C]Acetate incorporation into glycerolipids from cauliflower proplastids and sycamore amyloplasts. FEBS Lett **249:** 95–99

Alban C, Joyard J, Douce R (1989b) Comparison of glycerolipid biosynthesis in non-green plastids from sycamore (*Acer pseudoplantanus*) cells and cauliflower (*Brassica oleracea*) buds. Biochem J **259:** 775–783

Andrews J, Mudd JB (1984) Characterization of CDP-DG and PG synthesis in pea chloroplast envelope membranes. *In* P-A Siegenthaler, W Eichenberger, eds, Structure, Function and Metabolism of Plant Lipids. Elsevier North Holland Biomedical Press, Amsterdam, pp 131–134

Bahl J, Guillot-Salomon, Douce R (1970) Synthèse enzymatique du cytidine diphosphate diglycéride dans les végétaux supérieurs. Physiol Veg **8:** 55–74

Bertrams M, Heinz E (1981) Positional specificity and fatty acid selectivity of purified *sn*-glycerol-3-phosphate acyltransferases from chloroplasts. Plant Physiol **68:** 653–657

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extractions and purification. Can J Biochem Physiol **37:** *911–917*

Browse J, Somerville C (1991) Glycerolipid synthesis: biochemistry and regulation. Annu Rev Plant Physiol Plant Mol Biol **42:** 467–506

Douce R (1968) Mise en évidence du cytidine diphosphate diglycéride dans les mitochondries végétales isolées. C R Acad Sci **267:** 534–537

Emes MJ, England S (1986) Purification of plastids from higher plant roots. Planta **168:** 161–166

Fishwick MJ, Wright AJ (1980) Isolation and characterization of amyloplast envelope membranes from *Solanum tuberosum*. Phytochemistry **19:** 55–59

Harwood JL (1980) Plant acyl lipids: structure, distribution, and analysis. *In* PK Stumpf, ed, The Biochemistry of Plants, Vol 4, Lipids: Structure and Function. Academic Press, New York, pp 1–55

Heinz E, Roughan PG (1983) Similarities and differences in lipid metabolism of chloroplasts isolated from 18:3 and 16:3 plants. Plant Physiol **72:** 273–279

Hitchcock C, Nichols BW (1971) Plant Lipid Biochemistry. Academic Press, New York

Journet E-P, Douce R (1985) Enzymic capacities of purified cauliflower bud plastids for lipid synthesis and carbohydrate metabolism. Plant Physiol **79:** 458–467

Joyard J, Block MA, Malherbe A, Marechal E, Douce R (1993) Origin and synthesis of galactolipid and sulfolipid head groups. *In* TS Moore, ed, Lipid Metabolism in Plants. CRC Press, Boca Raton, FL, pp 231–258

Joyard J, Douce R (1979) Characterization of phosphatidate phosphohydrolase activity associated with chloroplast envelope membranes. FEBS Lett **102:** 147–150

Kennedy EP (1961) Biosynthesis of complex lipids. Fed Proc **20:** 934–940

Kleinig H, Liedvogel B (1978) Fatty acid synthesis by isolated chromoplasts from the daffodil. [^{14}C]Acetate incorporation and distribution of labelled acids. Eur J Biochem **83:** 499–505

Kleppinger-Sparace KF, Moore TS (1985) Biosynthesis of cytidine 5′-diphosphate-diacylglycerol in endoplasmic reticulum and mitochondria of castor bean endosperm. Plant Physiol **77:** 12–15

Kleppinger-Sparace KF, Stahl RJ, Sparace SA (1992) Energy requirements for fatty acid and glycerolipid synthesis in pea root plastids. Plant Physiol **98:** 723–727

Kolattakudy PE (1980) Cutin, suberin and waxes. *In* PK Stumpf, ed, The Biochemistry of Plants, Vol 4, Lipids: Structure and Function. Academic Press, New York, pp 571–645

Kuiper PJC, Stuiver B (1972) Cyclopropane fatty acids in relation to earliness in spring and drought tolerance in plants. Plant Physiol **49:** 307–309

Lord JM, Kagawa T, Moore TS, Beevers H (1973) Endoplasmic reticulum as the site of lecithin formation in castor bean endosperm. J Cell Biol **57:** 659–657

McCune LM (1995) Characterization of galactolipid synthesis in pea root plastids. MS thesis, McGill University, Quebec, Canada

Moore TS (1974) Phosphatidylglycerol synthesis in castor bean endosperm. Kinetics, requirements, and intracellular localization. Plant Physiol **54:** 164–168

Moore TS (1982) Phospholipid biosynthesis. Annu Rev Plant Physiol **33:** 235–259

Moore TS (1987) Phosphoglyceride synthesis in ER. Methods Enzymol **148:** 585–596

Mudd JB, DeZacks R (1981) Synthesis of phosphatidylglycerol by chloroplasts from leaves of *Spinacia oleracea* L. (spinach). Arch Biochem Biophys **209:** 584–591

Nakamura Y, Yamada M (1975) Fatty acid synthesis by spinach chloroplasts I. Property of fatty acid synthesis from acetate. Plant Cell Physiol **16:** 139–149

Nikolau BJ, Hawke JC (1984) Purification and characterization of maize leaf acetyl-coenzyme A carboxylase. Arch Biochem Biophys **228:** 86–96

Ohlrogge JB, Jaworski JG, Post-Bittenmiller D (1993) De novo fatty acid biosynthesis. *In* TS Moore, ed, Lipid Metabolism in Plants. CRC Press, Boca Raton, FL, pp 3–32

Pomeroy MK, Mudd JB (1993) Lipid composition and biosynthesis in protoplasts from cucumber cotyledons. Phytochemistry **34:** 1015–1020

Qi Q (1995) The role of glycolytic metabolism in fatty acid and glycerolipid biosynthesis in pea root plastids. PhD thesis, McGill University, Quebec, Canada

Qi Q, Kleppinger-Sparace KF, Sparace SA (1994) The role of the triose-phosphate shuttle and glycolytic intermediates in fatty-acid and glycerolipid biosynthesis in pea root plastids. Planta **194:** 193–199

Qi Q, Kleppinger-Sparace KF, Sparace SA (1995) The utilization of glycolytic intermediates as precursors for fatty acid biosynthesis by pea root plastids. Plant Physiol **107:** 413–419

Qi Q, Trimming B, Kleppinger-Sparace KF, Emes MJ, Sparace SA (1996) Pyruvate dehydrogenase complex and acetyl-CoA carboxylase in pea root plastids: their characterization and role in modulating glycolytic carbon flow to fatty acid biosynthesis. J Exp Bot **47:** 1889–1896

Quail PH (1979) Plant cell fractionation. Annu Rev Plant Physiol **30:** 425–484

Racker E (1950) Spectrophotometric measurements of the enzymatic formation of fumaric and *cis*-aconitic acids. Biochim Biophys Acta **4:** 211–214

Roughan PG, Slack CR (1982) Cellular organization of glycerolipid metabolism. Annu Rev Plant Physiol **33:** 97–132

Sparace SA, Kleppinger-Sparace (1993) Metabolism in nonphotosynthetic, nonoilseed tissues. *In* TS Moore, ed, Lipid Metabolism in Plants. CRC Press, Boca Raton, FL, pp 563–584

Sparace SA, Menassa R, Kleppinger-Sparace KF (1988) A preliminary analysis of fatty acid synthesis in pea roots. Plant Physiol **87:** 134–137

Sparace SA, Mudd JB (1982) Phosphatidylglycerol synthesis in spinach chloroplasts. Characterization of the newly synthesized molecule. Plant Physiol **70:** 1260–1264

Stahl RJ (1990) Fatty acid and glycerolipid biosynthesis in pea root plastids. MS thesis, McGill University, Quebec, Canada

Stahl RJ, Sparace SA (1991) Characterization of fatty acid biosynthesis in isolated pea root plastids. Plant Physiol **96:** 602–608

Stumpf PK (1984) Fatty acid biosynthesis in higher plants. *In* S Numa, ed, Fatty Acid Metabolism and Its Regulation. Elsevier, Amsterdam, pp 155–179

Trimming BA, Emes MJ (1993) Glycolytic enzymes in non-photosynthetic plastids of pea (*Pisum sativum* L.) roots. Planta **190:** 439–445