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Phosphatidylglycerol Synthesis in Spinach Chloroplasts: Characterization of the Newly Synthesized Molecule

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ABSTRACT

Intact chloroplasts from spinach (*Spinacia oleracea* L., hybrid 424) readily incorporate [^{14}C]glycerol-3-phosphate and [^{14}C]acetate into diacylglycerol, monoacylglycerol, diacylglycrol, free fatty acids (only when acetate is the precursor), phosphatidic acid, phosphatidylcholine, and most notably phosphatidylglycerol. The fraction of phosphatidylglycerol synthesized is greatly increased by the presence of manganese chloride in the reaction mixture. Glycerol-3-phosphate-labeled phosphatidylglycerol is equally labeled in the two glycerol moieties of the molecule. Acetate-labeled phosphatidylglycerol is equally labeled in both acyl groups. Position one contains primarily oleate, linoleate and small amounts of palmitate. Position two contains primarily palmitate. No radioactive *trans*-Δ^3-hexadecenoate was detected. The labeling patterns indicate that the radioactive phosphatidylglycerol is the product of *de novo* chloroplast lipid biosynthesis and furthermore, phosphatidylglycerol may be a substrate for fatty acid desaturation.

PG[2] is an important lipid component of chloroplasts. Depending on the tissue, it can comprise 60 to 70% of the total chloroplast phospholipid (10). Characterized by the presence of the unusual *trans*-Δ^3-hexadecenoic acid (11) and its increase during chloroplast development, this unique species of PG has been suggested to have a role in granal stacking (9).

While chloroplasts from *Euglena* can synthesize PG (4–6), conflicting reports (7, 16, 25) left some question as to whether or not chloroplasts from higher plants can synthesize their own PG. The earlier works of Douce and Guillot-Salomon (7) and Sastry and Kates (25) suggested that PG was synthesized by chloroplasts. These results were cast in doubt when Marshall and Kates (16) determined that PG was not synthesized by chloroplasts, and since then, chloroplasts from higher plants generally have been considered incapable of PG synthesis. Recently, Mudd and DeZacks (20) reexamined chloroplasts from spinach for their capacity to synthesize PG and presented evidence that these higher plant chloroplasts could in fact synthesize their own PG.

We have continued these investigations using both radiolabeled G3P and acetate to further establish optimal conditions for PG synthesis. We have also subjected the biosynthesized PG to a number of degradative procedures in order to understand the pathway to the newly synthesized PG molecule.

MATERIALS AND METHODS

Spinach Culture. Spinach seeds (*Spinacia oleracea* L., hybrid 424; Ferry Morse Seed Company, Mountain View, CA) were germinated and maintained for 10 to 12 d in vermiculite moistened with half-strength Hoagland nutrient solution. The seedlings were transferred to hydroponic culture dishes containing nutrient solution and supported by plastic sheets with 1-cm holes and cotton plugs. Seedlings and plants were grown in a growth chamber with a 12-h daylength and maintained at 23°C and 18°C during the daytime and nighttime, respectively.

Chloroplast Isolation. Spinach leaves were harvested and homogenized and chloroplasts isolated and purified as described previously (20). Chl determinations were as described by Holden (12).

Chloroplast Incubation Conditions and Lipid Extraction. The composition of the standard reaction mixture conditions of incubation and lipid extraction were all as described earlier (20). Variations of these procedures were the use of 2 mM $MnCl_2$ in the standard reaction mixture and substitution of 1 M KCl for the water of the extraction mixtures when phospholipase A_2 and *Rhizopus arrhizus* digestions were performed. When necessary, approximately 500,000 to 600,000 dpm of [2-^{14}C] acetate were substituted for the L-[U-^{14}C]G3P (Amersham Corp.).

Product Analysis. Aliquots of the lipid extracts were retained for scintillation counting to determine total substrate incorporation. The remainders of the samples were applied as 2-cm streaks to commercially prepared (Brinkman Instruments, Inc., Westbury, NY) TLC plates. For routine product separations, silica gel thin-layer plates were developed first with acetone:acetic acid:water (100:2:1, v/v) and allowed to dry under flowing N_2 for 3 h after which they were redeveloped in the same direction with chloroform:methanol:NH_4OH:water (65:35:2:2, v/v), but permitting the second solvent to transverse the plate approximately half as far as the first solvent. Radioactive products were initially identified by both cochromatography with known standards and the use of specific spray reagents and they were routinely located by autoradiography and iodine vapors. Regions of interest on the chromatograms were scraped directly into scintillation vials. Aqueous Counting Scintillant (Amersham) was added, and radioactivity measured using a Beckman model LS-230 liquid scintillation counter. When lipid samples were to be further analyzed (*e.g.* lipase digestion, GC), duplicate samples were handled in parallel, one sample was used to locate compounds of interest while the other was used for the analysis. Whenever possible, samples were maintained under N_2.

Lipase Digestions. Aliquots of either G3P-labeled or acetate-labeled PG were fortified with PG derived from egg lecithin

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[2] Abbreviations: PG, phosphatidylglycerol; G3P; glycerol-3-phosphate; GPG, glycerylphosphorylglycerol; FFA, free fatty acids; DG, diacylglycerol; PA, phosphatidic acid; MG, monoacylglycerol; LPA, lysophosphatidic acid; PC, phosphatidylcholine; SQDG, sulfoquinovosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; CDP-DG, cytidine-5′-diphosphatediacylglycerol.

(Sigma Chemical Co.) and digested with *Rhizopus arrhizus* lipase, phospholipase A_2, phospholipase C from *Bacillus cereus* (all from Sigma Chemical Co.) or crude phospholipase D from cauliflower. Prior to the addition of lipases, each reaction vessel containing the lipid sample and all components of the reaction mixture except the enzyme was sonicated. All digestions were initiated by the addition of the appropriate lipase solution, carried out at room temperature with vigorous shaking and finally terminated by the addition of the extraction mixture. The remaining conditions for each digest are as follows. *Rhizopus arrhizus* lipase: according to Fischer *et al.* (8) except that 4,000 units of lipase were used and the reaction allowed to proceed for 3 h. Phospholipase A_2: 100 mM Tris, pH 7.5; 10 mM $CaCl_2$; 2.5 μg Triton X-100; 1 mg cobra venom containing phospholipase A_2; in a final volume of 2 ml. Triton was added as a chloroform solution to the lipid sample to be digested. The mixture was taken to dryness with flowing N_2 and the remaining components of the reaction mixture were added. The digest was allowed to proceed for 30 min. Phospholipase C: 100 mM Tris, pH 7.5; 10 mM $CaCl_2$; 10 units phospholipase C; 1 ml diethylether; in a final aqueous volume of 2 ml (total liquid volume was 3 ml). The ether and the reaction mixture were added to a dried sample to be digested and the digestion allowed to proceed for 4 h. Phospholipase D: Crude phospholipase D was prepared from cauliflower inflorescence tips as described by Quarles and Dawson (22) and the reaction mixture consisted of 40 mM acetate buffer, pH 5.5; 40 mM $CaCl_2$; 0.35 mM SDS; 1 ml of the crude preparation phospholipase D; in a final volume of 2 ml. The reaction was allowed to proceed for 4 h.

The chloroform-soluble products released from the *Rhizopus arrhizus* and phosholipase A_2 digestions (2-acyl-GPG and 1-acyl-GPG, respectively, and FFAs) were separated by TLC using the double solvent system discussed earlier. The chloroform-soluble products of the phospholipase C and D digestions (DG and PA, respectively) were applied to silica gel thin-layer plates which were developed with hexane:diethyl ether:acetic acid (80:20:2, v/v) and chloroform:methanol:NH_4OH:water (65:35:2:2, v/v), respectively. Water-soluble products of these digestions (glycerol phosphate and glycerol, respectively) were applied to silica gel thin-layer plates which were developed with isopropanol:NH_4OH:water (6:3:1, v/v). All radioactive products of the lipase digestions were localized and identified as described earlier.

Gas Chromatography. Methyl esters of fatty acids were prepared with BF_3 as described by Beare-Rogers (2) and were analyzed with a Packard model 430 gas chromatograph equipped with flame ionization detectors and a stream splitter which diverted 95% of the sample effluent to a Packard model 895 gas proportional counter. Glass columns were packed with 10% EGSS-X on chromosorb W (Supelco, Inc.).

RESULTS

General. Spinach chloroplasts readily incorporate both ^{14}C-labeled G3P and acetate into neutral and polar lipids, most notable PG, under conditions designed to promote fatty acid synthesis and acylation of G3P. Under the conditions described by Mudd and DeZacks (20), acetate is incorporated into DG, MG, FFA, PA, LPA, PC, and PG. In addition, SQDG is also labeled to some extent with only trace amounts of radioactivity occurring in MGDG. Figure 1 shows a routine separation of acetate-labeled lipids using the double solvent system discussed in "Materials and Methods."

Under the conditions described previously (20), total acetate incorporation into lipid was linear up to 1 h after which the incorporation tapered off and by 3 h further incorporation ceased. With respect to Chl concentration, acetate incorporation was essentially linear from 0 to 75 μg/ml.

Manganese Effects. Manganese chloride was found to stimulate greatly the relative amount of label occurring in PG using either

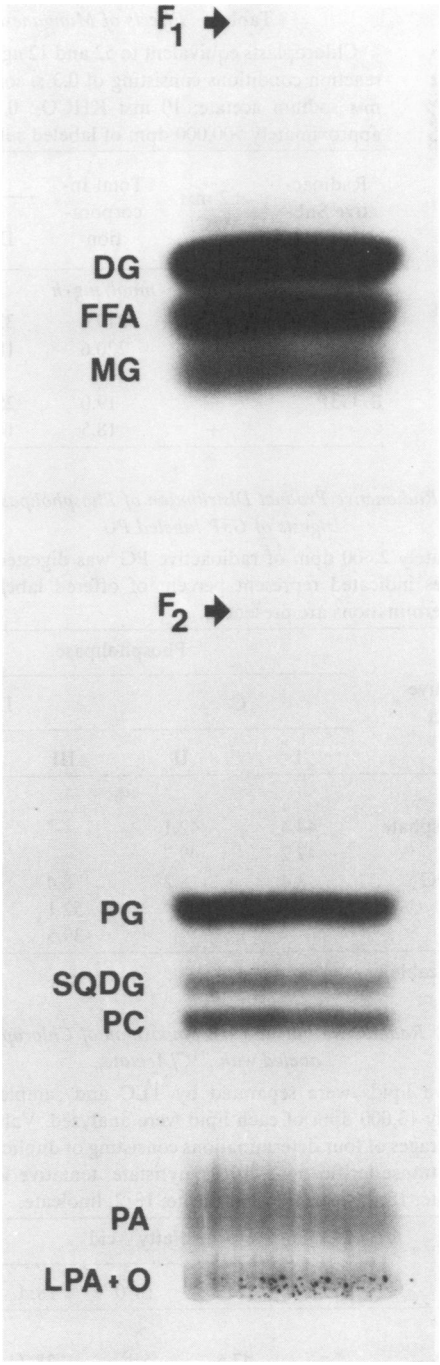

FIG. 1. Autoradiogram of routine separation of radioactive chloroplast lipids labeled with [^{14}C]acetate. Chloroplasts equivalent to 87 μg Chl were incubated in the standard reaction mixture and resulted in the incorporation of approximately 80,000 dpm into lipid of the chloroform phase of the extraction mixture which was applied as a 3-cm streak to a thin layer plate developed with the double solvent system described in text. X-ray film was exposed to the plate for 15 d. F_2 and $_2$, solvent fronts of solvents 1 and 2; O, origin.

G3P or acetate as the lipid precursor (Table I). At 2 mM $MnCl_2$, incorporation of either precursor into PG was maximal even though overall incorporations of label into total lipid were slightly reduced. The increase in the percent PG synthesized was invariably accompanied by a decrease in the amount of DG formed. Higher concentrations of Mn^{2+} gave greater proportions of PG synthesized (as high as 40% for acetate) but further reduced overall incorporation of substrate into lipid. The manganese requirement

Table I. *Effects of Manganese Chloride on PG Synthesis from [^{14}C]Acetate and [^{14}C]G3P*

Chloroplasts equivalent to 52 and 12 μg Chl were used in experiment A and B, respectively, under the standard reaction conditions consisting of 0.3 M sorbitol; 33 mM Tricine-NaOH, pH 7.9; 2 mM $MgCl_2$; 1.5 mM ATP; 0.15 mM sodium acetate; 10 mM $KHCO_3$; 0.2 mM K_2HPO_4; 0.2 mM CoA; 0.5 mM DTT; 0.4 mM DL-G3P; and approximately 500,000 dpm of labeled substrate.

Radioactive Substrate	±2 mM $MnCl_2$	Total Incorporation	Product Distribution						
			DG	FFA + MG	PG	SQDG	PC	PA	LPA + Origin
		nmol/mg·h				%			
A. Acetate	−	319.6	32.1	38.2	8.5	1.5	4.7	10.7	4.2
	+	220.6	18.7	26.5	32.2	2.1	5.8	10.8	4.0
B. G3P	−	19.0	25.5	32.8	4.0	3.0	1.9	19.8	13.0
	+	18.5	14.9	31.5	17.8	2.9	2.1	21.1	9.6

Table II. *Radioactive Product Distribution of Phospholipase C and D Digests of G3P-labeled PG*

Approximately 2,600 dpm of radioactive PG was digested per sample (I–IV). Values indicated represent percent of offered label. Results of duplicate determinations are presented.

Radioactive Product	Phospholipase			
	C		D	
	I	II	III	IV
		%		
Glycerol phosphate	44.4	42.1	—[a]	—
DG	47.2	49.7	—	—
Undigested PG	8.4	8.2	8.4	8.6
Glycerol	—	—	52.1	47.9
PA	—	—	39.5	43.6

[a] Not applicable.

Table III. *Radioactive Fatty Acid Composition of Chloroplast Lipids Labeled with [^{14}C]Acetate*

Radioactive lipids were separated by TLC and samples containing approximately 13,000 dpm of each lipid were analyzed. Values indicated represent averages of four determinations consisting of duplicate injections of duplicate transesterifications. 14:0, myristate, tentative identification; 16:0, palmitate; 18:0, stearate; 18:1, oleate; 18:2, linoleate.

Lipid	Fatty Acid				
	14:0	16:0	18:0	18:1	18:2
			%		
PG	—[a]	47.5	5.1	28.7	18.7
DG	Trace[b]	43.2	6.9	48.6	—
FFA + MG	Trace	29.5	11.8	57.1	—
PC + SQDG	—	30.3	Trace	57.6	10.1
PA + LPA + O	—	36.3	3.2	57.8	Trace

[a] None detected.
[b] Less than 3%.

for high rates of PG synthesis was in addition to the already-present Mg^{2+} of the standard reaction mixture (20). With [^{14}C]-acetate as precursor, substitution of Mn^{2+} for Mg^{2+} decreased total incorporation as well as the proportion of PG synthesized.

Phospholipase C and D Digests. Radioactive PG was isolated from chloroplasts which were allowed to incorporate [^{14}C]G3P, and digested with phospholipases C and D to ascertain the nature of labeling by this lipid precursor. Phospholipase C released radioactive glycerol phosphate and radioactive DG to the aqueous and organic phases, respectively, of the extraction mixture. Phospholipase D released radioactive glycerol and radioactive PA to the aqueous and organic phases, respectively, of the extraction mixture. The results of these digests, summarized in Table II, are in good agreement and indicate that the PG newly synthesized from radioactive G3P is essentially equally labeled in the glycerol 'backbone' and polar 'head' moieties of the molecule.

Table IV. *Distribution of Radioactivity among Products of Phospholipase A_2 and Rhizopus arrhizus Lipase Digestions of [^{14}C]Acetate-Labeled PG*

Approximately 6,600 dpm of labeled PG was digested per sample (I–IV). Unaccounted-for radioactivity was assumed to be in LPG lost to aqueous phase during extraction (see text). Values indicated represent duplicate determinations of percent of label offered.

Radioactive Product	Phospholipase A_2		Rhizopus Lipase	
	I	II	III	IV
		%		
1-Acyl-GPG	16.0	19.4	—[a]	—
2-Acyl-GPG	—	—	18.7	20.7
FFA	51.0	53.9	38.6	40.3
Undigested PG	1.5	1.8	2.9	3.6
Lost to aqueous	31.5	24.9	39.8	35.9

[a] Not applicable.

^{14}C-Fatty Acid Composition of Chloroplast Lipids. Radioactive PG was isolated from chloroplasts which were allowed to incorporate [^{14}C]acetate and was analyzed for its radioactive fatty acid composition by GC. The remaining radiolabeled lipids were variously combined and similarly analyzed as a comparison. A summary of these data is shown in Table III. As expected for all lipids, the fatty acids most highly labeled were palmitate and oleate. However, it was somewhat surprising to find as much as 19% linoleate associated with the PG as well as the lesser amounts of this acid with the PC and PA fractions particularly since the chloroplasts used in this investigation are essentially free from microsomal contamination (20) and are not supplied with UDP-galactose and consequently not in a galactolipid biosynthetic mode as described by Roughan *et al.* (23). Only small amounts of stearate were found except in the FFA + MG fraction which contained almost 12% stearate. Throughout this investigation, none of the characteristic *trans*-Δ^3-hexadecenoate of PG was ever labeled with [^{14}C]acetate although the mass of this fatty acid was readily detected.

[^{14}C]Acetate-labeled PG was also digested with phospholipase A_2 and lipase from *Rhizopus arrhizus* to determine the relative distribution of label between the number one and number two acyl positions of the newly synthesized PG molecule as well as the fatty acid composition at these two positions. The results of these digests are summarized in Tables IV and V. The recovery of the radioactive digestion products in the organic phase of the extrac-

tion mixture was about 70% of the originally offered radioactive PG (approximately 6,600 dpm), although 100% of the radioactive products applied to the TLC plates was recovered. It was concluded that the unaccounted-for radioactivity was in LPG that was lost to the aqueous phase of the extraction mixture. The detergent-like nature of lyso lipids increases their solubility in aqueous media. The loss of radioactivity occurred despite attempts to force all products into the chloroform phase by means of an acidified extraction mixture (20) which also incorporated the use of 1 M KCl. The presence of Triton X-100 in the digestion mixture and throughout the extraction may have enhanced retention of LPG in the aqueous phase. The data in Table IV were adjusted to 100% recovery by attributing unrecovered radioactivity to LPG lost to the aqueous phase. In any case, lipases released 50% of the labeled fatty acid from position two and 40% from position one and indicate that PG newly synthesized from [^{14}C]acetate is nearly equally labeled in each of its acyl positions.

FFA and LPG products of both the *Rhizopus arrhizus* and phospholipase A_2 digestions were recovered and individually analyzed for their radioactive fatty acid compositions, and the results of these analyses are shown in Table V. The high degree of similarities between the fatty acid compositions of the LPG from the phospholipase A_2 digest and the FFA from the *Rhizopus arrhizus* digest as well as that between the FFA from the phospholipase A_2 and LPG from the *Rhizopus arrhizus* digests indicate that there was no apparent selective loss of the various species of the LPGs generated. These data further indicate that virtually all of the 18-carbon fatty acids were associated with position one of the PG molecule while only palmitate was associated with position two. In addition, small amounts of palmitate were also detected at position one.

DISCUSSION

Owing to the relatively high amounts of the unique molecular species of PG associated with chloroplasts and the somewhat doubtful aspect of total dependence of this organelle on extrachloroplastic sources of PG, Mudd and DeZacks (20) recently reexamined isolated spinach chloroplasts for their capacity to synthesize this important lipid component. Using purified chloroplasts under reaction conditions designed to promote photosynthesis, fatty acid biosynthesis and acylation of G3P, they were able to demonstrate PG synthesis which accounted for as much as 40% of the total newly synthesized glycerolipid.

We have continued these investigations using both [^{14}C]G3P and [^{14}C]acetate as lipid precursors and have found that PG synthesis from either precursor is stimulated by the addition of $MnCl_2$ to the standard reaction mixture as described earlier (20). The increases in amounts of PG synthesized were always accompanied by decreased amounts of DG formed and when sums of the amounts of oleate and linoleate in these lipids are viewed, the high degree of similarity in their radioactive fatty acid compositions becomes quite evident (Table III). These results strongly suggest that Mn^{2+} may be acting to divert the products of chloroplast lipid metabolism from DG to PG by either directly inhibiting PA phosphatase or stimulating PG synthesis presumably via CDP-DG and possibly a combination of both actions. Indirect evidence for the former comes from the work of Joyard and Douce (14) who showed that Mg^{2+} inhibited, and EDTA stimulated, PA phosphatase of isolated chloroplast envelopes. Mg^{2+} has the same effect on the castor bean PA phosphatases (T. S. Moore, personal communication). As a divalent cation, Mn^{2+} may be acting in a similar fashion. Evidence in regard to the involvement of Mn^{2+}-related CDP-DG synthesis is also indirect. While we were unable to detect any accumulation of radioactive CDP-DG with [^{14}C]acetate, [^{14}C]G3P or [^{3}H]CTP (S. A. Sparace and J. B. Mudd, unpublished results), the participation of this important lipid intermediate cannot be ruled out. The presumably undetectable levels of CDP-DG can be explained by the possibility that, once synthesized, it is rapidly and efficiently converted to PG. If this is true, Mn^{2+} may be serving as cofactors for both CDP-DG synthesis and its conversion to PG, as is true in other systems where these activities are known to occur (1, 15, 16, 19, 28). However, further work is necessary to better understand the relationship between Mn^{2+} and CDP-DG synthesis and their role in chloroplast lipid metabolism. More recently (17), divalent cations have been shown to alter stromal pH of isolated chloroplasts, which could ultimately affect activities of enzymes involved in chloroplast lipid metabolism (13).

When [^{14}C]G3P is the lipid precursor, the newly synthesized PG is approximately equally labeled in the glycerol backbone and polar head portions of the molecule. When [^{14}C]acetate is the precursor, both acyl positions become essentially equally labeled. These data collectively indicate that the PG synthesized under our experimental conditions is the product of *de novo* lipid metabolism and rule out the possibilities of PG synthesis by transphosphatidylation catalyzed by phospholipase D or acylation of LPG.

It was somewhat unexpected to find that approximately 30% of the 18-carbon fatty acids associated with position one of the PG molecule was in the form of linoleate with considerably lesser amounts of this fatty acid detected in the PC, DG, and PA fractions. At the present time, there appear to be only two complex lipids on which fatty acid desaturation is thought to occur in higher plants. These are microsomal PC (26, 27) and chloroplastic MGDG (23). Inasmuch as our chloroplasts are essentially free from microsomal contamination (20) and they are not in the presence of UDP-galactose for galactolipid biosynthesis (23), we feel that the observed desaturation of oleate to linoleate most likely occurred *in situ* on the newly synthesized PG molecule. This would suggest that under conditions favoring high rates of PG synthesis, the chloroplast desaturase system already described (23) might also accept PG as a substrate.

The fatty acid labeling patterns shown in Table III confirm previous reports of equal amounts of label in 16:0 and 18:1 in DG when G3P is present in the incubation medium (18, 24). Our results are also consistent with the findings of Bertrams and Heinz (3) on the order of acylation of G3P in the chloroplast. The data in Table III show that the presence of monoacyl lipids in an analyzed fraction (MG or LPA) increases the proportion of 18:1, consistent with the evidence that the first acylation of G3P is at position 1 and the preferred fatty acid is 18:1 (3).

The occurrence of approximately 2% G3P-labeled PC (Table I)

Table V. *Radioactive Fatty Acid Composition of Products of Phospholipase A_2 and Rhizopus arrhizus Digests of [^{14}C]Acetate-Labeled PG*

Approximately 13,000 dpm of radioactive PG was digested in duplicate for each lipase. The products were recovered by extraction and TLC. 16:0, palmitate; 18:0, stearate; 18:1, oleate; 18:2, linoleate.

Fatty Acid	Phospholipase A_2				*Rhizopus* Lipase			
	Position 1 (LPG)		Position 2 (FFA)		Position 1 (FFA)		Position 2 (LPG)	
	I	II	I	II	III	IV	III	IV
				%				
16:0	7.8	10.1	92.6	84.4	15.6	13.0	100	100
18:0	—[a]	Trace[b]	7.4	15.5	9.5	—	—	—
18:1	64.6	62.0	Trace	—	44.9	64.1	—	—
18:2	27.5	27.9	—	—	30.0	22.9	—	—

[a] None detected.
[b] Not computed.

is unexpected. At that level, it is barely detectable on the autoradiographs. The value was included merely to provide a complete comparison between the patterns of G3P- and acetate labeling. CDP-choline was not metabolized by the chloroplast preparations (S. A. Sparace and J. B. Mudd, unpublished results).

Throughout this investigation, no radioactive *trans*-Δ^3-hexadecenoate of PG ever became labeled enough to be detected. This fact was not surprising since fully matured chloroplasts were used in this investigation, whereas the available evidence suggests that the biosynthesis of *trans*-Δ^3-hexadecenoate may be tightly coupled to the early stages of chloroplast development (9, 21, 29).

The data presented here and previously (20) are a clear indication that PG synthesis is a normal component of chloroplast lipid metabolism and that chloroplasts may be autonomous in this regard.

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