Lipid biosynthesis by isolated plastids from greening pea, *Pisum sativum*

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Abstract Isolated etioplasts from 8-day-old dark-grown pea seedlings incorporated [1-^{14}C]acetate into lipid at a relatively low rate. Plastids from seedlings that had been illuminated for at least 2 hr showed an enhanced incorporation provided the plastids were illuminated during incubation with the labeled acetate. Dark incubation or the addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) decreased the acetate-incorporating activity of the developing chloroplasts to the level observed with etioplasts. Light had a marked effect on the type of fatty acid into which acetate was incorporated by the developing chloroplasts. Unsaturated fatty acids (mostly oleic acid) accounted for 60–80% of the incorporated label if the plastids were illuminated, but in the dark or in the presence of DCMU the unsaturated acids accounted for only 0–15% of the label incorporated into lipid. The effect of ATP on incorporation was dependent on the maturity of the chloroplasts; mature pea chloroplasts were inhibited by ATP, whereas in developing plastids there was a slight stimulation by ATP. Inhibition of acetate incorporation into lipid by DCMU appears to be due to inhibition of noncyclic phosphorylation. Incorporation was restored by reduced 2,3,5,6-tetramethylphenylenediamine, which restored phosphorylation, but not by reduced *N,N,N′,N′*-tetramethylphenylenediamine.

Supplementary key words developing photosystems · desaturation · phosphatidylcholine · subcellular distribution · 3-(3,4-dichlorophenyl)-1,1-dimethylurea

The levels of lipid in the leaves of castor bean (1), barley (2, 3), clover (4), pea (5, 6), and bean (6) seedlings during greening have been reported. The most noticeable differences between the leaves of dark-grown plants and green leaves are in the levels of MGD and DGD, which are the dominant complex lipids of green tissues, and in the amount of the polyunsaturated fatty acid linolenic acid. The increase in linolenic acid during greening is due largely to the higher levels of the galactolipids, since linolenic acid is the major fatty acid of these lipids in higher plants. The galactolipids are confined mainly to the plastids, and their increase during greening is a reflection of the synthesis of thylakoid membranes. Another distinctive feature of green leaves is the presence of *trans*-Δ^3-hexadecenoic acid as one of the major fatty acids of PG. Tevini (3) found an increase in PC relative to leaf weight in greening barley, and he suggested that this phospholipid has a role in chloroplast development. With bean, on the other hand, the level of PC falls with greening, on the basis of both total lipid and leaf weight (6).

Roughan and Boardman (6) have shown that in the first 6–8 hr of illumination of dark-grown pea and bean seedlings, there is no significant change in concentration of the major leaf lipids, even though differentiation of the chloroplast and photosynthesis begins within this period (7, 8). The increased levels of lipid observed after 6–8 hr of greening are probably fulfilling a requirement for new chloroplast membranes in grana, rather than being essential for the conversion of the etioplast membranes to photosynthetically active membranes.

In addition to assays of total lipids, the incorporation of radioactive acetate into lipids by leaves has also been studied under dark and light growth conditions (2, 4). In general, the patterns of labeled lipids are surprisingly different from those expected on consideration of the total lipid assays. In clover, for example, the galactolipids are relatively poorly labeled in the light, whereas the phospholipids PE and PC are heavily labeled (4). Also, there is more incorporation of acetate into palmitic and oleic acids than into linoleic and linolenic acids. It is not known whether the large stimulation of de novo lipid synthesis by light (2, 4) is due to increased enzyme synthesis, increased ATP or NADPH from photosynthesis, or other light-dependent factors.

The biosynthesis of fatty acids from acetate by isolated chloroplasts from green leaves is dependent on light (9–11) and is stimulated by the detergents Triton X-100 and digitonin (12). The major labeled fatty acids are oleic and

Abbreviations: DAD, 2,3,5,6-tetramethylphenylenediamine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DGD, digalactosyldiglyceride; FFA, free fatty acids; MGD, monogalactosyldiglyceride; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; POPOP, 1,4-bis-2-(5-phenyloxazolyl)-benzene; PPO, 2,5-diphenyloxazole; TMPD, *N,N,N′,N′*-tetramethylphenylenediamine.

palmitic acids. Recently, Kannangara et al. (13) have reported on the biosynthesis of lipids by developing barley plastids. A light stimulation of [1-^{14}C]acetate incorporation into lipid was observed with isolated plastids provided the dark-grown seedlings had been illuminated for at least 6 hr. Palmitic acid was the major labeled product with plastids from dark-grown seedlings, but there was a marked increase in the relative labeling of oleic acid if the seedlings were illuminated for several hours prior to plastid isolation.

In this communication, we describe the incorporation of [1-^{14}C]acetate into lipid by developing pea plastids, which were considerably more active than developing barley plastids (13). We confirm the observation of Kannangara et al. (13) that incorporation by developing plastids is stimulated by light. In an attempt to understand the nature of the light effect, we have examined the effects of DCMU, ATP, and electron donation to photosystem I on both the total incorporation and the relative amount of label in the unsaturated fatty acids.

MATERIALS AND METHODS

Pea seeds (*Pisum sativum* L. var. Greenfeast) were soaked overnight in running water and planted in vermiculite wetted with Hoagland's nutrient solution. After the stated number of days in the dark at 25°C, the leaf buds were harvested either in the dark or after a period of continuous illumination of the whole plants (greening) under fluorescent white light (Condor daylight, 1000 ft-c at the level of the plants).

The harvested leaf material was homogenized in a Sorvall Omni-Mixer with buffer (5 ml of 0.05 M KH_2PO_4, 0.3 M sucrose, 0.01 M KCl, pH 7.2, per g of tissue, fresh wt) for 10 sec at 90 V followed by 15 sec at 140 V (line voltage 240 V). The homogenate was squeezed through one layer of Miracloth (Calbiochem, San Diego, Calif.) and filtered through another layer, and the filtrate was centrifuged at 2000 *g* for 5 min in a Sorvall SS-3 machine equipped with an SS-34 rotor. The resulting pellet was washed once with the original volume of buffer and then dispersed in incubation buffer (0.05 M KH_2PO_4, 0.3 M sucrose, 0.01 M KCl, 1 mM dithiothreitol, 10 mM $NaHCO_3$, pH 7.2) in the proportion of 2 ml of buffer/5 g of tissue (fresh wt). In some of the later experiments, the plastids were resuspended in a buffer containing 0.05 M Tricine–KOH, pH 8.3, 0.2 M KCl, and 7 mM $MgCl_2$, and they were washed once in the same medium. This KCl-containing medium was used by Ramírez, del Campo, and Arnon (14) in their studies on the light stimulation of protein synthesis by isolated chloroplasts. Incorporation of [1-^{14}C]acetate into lipid was considerably higher (up to 10-fold) if the plastids were resuspended and assayed in Tricine–KCl medium, but the distribution of label among the fatty acids was the same as in comparable experiments in sucrose–phosphate medium. The time of illumination of the plants before homogenization is indicated in the text. For example, plastids isolated from plants greened for 4 hr are termed "4-hr plastids." All operations after harvesting were carried out at 0–5°C. Unless otherwise stated, incubation mixtures contained ATP (400 nmoles, P-L Biochemicals, Inc., Milwaukee, Wis.), CoA (200 nmoles, Calbiochem), $MgCl_2$ (400 nmoles), sodium [1-^{14}C]acetate (13 nmoles, 1.74×10^6 dpm, Radiochemical Centre, Amersham, England), and 0.5 ml of chloroplast fraction in a total of 0.8 ml of incubation buffer, pH 7.2. The mixtures were placed in 16 × 100 mm tubes and incubated at 30°C with shaking (100 strokes/min) over white light (40-W incandescent lamps, 1200 ft-c at base of tubes). For the dark incubations, the tubes were wrapped in three layers of aluminum foil. DCMU was donated by Dr. C. W. Todd of du Pont & Co. Unless otherwise stated, the reactions were stopped after 5 min with 3.0 ml of chloroform–methanol 1:2, and the total lipid fractions were extracted from the incubation mixtures by the method of Bligh and Dyer (15). The extracts were taken to dryness, bleached under a lamp (Philips IR, 250 W), then dissolved in 0.5% PPO and 0.03% POPOP in toluene (5 ml) and placed in a Tri-Carb liquid scintillation counter. The efficiency of counting was 83% and constant, using an internal standard and channel ratios.

Polar lipids were separated from total lipids by two-dimensional thin-layer chromatography on 20 × 20 cm glass plates coated with a 0.25-mm layer of silica gel G (E. Merck A.G., Darmstadt, West Germany). The solvent was chloroform–methanol–7 M NH_4OH 65:30:4 for the first direction and chloroform–methanol–acetic acid–water 170:25:25:6 for the second direction (16). Glycolipids were detected with orcinol–sulfuric acid spray (17), amino lipids with ninhydrin (18), phospholipids with molybdate reagent (19), and choline lipids with Dragendorff reagent (20). Iodine was used as a nonspecific removable detector. Palmitic acid, dipalmitin, and triolein, all supplied by Calbiochem, were used as standards for FFA, diglycerides, and triglycerides, respectively, in the assay of neutral lipids by one-dimensional chromatography with hexane–diethyl ether–acetic acid 70:30:1 (21). For counting polar lipids and the FFA fraction, the spots located with iodine were scraped off the plates into 4% Cab-O-Sil in scintillation fluid. The efficiency of counting was constant from channel ratios.

In the analysis of labeled fatty acids, extracts from the incubation mixtures were taken to dryness under nitrogen, 4 ml of methanol–benzene–sulfuric acid 150:75:10 was added, and the mixtures were heated for 1 hr at 100°C in capped tubes previously flushed with nitrogen. The methyl esters were extracted with 3 × 3 ml of light petroleum ether (bp 80–100°C), and the extracts were concentrated

TABLE 1. Dependence of [1-^{14}C]acetate incorporation into lipid by developing chloroplasts on added ATP, CoA, $MgCl_2$, and $NaHCO_3$

System	Relative dpm
	%
Complete	100
−ATP	35
−CoA	55
−$MgCl_2$	78
−$NaHCO_3$	11

The developing chloroplasts were incubated with [1-^{14}C]acetate, as described in Materials and Methods, after isolation from 8-day dark-grown plants exposed to light for 11 hr.

to about 50 μl under nitrogen and placed on 5 × 20 cm plates for argentation thin-layer chromatography (22) using light petroleum ether (bp 80–100°C)–diethyl ether 80:20 as solvent. A mixture of methyl esters of palmitic, stearic, oleic, linoleic, and linolenic acids (K108 standard, Applied Science Laboratories, Inc., State College, Pa.) was run alongside the labeled samples, and the spots were detected under ultraviolet light after spraying the plates with 0.2% sodium dichlorofluorescein. The labeled compounds were detected on the plates with a Packard strip scanner. To further identify the methyl esters, radioactive bands were eluted from the $AgNO_3$ thin-layer chromatograms with diethyl ether. The ether solutions were concentrated and the esters analyzed by gas–liquid chromatography (12% ethylene glycol succinate–Chromosorb W) on a Varian Aerograph model 200 instrument. After detection by thermal conductivity, the methyl esters were counted on a Nuclear-Chicago detector and ratemeter. The retention times of the resulting peaks were compared with those obtained with the K108 mixture of esters.

Total chlorophyll (a + b) was estimated with the equation of Bruinsma (23) and protein by the method of Lowry et al. (24) with bovine serum albumin as a reference standard.

RESULTS

Incorporation of [1-^{14}C]acetate into the total lipid fraction

The rate of acetate incorporation by developing pea chloroplasts was dependent on ATP, CoA, Mg^{2+}, and bicarbonate ions (Table 1). The incorporation of [1-^{14}C]acetate was proportional to protein concentration up to about 4 mg of protein/incubation, but above this concentration there was an inhibition of lipid synthesis. In most experiments the incubation mixture contained plastids derived from 1.25 g of leaves (fresh wt), which was within the linear range with respect to protein concentration. Incorporation into lipid increased with incubation time up to about 20 min (Table 2), but most incubations gave satisfactory incorporation in 5 min. The plastid fraction lost about one-half of its activity at ice-bucket temperatures from the first to the fourth hour after homogenizing the leaves.

TABLE 2. Effect of time of incubation on [1-^{14}C]acetate incorporation into lipid by developing chloroplasts

Time of Incubation	^{14}C in Lipid
min	*dpm × 10^{-3}/mg protein*
0	0.02
2.5	1.5
5	3.6
10	4.8
15	6.3
20	7.1

The chloroplasts were incubated with [1-^{14}C]acetate, as described in Materials and Methods, after isolation from 8-day dark-grown plants exposed to light for 10 hr. The average values of duplicate tubes are shown.

TABLE 3. Comparison of effects of DCMU[a] and dark incubation on [1-^{14}C]acetate incorporation into lipid and into unsaturated fatty acids by developing pea chloroplasts

Leaf Illumination Time	DCMU	Incubation of Plastids	^{14}C in Lipid	Unsaturated Acid/Saturated Acid
			dpm × 10^{-3}/g fresh wt/min	
hr				*cpm*
0	+	Light	2.50	0.16
0	−	Light	2.37	0.11
0	+	Dark	2.53	0.18
0	−	Dark	2.29	0.06
2	+	Light	1.73	0.18
2	−	Light	5.40	1.90
2	+	Dark	1.48	0.08
2	−	Dark	1.51	0.09
4	+	Light	2.62	0.12
4	−	Light	20.7	2.60
4	+	Dark	2.47	0
4	−	Dark	2.16	0

Incubations of etioplasts and developing chloroplasts from 8-day dark-grown plants with [1-^{14}C]acetate were as described in Materials and Methods except that, where indicated, the reaction mixture contained DCMU (1.25×10^{-5} M) or the incubation was in the dark.

[a] 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

The effects of DCMU, dark incubation, and time of greening on (a) incorporation into the total lipid fraction and on (b) percentage incorporation into unsaturated acids (monoenoic plus dienoic plus trienoic acids) are shown in Table 3. With regard to the distribution of label among the various fatty acids, gas–liquid chromatography showed that of the saturated labeled acids, palmitic acid accounted for at least 86% of the counts in etioplasts and developing chloroplasts; the remaining radioactivity in the saturated acids was in lauric and myristic acids. In etioplasts the only unsaturated labeled acid was oleic acid. Oleic acid was also the major unsaturated acid labeled by developing chloroplasts, a lesser component being a hexadecenoic acid (rather variable during greening; it was possibly *trans*-Δ^3-hexadecenoic acid as judged by its behavior on $AgNO_3$ thin-layer chromatograms). Gas–liquid chromatography indicated that linolenic acid was labeled by 6-hr plastids (Fig. 1), but labeling of this acid did not account for more than about 3% of total fatty acid radioactivity.

By comparison with incorporation into the total lipid fraction by illuminated 4-hr plastids (Table 3), it can be seen that the addition of DCMU, dark incubation, or no prior illumination of the seedlings (0-hr plastids) each re-

TABLE 4. Incorporation of [1-^{14}C]acetate into the total lipid fraction, free fatty acids, diglycerides, and triglycerides on greening

Leaf Illumination Time	^{14}C in Lipid	[1-^{14}C]Acetate Incorporation		
		FFA	Diglyceride	Triglyceride
hr	*dpm × 10^{-2}/g fresh wt/min*	*cpm × 10^{-2}/g fresh wt/min*		
0	9.4	2.9	0.3	0.3
1	10.7	3.2	0.3	0.2
2	40.5	14.8	0.7	0.5
3	52.7	19.4	0.9	0.6
4	73.7	30.8	1.3	0.6

The etioplasts and developing chloroplasts were incubated with [1-^{14}C]acetate, as described in Materials and Methods, after isolation from 8-day dark-grown plants exposed to light for the indicated times. The average values of duplicate tubes are shown.

TABLE 5. Incorporation of [1-^{14}C]acetate into lipid by various subcellular fractions

Leaf Illumination Time	Fraction	Protein per Fraction	^{14}C in Lipid
hr		*mg/ml*	*dpm × 10^{-3}/fraction/min*
0	500 *g*, 2-min pellet (unwashed)	0.8	2.80
0	2,000 *g*, 5-min pellet (one wash)	2.9	5.46
0	15,000 *g*, 30-min pellet (one wash)	5.4	1.39
0	15,000 *g*, 30-min supernate	3.1	10.5
6	500 *g*, 2-min pellet (unwashed)	3.6	35.3
6	2,000 *g*, 5-min pellet (1st wash)	4.9	31.3
6	2,000 *g*, 5-min pellet (2nd wash)		31.6
6	2,000 *g*, 5-min pellet (3rd wash)		26.2
6	15,000 *g*, 30-min pellet (one wash)	7.3	1.05
6	15,000 *g*, 30-min supernate	4.1	11.2

Fractions were washed with homogenization buffer where indicated after isolation from 8-day dark-grown plants. Incubations of the fractions with [1-^{14}C]acetate were as described in Materials and Methods.

sulted in a large and similar inhibition of labeling of the lipids. The incubation, moreover, was not additive, since combinations of the above conditions resulted in approximately the same low rate of incorporation. Table 4 indicates that the seedlings required between 1 and 2 hr of light before incorporation was stimulated.

Whereas the major proportion of the fatty acids labeled by etioplasts were saturated (Table 3), two-thirds of the label with 2-hr plastids was found in unsaturated acids. The proportion increased to about three-quarters with 4-hr plastids and remained at this level for 6- and 8-hr plastids. This stimulation of incorporation into unsaturated acids was completely abolished by DCMU.

Several attempts were made to increase lipid synthesis in etioplasts by suspending them in a sucrose-free medium and subjecting them to various treatments. The results were as follows: (*a*) the addition of NADPH (to a final concentration of 125 μM) had no effect on incorporation, either by etioplasts or chloroplasts, and the activities were similar to those obtained with plastids in sucrose; (*b*) digitonin (final concentration of 100 μM) stimulated incorporation by etioplasts, but the effect was greater with chloroplasts; and (*c*) sonication or freezing and thawing was strongly inhibitory for both etioplasts and chloroplasts.

The capabilities of various subcellular fractions from etiolated and from 6-hr-greened pea leaves to incorporate acetate into lipid are shown in Table 5. Phase-contrast microscopy indicates that the fractions from centrifugation of green leaf homogenates contain the following major constituents: 500 *g*, 2-min pellet, mainly intact chloroplasts with a few nuclei and cell wall fragments; 2000 *g*, 5-min pellet, intact and broken chloroplasts; 15,000 *g*, mitochondria and chloroplast fragments. The 15,000 *g*, 30-min supernate contained soluble proteins and membrane fragments. With the greening plants, most of the incorporation into lipid occurred in the chloroplast-enriched fractions (500 and 2000 *g* pellets), whereas with the etiolated plants, the 15,000 *g* supernate showed the highest incorporation. The stimulation of incorporation on greening was a feature of the chloroplast-enriched fractions, the 15,000 *g* pellet and supernate activities showing no significant change for etiolated or greened plants. Repeated washing of the 2000 *g* fraction from greened plants had no great effect on incorporation into lipid, supporting the conclusion that the activity was not due to the presence of nonchloroplast contaminants.

^{14}C incorporated by developing chloroplasts was distributed among a number of lipid fractions, including FFA, diglycerides, triglycerides, and the six polar lipids, PC, PA, MGD, DGD, PG, and PE. Each of these fractions was more highly labeled in developing chloroplasts than in etioplasts (Tables 4 and 6). The highest proportion of label among the polar lipids was in PC, 28% in

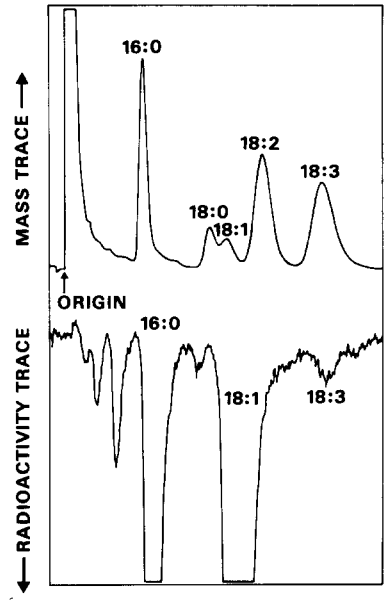

Fig. 1. Gas–liquid chromatography of radioactive products extracted by light petroleum ether after transmethylation of total lipid. The developing chloroplasts were incubated with [1-^{14}C]acetate, as described in Materials and Methods, after isolation from 8-day dark-grown plants exposed to light for 6 hr. Mass peaks were designated after comparison of the retention times with those of commercial standards. Because of the time of gas transit from the thermal conductivity detector to the counter, there is a slight lag in the radioactivity trace.

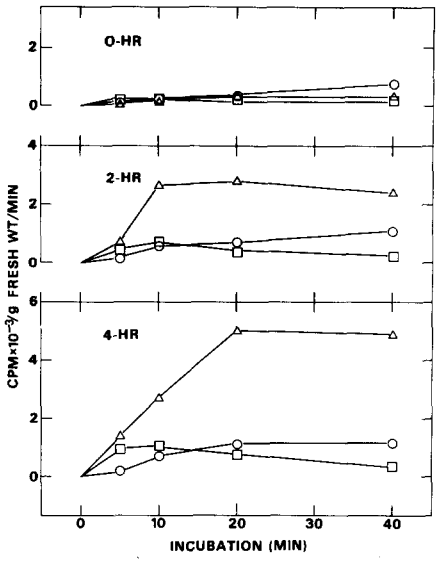

Fig. 2. Labeling of PC, PA, and MGD by etioplasts and developing chloroplasts as a function of time of greening of 8-day dark-grown plants and time of incubation. In this experiment, the plastid fraction was obtained after discarding the 500 *g*, 2-min pellet, centrifuging the supernate at 2000 *g* for 5 min, and collecting the pellet. The incubation mixture contained ATP (400 nmoles), CoA (200 nmoles), $MgCl_2$ (400 nmoles), NADPH (100 nmoles), D-galactose (800 nmoles), sucrose (240 μmoles), KCl (8 nmoles), dithiothreitol (800 nmoles), $NaHCO_3$ (8 μmoles), KH_2PO_4 (40 μmoles, pH 7.2), sodium [1-^{14}C]acetate (13 nmoles, 1.74 × 10^6 dpm), and the plastid fraction from 1.25 g of leaves in a total volume of 0.8 ml. Incubation was at 30°C. Symbols: ▲, PC; □, PA; ○, MGD.

etioplasts and 73% in 24-hr plastids. Repeated washing of the 2000 *g*, 5-min pellet of 6-hr plastids increased rather than decreased PC labeling, suggesting that the incorporation into PC was not due to contamination of the plastids by other subcellular fractions. The dominance of PC labeling was greatly emphasized when developing chloroplasts were incubated for periods greater than 5 min (Fig. 2). Labeling of PA in 2- and 4-hr plastids showed a different time course from that of PC and MGD. Maximum incorporation of [1-^{14}C]acetate into PA was reached at about 10 min of incubation, after which there was a significant decline. Labeling of PC and MGD increased for 10–20 min, but no decline was observed at the longer incubation times. PG, PE, and DGD were relatively poorly labeled irrespective of incubation time. In the experiment of Fig. 2, the 500 *g*, 2-min pellet was discarded before centrifugation at 2000 *g* for 5 min. PC, however, was still the major labeled polar lipid if the complete 2000 *g* 5-min pellet was used. In a similar experiment to that of Fig. 2 except that the incubation mixtures did not contain D-galactose, the labeling of MGD and DGD was not noticeably affected.

TABLE 6. Distribution of label among free fatty acids and various polar lipids synthesized by developing chloroplasts

Leaf Illumination Time	^{14}C in Lipid						
	FFA	PC	PA	PE	PG	MGD	DGD
hr	*cpm × 10^{-2}/g fresh wt/min*						
0	4.8	0.7	0.9	0.2	0.2	0.3	0.2
2	11.6	2.9	1.7	0.4	0.3	2.2	0.4
4	41.5	4.7	4.3	0.3	0.4	2.1	0.5
6	71.8	9.9	6.1	0.5	0.4	5.8	0.7
8	80.7	15.8	11.4	0.7	0.8	4.8	1.0
24	159	33.6	5.1	1.2	3.7	1.5	1.1

Incubations of etioplasts and developing chloroplasts from 8-day dark-grown plants with [1-^{14}C]acetate were as described in Materials and Methods. Abbreviations: FFA, free fatty acids; PC, phosphatidylcholine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; MGD, monogalactosyldiglyceride; DGD, digalactosyldiglyceride.

TABLE 7. Effect of ATP on acetate incorporation into lipid by developing and mature pea chloroplasts

Plant Material	ATP	^{14}C in Lipid	Unsaturated Acid/ Saturated Acid	^{14}C in Lipid in Presence of DCMU[a]
	mM	*dpm × 10^{-2}/g fresh wt/min*	*cpm/cpm*	*dpm × 10^{-2}/g fresh wt/min*
8-day dark-grown, then 8 hr light	0	45.8	1.70	1.1
	0.5	85.7	0.85	3.8
	1.0	87.0	1.20	8.5
	1.5	68.1	1.30	9.7
	2.0	65.4	1.20	12.6
Grown for 2 wk in light	0	24.3	1.30	
	0.5	26.1	0.61	
	1.0	17.8	0.53	
	1.5	12.9	0.40	
	2.0	9.9	0.30	

The developing and mature chloroplasts were incubated with [1-^{14}C]-acetate, as described in Materials and Methods, after isolation from 8- and 14-day plants as indicated.

[a] These incubations were carried out in the presence of 1.25 × 10^{-5} M DCMU.

Incorporation of [1-^{14}C]acetate into lipid of mature pea chloroplasts was markedly inhibited by ATP, which also caused a decrease in the relative labeling of unsaturated fatty acids (Table 7). This confirms previous work with spinach chloroplasts (12, 25). However, incorporation into lipid of developing pea chloroplasts was stimulated slightly by ATP, and there was only a small effect on the relative incorporation into unsaturated fatty acids (Table 7). DCMU almost completely inhibited incorpora-

TABLE 8. Effects of TMPD and DAD on inhibition of lipid synthesis by DCMU

Addtions	DCMU	^{14}C in Lipid	18:1/16:0
		dpm × 10^{-3}/g fresh wt/min	*cpm/cpm*
None	−	4.27	3.65
DAD plus ascorbate	−	1.35	1.27
TMPD plus ascorbate	−	0.19	
None	+	0.11	
DAD plus ascorbate	+	1.90	1.67
TMPD plus ascorbate	+	0.06	

Developing chloroplasts were isolated from 8-day plants and resuspended in 0.05 M Tricine–KOH, pH 8.3, 0.2 M KCl, and 7 mM $MgCl_2$, and then they were washed in the same medium. [1-^{14}C]Acetate, ATP, and CoA were in each incubation, as well as DCMU (12.5 μM), DAD (1.5 mM), ascorbate (3 mM), and TMPD (1.5 mM) where indicated. Incubation was at 30°C for 10 min in the light. The average values of duplicates are shown. Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DAD, 2,3,5,6-tetramethylphenylenediamine; TMPD, *N,N,N′,N′*-tetramethylphenylenediamine.

tion into total lipid by developing chloroplasts in the absence of ATP. Addition of increasing concentrations of ATP restored some of the incorporation, so that in the presence of 2.0 mM ATP, DCMU caused only an 80% inhibition of acetate incorporation. This result suggested that inhibition of acetate incorporation by DCMU may be related to inhibition of noncyclic phosphorylation.

Table 8 shows the effect on acetate incorporation into lipid of electron donors that feed electrons into photosystem I. Ascorbate *plus* DAD and ascorbate *plus* TMPD were chosen as electron donors because electron flow from reduced DAD to photosystem I is coupled to photophosphorylation, whereas no phosphorylation is associated with electron flow from reduced TMPD (26). In the presence of DCMU, which almost completely inhibited acetate incorporation in the control plastids, ascorbate *plus* DAD restored substantial incorporation while ascorbate *plus* TMPD was completely ineffective. Incorporation into unsaturated fatty acid was also restored by DAD *plus* ascorbate. In the absence of DCMU, ascorbate *plus* TMPD substantially inhibited acetate incorporation.

In the presence of methyl viologen, which accepts electrons directly from photosystem I, acetate incorporation into oleic acid by developing pea chloroplasts was inhibited and stearic acid accumulated (data not shown). This confirms the observation of Givan and Stumpf (27) with spinach chloroplasts and suggests that desaturation occurs after elongation of palmitic to stearic acid.

DISCUSSION

Some interesting comparisons can be made between lipid synthesis by developing pea chloroplasts, described in this communication, and synthesis by spinach chloroplasts from immature and mature leaves, results of which have been previously reported (12, 25, 27). Whereas DCMU had little effect upon incorporation into unsaturated fatty acids and reduced total incorporation into lipid by less than half in the case of spinach, with developing pea chloroplasts both total incorporation into lipid and the proportion of label in unsaturated acid were lowered by DCMU to the level of etioplasts or dark-incubated plastids. We do not have an explanation for this difference between developing pea chloroplasts and spinach chloroplasts. Perhaps there is sufficient noncyclic electron flow in DCMU-inhibited spinach chloroplasts (electron flow is inhibited about 98% by 10 μM DCMU) to support the observed rates of acetate incorporation.

With respect to ATP, it now appears that the results obtained depend on the maturity of the chloroplast. Using mature spinach chloroplasts, Stumpf and Boardman (12) showed that ATP depresses total incorporation into fatty acids and specifically incorporation into oleic acid, the major labeled unsaturated fatty acid. Apparently, ATP inhibits the elongation step (palmitic→stearic) rather than desaturation itself (27). In contrast with these findings, ATP had little effect on the relative proportions of palmitic and oleic acids synthesized by plastids from young spinach leaves (25). In fact, ATP increased [1-^{14}C]acetate incorporation into the polyunsaturated fatty acids, linoleic and linolenic acids. A similar difference in ATP sensitivity was observed in the present work when developing pea chloroplasts were compared with mature pea chloroplasts. We have no plausible explanation for this result at present, except to suggest that it may be related to the capacity of the isolated plastids to synthesize ATP.

The current studies support the view that the light effect on lipid synthesis is related to photosynthetic electron transport. No light stimulation of lipid synthesis was observed with etioplasts or plastids isolated from seedlings greened for less than 2 hr. Plastids from 10-day-old dark-grown pea seedlings are functional in the Hill reaction if the plants are illuminated for at least 5 hr prior to isolation of the plastids (8). Plants grown for 8 days before illumination, as used in the present work, green more rapidly than 10-day-old plants, and Hill activity of plastids is first observed at about 3 hr of greening. Measurements of light-induced redox changes of cytochromes in developing pea plastids, however, suggest that photosystem I is functional after a greening period of about 30 min, and photosystem II after 1–2 hr (28), the latter corresponding to the end of the lag phase in chlorophyll synthesis. Light-induced absorbance measurements with intact leaves of greening bean seedlings also indicate that both photosystems are functional soon after the lag phase in chlorophyll formation (29). The 1–2-hr lag in the light stimulation of acetate incorporation into lipid and preferentially into unsaturated fatty acids by pea plastids (Tables 3 and 4) suggests that the light effect in lipid synthesis by chloroplasts is dependent on an active photosystem II. This conclusion is supported by the observation that acetate incorporation by developing plastids in the presence of DCMU, an inhibitor of electron flow from photosystem II, was no higher than incorporation by etioplasts.

It would seem logical to infer that the role of light in lipid synthesis by the developing pea chloroplasts is to provide the ATP and reducing equivalents required for fatty acid synthesis from acetate. There is a difficulty in concluding that the light effect is related to the production of NADPH, because pea plastids from 10-day-old seedlings do not show a measurable rate of photoreduction of NADP until about 6 hr of greening. The rates of lipid synthesis (about 200 pmoles of acetate incorporated/mg of chlorophyll/min) by developing chloroplasts, however, are low, and the levels of NADPH required for the synthesis of these amounts of lipid would be below the limits of detection by the spectrophotometric method.

However, our experiments with DCMU and electron donors to photosystem I strongly suggest that noncyclic

phosphorylation is necessary for acetate incorporation into lipid. Reduced TMPD provides electrons to photosystem I for the photoreduction of $NADP^+$, but ATP formation is not coupled to this noncyclic electron flow through photosystem I (26), and acetate incorporation into lipid is very low. On the other hand, ascorbate *plus* DAD, which also provides electrons for $NADP^+$ photoreduction by photosystem I, supports phosphorylation and acetate incorporation into lipid.

Etiolated pea leaves contain relatively high contents of galactolipids and polyunsaturated fatty acids (6), which are thought to be confined mainly to the plastids. Furthermore, the amounts and relative proportions of the various glycerolipids of pea leaves do not change significantly during the first 8 hr of greening (6). Because light appears to control the degree of incorporation into unsaturated fatty acids by the developing chloroplast, and because there is no light effect on total incorporation into lipid by our pea etioplasts, the synthesis of lipids and polyunsaturated fatty acids by etiolated leaves presents an interesting problem. For *Zea mays*, it is known that the rate of incorporation of [^{14}C]glycerophosphate into lipids by etioplasts is higher than that by chloroplasts (30). The incorporation is not dependent on light during the incubation. It is possible that the complex lipids of the etioplast are assembled from, for example, triglycerides either present in the etioplasts or transported there. This lipid could originate from seed reserves or be synthesized by the microsomes of the etiolated plant. Another possibility is that the fatty acid synthetase complex of etioplasts is more sensitive to damage during isolation than that of more developed chloroplasts. This does not seem to be a likely explanation for the poor activity of etioplasts in view of the fact that etiolated barley leaves poorly incorporate [1-^{14}C]acetate into fatty acids as compared with green leaves (2).

Although PC was rapidly labeled by acetate in greening clover leaves (4), developing pea chloroplasts seem to be the first preparation of plastids, as distinct from leaf preparations, in which PC was labeled to a greater extent than the major chloroplast lipids MGD, DGD, and PG. Of the polar lipids, PC also showed the greatest increase in rate of labeling during greening. Because PC has been implicated as an intermediate in polyunsaturated fatty acid synthesis in pumpkin leaves (31) as well as in algae (32), it is tempting to suggest that the increase in PC labeling by developing pea chloroplasts compared with other polar lipids may be related to desaturation in this system. It appears, however, that PC plays no role in the conversion of oleyl CoA to linoleyl CoA by microsomes from *Carthamus tinctorius* seeds (33). Because there is little or no light stimulation of microsomal lipid synthesis in greening peas, the possible role of PC in desaturation is not necessarily ruled out. In view of the rather large pool of labeled FFA, however, the possibility remains that the incorporation of fatty acids into PC was a "scavenging" reaction not necessarily of physiological importance.

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