Lipid biosynthesis in relation to chloroplast development in barley

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ABSTRACT During greening of detached leaves from dark-grown barley seedlings, the linolenic acid content of the lipids increases in the early stages of the formation of the chloroplast lamellar system. Primarily the fraction containing monogalactosyl diglyceride is enriched with linolenic acid.

Incorporation of ^{14}C-labeled acetate into the leaf lipids of detached whole leaves is low, but increases 10- to 20-fold during greening. Increasing percentages of label appear in linolenic acid during the first 15 hr of greening, whereafter they remain constant.

A constant, relatively high amount of acetate is incorporated into lipids when slices of leaves at various stages of greening are incubated by submersion in acetate solution, a treatment that blocks further chlorophyll synthesis during incubation. At the initial greening stages 75% of the label is channeled into steroids and other unsaponifiable lipids, but in advanced stages of chloroplast development 75% of the incorporated acetate is built into phospho-, sulfo- and galacto-lipids, and only 25% is channeled into unsaponifiable lipids.

Experimental variation of the physiological conditions of the tissue during incubation resulted in differences in the amount of label found in the various phospho- and galacto-lipids. The amounts of labeling of the individual fatty acids in the lipid classes studied differ markedly and could be changed by varying the conditions of incubation. Labeling of linolenic acid was found to be highest in the monogalactosyl diglyceride fraction at all stages of greening.

KEY WORDS barley · chloroplast development · chlorophyll synthesis · acetate incorporation · lipid biosynthesis · monogalactosyl diglyceride · digalactosyl diglyceride · steroids · radio–gas chromatography · linolenic acid

Greening of dark-grown leaves in higher plants is accompanied by an increase in the linolenic acid contents of the leaf lipids (1–4). A high linolenic acid content is characteristic of the monogalactosyl diglycerides and digalactosyl diglycerides, major constituents of the chloroplast lipids (5–9). Nichols, Stubbs, and James (2) have indeed demonstrated that the increase in linolenic acid content observed upon illumination of dark-grown *Ricinus* seedlings occurs primarily in these galactolipids.

This poses the question of whether a net synthesis of the lamellar galacto-lipids accompanies the light-induced formation of the membranes and grana structure, which occurs during the differentiation of the proplastids into chloroplasts. Since a synchronous development of proplastids can be induced by illumination in dark-grown leaves, and chlorophyll accumulation can be used as an indicator for the amount of grana formed in such a system (10), we have aimed to follow the course of synthesis of galactolipids and other lipids in dark-grown barley leaves exposed for various times to white light. A quantitative analysis of the capacities of greening barley leaves to synthesize the various lipid classes is also desirable as the basis for a study of gene mutants with impairments in the formation of the chloroplast membranes (11, 12). It is conceivable that the absence of chloroplast membranes or the formation of abnormal chloroplast structure in some of the mutants is associated with a defect in the capacity to synthesize chloroplast lipids.

EXPERIMENTAL PROCEDURE

Seeds of the barley cultivar Svalöf's Bonus were germinated at 20°C in the dark in trays containing moist

Fatty acids are designated by number of carbon atoms:number of double bonds.

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vermiculite. For greening, the upper 13–15-cm long parts of primary leaves were detached from 7–9-day old dark-grown seedlings, divided into groups of 50, and placed in small glass vials containing 1 or a few ml of distilled water or experimental solution. Thus the solutions covered about 1 cm portions of the leaf bases. The vials with the leaves were placed in a humid chamber which was equipped with an air circulation system and a CO_2 trap, and illuminated for 3–33 hr from above with cool white fluorescent lights giving approximately 500 foot candles. 8-day old light-grown seedlings raised under the same light and temperature conditions were used for some controls.

For the study of lipid biosynthesis from labeled acetate five different techniques were used.

(*1*) At various stages of greening, the 50 leaves (about 3 g) were placed for 3 hr in vials containing 1 ml of aqueous, bicarbonate-buffered solution of 30 μC sodium acetate-1-^{14}C. The leaves were used for lipid analysis either directly after incubation in light, or after incubation with unlabeled acetate in the light for an additional 3 hr. In harvesting, 1 cm of the leaf tips and 3 cm of the bases were discarded and thus excluded from the lipid analysis.

(*2*) At various stages of greening, 2.5 g of leaf tissue was sliced cross-wise into 1–2-mm long pieces, suspended in 5.2 ml of 0.2 M bicarbonate buffer containing 4 μC sodium acetate-1-^{14}C (72 mμmoles), infiltrated in vacuo, saturated with O_2, and incubated for 3 hr in a Warburg incubator at 30°C with a light intensity of about 1000 foot candles.

(*3*) After 5 hr greening, 3 g of 8-cm long leaf pieces were floated in the light for 3 hr on 10 ml of a bicarbonate-buffered solution containing 40 μC sodium acetate-1-^{14}C.

(*4*) After 5 hr greening, 3 g of 0.5-cm long leaf pieces were floated for 3 hr in the light on the solution described under (*3*).

(*5*) After 5 hr greening, 3 g of 15-cm long leaf pieces were dipped into the solution described under (*3*) for 30 sec, four times per hr, during a period of 3 hr in the light. 1 cm of the leaf tips and 3 cm of the bases were discarded prior to lipid analysis.

Radioisotopes used included: sodium acetate-1-^{14}C (New England Nuclear Corp.), and an equimolar mixture of glucose-U-^{14}C, fructose-U-^{14}C (New England Nuclear Corp.), and potassium bicarbonate-^{14}C prepared from $Ba^{14}CO_3$ (Oak Ridge National Laboratories). Leaf lipids were extracted according to Bligh and Dyer (13) in a Sorvall Omni-mixer with chloroform–methanol–water 2:2:0.8. Thereafter 1 volume of water was added. To prevent the formation of emulsions that separate slowly into the chloroform and aqueous methanol phases, we sometimes substituted dilute salt solutions for water. After separation, the chloroform phase was removed and dried with anhydrous sodium sulfate.

Radioactivity of the chloroform phase containing the total lipids was determined by counting aliquots in a liquid scintillation counter (Packard Tri-Carb model 314 Ex or Nuclear-Chicago Unilux model) after evaporating them to dryness and adding a scintillator composed of 0.6% 2,5-diphenyloxazole (PPO) and 0.05% 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) in toluene. Quenching corrections were made by means of an internal standard.

For separation of lipid classes, ascending thin-layer chromatography was carried out on glass plates coated with Merck's Silica Gel G by the technique of Lees and DeMuria (14). Three solvent systems were utilized: petroleum ether–diethyl ether–acetic acid 70:30:1, toluene–ethyl acetate–ethanol 2:1:1, and chloroform–methanol–acetic acid 65:25:8. For analytical purposes, 5 × 20 cm plates were used and the distribution of radioactivity was recorded with a Nuclear-Chicago chromatogram scanner. The lipid bands were made visible by applying one or a combination of the following reagents: Iodine vapor, 50% sulfuric acid followed by charring at 150°C, 10% phosphomolybdic acid in diethyl ether–ethanol 1:1 followed by heating to 110°C for 5 min, or 0.2% 2′,7′-dichlorofluorescein in 50% ethanol for inspection under UV light.

The separated lipid components were identified by specific spray reagents, and by cochromatography with known marker substances.

The molybdate spray of Dittmer and Lester (15) was used for phosphorus-containing lipids, a periodate–benzidine spray (16) slightly modified for lipid-bound galactose, Dragendorff's reagent (16) for choline-containing lipids, and ninhydrin (17) for phosphatidyl ethanolamine and(or) phosphatidyl serine. The relative locations found for various phospholipids and galactolipids with the solvent systems used agreed with those reported by Nichols (18). A diagram of a typical thin-layer chromatogram obtained with the toluene–ethyl acetate–ethanol solvent system is shown in Fig. 1.

Phosphatidyl inositol and phosphatidyl choline are associated with phospholipid fraction named *A*. Phosphatidyl glycerol and sulfoquinovosyl diglyceride are contained in fraction *B*. Digalactosyl diglycerides and monogalactosyl diglycerides move into fractions *C* and *D*, respectively. Free fatty acids and steroids are found in band *E*, and triglycerides and hydrocarbons at the solvent front, *F*. The minor components in fractions *C* and *D* had fatty acid compositions and labeling patterns very similar to those of the digalactosyl and monogalactosyl diglycerides, respectively.

This toluene solvent system was used for preparative separation of the total lipid extract, which was applied

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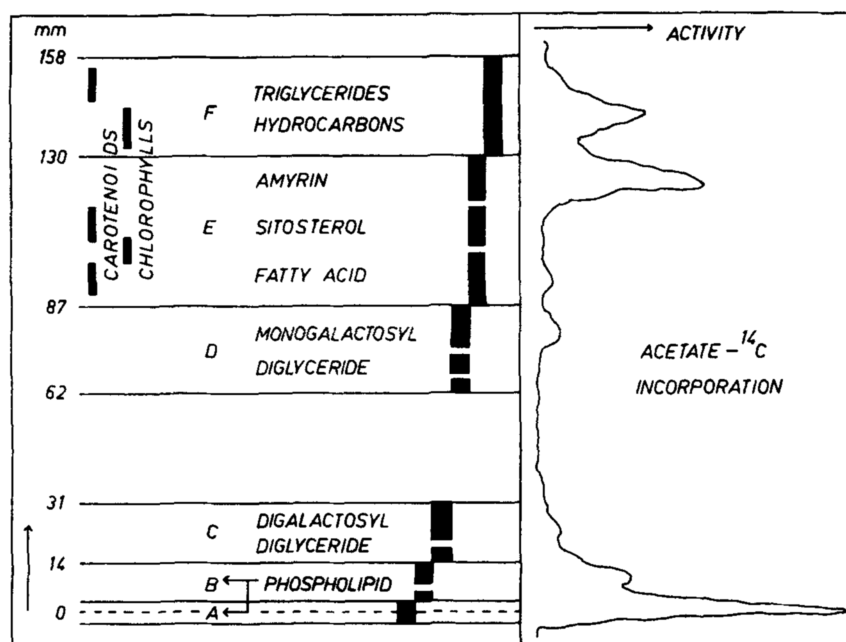

FIG. 1. Diagram of a typical thin-layer chromatogram showing the separation of the total lipid extract from barley leaves into six fractions (*A–F*) in toluene–ethyl acetate–ethanol 2:1:1. Black bars represent the range of the well-separated bands consistently obtained. Known compounds or characterized fractions are named. Distribution of the ^{14}C label in the lipid fractions of one sample is depicted at the right.

to a 20 × 20 cm plate as a series of partially overlapping spots. To protect the lipids from autoxidation during chromatography, we added butylated hydroxy anisole to the lipid extract and carried out the whole separation procedure in dim light. After development of the plate, we located bands *A* to *F* (Fig. 1) by spraying the plate with 0.25% 2′,7′-dichlorofluorescein in ethanol and then viewing it under a UV lamp with maximal emission at 254 mμ. Each band was scraped off the glass plate with a razor blade and collected in a funnel which contained a cotton plug in the lower end of the stem. The silica gel was thus packed into a column with a minimum of loss and delay. Bands containing phospholipids and galactolipids were eluted with chloroform–methanol 1:2, those containing nonpolar lipids, with diethyl ether. The solvent volume necessary for quantitative elution was determined by analyzing radioactivity in consecutive fractions of the eluates from a model plate.

The radioactivity residing in the various lipid bands was determined by assaying aliquots of the eluates on planchettes with a thin-window Geiger-Müller gas-flow counter (Nuclear-Chicago).

Fatty acid methyl esters were prepared from total lipid extracts or from chromatographic fractions with the aid of the BF_3–methanol reagent. Lipid extracts were evaporated under a stream of nitrogen in culture tubes with screw caps as described by Morrison and Smith (19). To avoid an increase in the concentration of BF_3 in the methanol, the rubber-lined screw caps had to be carefully tightened before the tubes were placed in a hot bath at 70–80°C. Since we used a lower temperature than Morrison and Smith (19), the reaction time necessary for the conversion of all fatty acids in the lipid extract to fatty acid methyl esters was reinvestigated. Thin-layer and gas chromatographic analysis of the reaction products indicated 20 min to be sufficient when not more than 20 mg of lipids react with 2 ml of 14% BF_3 in methanol. When methyl esters are prepared in this way, the unsaponifiable lipids (hydrocarbons, sterols, etc.) remain together with the methyl esters.

Gas chromatographic analysis was performed on an Aerograph A 90 P 2 model equipped with a 5 ft × 1/4 inch stainless steel column containing 12% diethylene glycol succinate (DEGS) on Anakrom ABS, 60–70 mesh. The column was operated at 160–170°C with a helium flow rate of about 60 ml/min. Radioactivity of the effluent was monitored with a Nuclear-Chicago Biospan proportional counter model 4998. Methyl palmitate-1-^{14}C (New England Nuclear Corp.) of known activity was used as the standard for radioactivity determinations. For calibration of the chromatograph and identification of the fatty acids peaks, mixtures of pure fatty acid methyl esters (Applied Science Laboratories Inc., State College, Pa.) were injected. Amounts of fatty acids were determined by calculating the peak areas as the product of peak height times width at half height.

Chlorophyll determinations were performed as follows. Approximately 0.1 g of leaf tissue was weighed, cut in

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small pieces in 90% acetone, and stored in the dark at −20°C. The samples were later ground in 10 ml of 90% acetone containing 3 drops of a 1% solution of $MgCO_3$, with a Teflon tissue grinder in an ice bath in dim light. The homogenate was centrifuged at 20,000 *g* for 20 min. Optical density of the solution was measured at 645, 663, and 750 mμ on a Beckman model DU spectrophotometer. Total chlorophyll was calculated by the equation of MacKinney (20) and Arnon (21).

RESULTS

Acetate Uptake and Lipid Synthesis in Detached Whole Leaves during Greening

Detached leaves from dark-grown barley seedlings will green and develop the lamellar system in their chloroplasts when illuminated in a moist chamber with their bases immersed in water. Greening leaves were fed with sodium acetate-^{14}C as lipid precursor, provided in a 3 hr pulse, at various times after the onset of greening. As shown in Table 1, chlorophyll synthesis occurred in the normal way with an initial lag period followed by an exponential phase. After 27 hr no additional greening was observed. About 50% of the acetate supplied was taken up by the leaves and this uptake remained constant throughout the greening period (Table 1). However, only 0.1–1.5% of the acetate-^{14}C taken up by the leaf was actually incorporated into lipids. The amount of labeling of lipids increases as the chloroplast structure develops: 10 to 20 times as much acetate is incorporated in later stages of greening as during the first 3 hr of illumination. Dark-grown barley leaves fed in the same way with acetate-^{14}C in the dark will incorporate very little acetate into lipids, only 10–20% of that observed with a comparable 0–3 hr light sample and less than 2% of that with a 0–12 hr light sample.

Since only a small amount of label was incorporated into the leaf lipids a quantitative analysis of the individual lipid classes was not feasible. By measuring the distribution of radioactivity on analytical thin-layer chromatograms of the lipid extracts from leaves illuminated for 27- and 33-hr, we observed that approximately one half of the total ^{14}C label incorporated resided in the phospho- and galactolipids, with an equal distribution between the two classes. However, in the leaves illuminated for 15-hr, only about one third of the total label appeared in these two lipid classes. In this greening experiment the whole leaves divert a relatively greater percentage of labeled acetate into the monogalactosyl diglyceride fraction than do leaf slices (cf. below).

The fatty acid composition of the total leaf lipid extract at successive stages of greening is presented in Table 2. During the first 15 hr of greening the linolenic acid content in the lipids increased from about 40 to 55% and remained thereafter at this higher level. This increase is balanced mainly by a decrease in the percentage of linoleic acid. If the assumption is made that the major site for linolenic acid is monogalactosyl diglyceride, these findings suggest that relatively large amounts of monogalactolipids have been synthesized by the detached greening leaves.

The distribution of the ^{14}C label among the fatty acids synthesized during successive stages of greening (Table 3) reveals an increasing amount of labeled linolenic acid in the lipids. Further incubation with unlabeled acetate for 3 hr in the light shifts the percentage of label markedly. An increase in linolenic acid and a corresponding decrease in oleic acid are observed. Although considerable acetate incorporation into linolenic acid has occurred, the incorporation into linoleic acid dominates.

In an attempt to obtain a higher and more representative incorporation of label into leaf lipids, other ^{14}C pre-

TABLE 1 Sodium Acetate-^{14}C Incorporation into Lipids by Detached Barley Leaves at Various Stages of Greening

Time of Illumination of Dark-Grown Leaves	Period of Acetate Feeding	Chlorophyll	Uptake of Acetate by the Leaves	Acetate Incorporation into Lipids	
	hr	*mg/g fresh weight*	*%*	*% of uptake*	*mμmoles/g fresh weight*
3	0–3	0.02	52	0.08	0.10
9	6–9	0.16	55	0.14	0.17
15	12–15	0.29	51	1.05	1.21
27	24–27	0.59	58	1.26	1.76
33	30–33	0.49	49	1.53	1.53

Incubation: 3 hr in light at 20°C.

TABLE 2 Fatty Acid Composition of Lipids in Detached Barley Leaves at Various Stages of Greening

Time of Illumination of Dark-Grown Leaves	Fatty Acids					
	16:0	16:u*	18:0	18:1	18:2	18:3
hr	%					
3	20	1	2	7	29	41
6	23	1	2	5	25	43
9	19	2	2	6	26	46
12	17	1	2	5	26	50
15	17	2	3	6	19	54
23	14	2	2	4	24	54
27	16	1	2	4	21	57
30	22	tr.	3	5	20	51
33	18	2	2	4	20	55
36	19	1	2	4	19	55

Small amounts of C_{14} fatty acids have not been considered in the calculations.

* 16:u, C_{16} unsaturated fatty acids.

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TABLE 3 Distribution of ^{14}C Label among Fatty Acids of Lipids Synthesized by Detached Barley Leaves at Various Stages of Greening

Time of Illumination: Prior to Incubation	Time of Illumination: Second Incubation Unlabeled Acetate	Total	Label in Fatty Acids: 16:0	18:1	18:2	18:3
	hr			%		
0	0	3	tr.	tr.	0	0
0	3	6	15	28	42	16
6	0	9	28	28	31	0
6	3	12	37	0	32	31
12	0	15	19	25	35	16
12	8	23	22	6	33	34
24	0	27	17	15	49	16
24	3	30	16	11	45	25
30	0	33	29	20	34	17
30	3	36	24	17	35	24

Incubation: 3 hr at 20°C in light. In some samples, small amounts of 18:0 and C_{16} unsaturated fatty acids were synthesized. They are included in the calculations but not listed in the table.

TABLE 4 Sodium Acetate-^{14}C Incorporation into Lipids by Leaf Slices of Barley Seedlings at Various Stages of Greening

Time of Illumination Prior to Incubation	Chlorophyll Content of: Whole Leaves Prior to Incubation	Chlorophyll Content of: Leaf Slices after Incubation	Acetate Incorporation into: Total Lipids	Acetate Incorporation into: Phospho-, Sulfo-, and Galactolipids
hr	mg/g fresh weight		mμmoles/g fresh weight	
3	0.01	0.04	8.5	2.2
6	0.04	0.06	9.2	3.0
9	0.10	0.09	10.4	5.6
12	0.21	0.15	11.6	7.0
18	0.33	0.30	9.6	7.1

Incubation: 3 hr in light at 30°C.

cursors were supplied to detached greening leaves by the same technique. The incorporation into lipids of a ^{14}C-labeled equimolar mixture of glucose and fructose fed for 3 hr, after 3 and 9 hr of greening, was of the same order of magnitude (0.06% and 0.13% of the uptake) as for sodium acetate-^{14}C. Labeled potassium bicarbonate proved to be as inefficient a lipid precursor in this system as acetate and hexose. With both hexose and bicarbonate as substrates, the incorporation of label into lipid increases during greening in a manner similar to that observed for acetate.

Acetate Uptake and Lipid Synthesis in Leaf Slices at Various Stages of Greening

Relatively high levels of acetate incorporation into leaf lipids have been attained previously by submerging small leaf slices of light-grown barley seedlings in the labeled acetate solution, and by incubating them for 3 hr in the light at 30°C. We therefore used this method for incubating 1–2 mm leaf slices of barley seedlings which had been greened for 3, 6, 9, 12, and 18 hr, respectively (Table 4).

Chlorophyll synthesis did not continue during the incubation of the leaf slices. The slight increase of chlorophyll content during incubation apparent in Table 4 for the samples greened for 3 and 6 hr is not significant. In leaf slices of samples from later greening stages, a loss and(or) destruction of chlorophyll occurred during incubation.

An almost constant amount of 9–12 mμmoles of labeled acetate was incorporated into the lipids of 1 g of leaf tissue at all stages of greening. The extent of incorporation is 5 to 10 times greater than in the detached leaves at later greening stages. If incorporation into only the phospho-, sulfo-, and galactolipids is considered (last column of Table 4) the increasing capacity of the tissue to insert the de novo-synthesized acyl moieties into the complex lipids at successive stages of greening becomes clear. In the tissue greened for 3 hr, 25% of the total incorporated acetate appears in the phospho-, sulfo-, and galactolipids, whereas 75% is built into steroids and other unsaponifiable lipids. After 18 hr of greening the situation is reversed: now 75% of the incorporated acetate is channeled into the phospho-, sulfo-, and galactolipids, whereas only 25% serves as the precursor for the syntheses of steroids and other unsaponifiable lipids.

This method of incubating leaf slices is highly effective for supplying exogenous acetate to leaf tissue for lipid synthesis and for studying the capacities of synthetic pathways in leaves containing chloroplasts at a given developmental stage. However, the method effectively blocks chlorophyll synthesis during incubation and thereby the further development of the chloroplast lamellar system and its grana structure. The fatty acids synthesized in leaf slices, therefore, may also fail to fulfill the requirements of the complex lipids necessary for the synthesis of the chloroplast disc membranes or the grana structure.

Fig. 2 presents the changes of the incorporation of acetate into the six lipid classes which occur when leaves at successive stages of greening are studied. For up to 12 hr of greening, gradually increasing amounts of labeled acetate were incorporated into the phospholipids of fraction *A* which include phosphatidyl inositol and phosphatidyl choline, into the phospholipids of fraction *B* with the chloroplast lipids phosphatidyl glycerol and sulfoquinovosyl diglyceride, as well as into the two galactolipid fractions *C* and *D*. (The small amount of label incorporated into the monogalactosyl diglycerides by the 12-hr greened leaf slices is considered atypical.) A similar increase of incorporation is observed for the triglycerides and hydrocarbons (fraction *F*). After 12 hr of greening,

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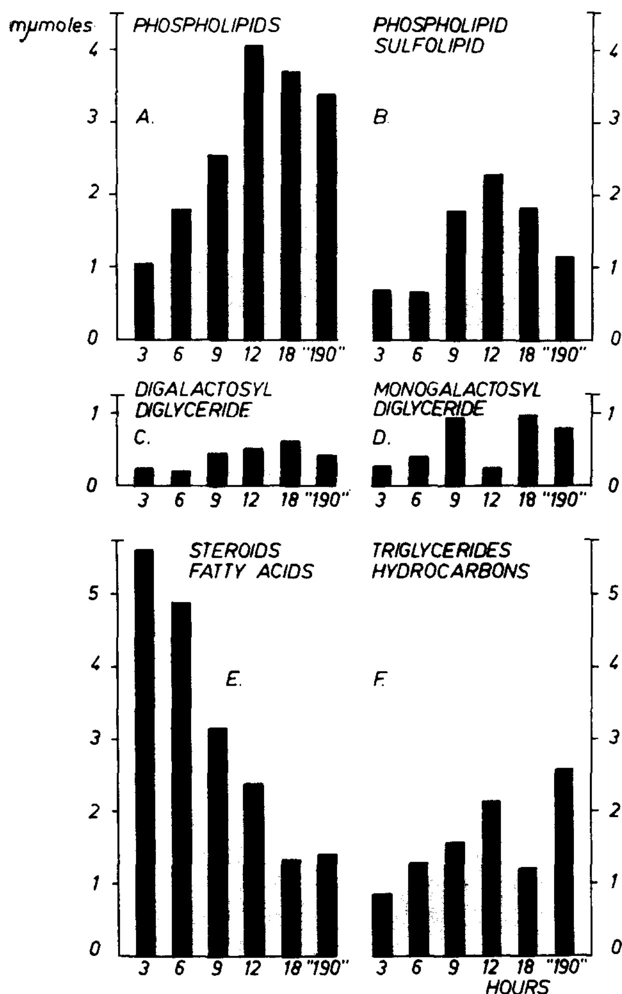

Fig. 2. Incorporation of sodium acetate-^{14}C into the various lipid classes by leaf slices of dark-grown barley seedlings greened for different lengths of time (abscissa) and by leaf slices of light-grown seedlings ("190" hr). Incubation: 3 hr in light at 30°C.

the full capacity for ^{14}C labeling in these five lipid classes (*A*, *B*, *C*, *D*, *F*) has been reached. Incorporation of label at this stage is of the same order of magnitude as that obtained with fully green leaves from "190"-hr old light-grown seedlings (Fig. 2). The increased incorporation of labeled acetate into these lipid classes is accompanied by a corresponding drop in the labeling of fraction *E* which contains the steroids and free fatty acids. Compared to whole leaves (p. 428), the incorporation of acetate-^{14}C into galactolipids is limited. This may be the result of the effective blocking of chlorophyll and grana synthesis.

The fatty acid compositions of fractions *A* to *D* for the various greening stages are compiled in Table 5. The increase in the relative amount of linolenic acid which was observed in the total lipid extract concomitant with greening (Table 2) can be traced to an enrichment of this fatty acid in the monogalactosyl diglyceride fraction and to a lesser extent in the phospho- and sulfolipid fraction *B*. No striking changes occurred in the relative frequency of the fatty acids in the other two lipid classes.

The gradually increased labeling of the lipid classes *A* to *D* at successive stages of greening is primarily due to a gradually increased synthesis of oleic and linoleic acids (Fig. 3). A significant increase in the synthesis of palmitic acid can be demonstrated for the phospholipid *B* fraction and of linolenic acid for the monogalactosyl diglycerides. A small amount of label was noted in C_{16} unsaturated fatty acids of the phospholipid *B* fraction which contains phosphatidyl glycerol.

It is apparent from both Fig. 3 and Table 6 that the proportions of the newly synthesized, labeled fatty acids within the four lipid classes do not change drastically or consistently at different stages of greening. A possible exception to this is the disproportionally large amount of oleic and linoleic acid synthesized in leaves at later stages of greening for the phospholipid fraction *A*.

However, the composition of the newly synthesized fatty acids in fractions *A*, *B*, *C*, and *D* is very different from that of the endogenous fatty acids present in the leaves during greening. This is clearly seen in Tables 5 and 6. The compositional differences between the newly synthesized fatty acids (^{14}C-labeled) and the endogenous fatty acids are greater in leaf slices than in whole leaves.

The large amounts of steroids synthesized during the incubation of leaves at early stages of greening comprise mainly a substance tentatively identified as β-amyrin. A major portion of the labeled free fatty acids in fraction *E* and the labeled fatty acids of the triglycerides in fraction *F* consisted of oleic and linoleic acids.

The experiments with the leaf slices demonstrate a strongly increasing capacity to synthesize fatty acids and to insert them into phospho- and sulfolipids and to a lesser extent, into galactolipids as the chloroplasts develop in illuminated leaves of dark-grown seedlings. Nevertheless, the fatty acids labeled in leaf slices incubated at 30°C are not similar to the majority of those assembled into lipids during the formation of the lamellar system and the grana in the chloroplast.

Acetate Uptake and Lipid Synthesis in Detached Greening Leaves under Different Conditions of Acetate Feeding

In order to determine the conditions under which normal chlorophyll synthesis and chloroplast development can proceed together with a high rate of acetate incorporation, detached leaves from dark-grown seedlings were greened for 5 hr and then incubated at 20°C with acetate-^{14}C for 3 hr in the light by three different procedures: (*a*) 8-cm long leaf pieces were floated on the acetate solution; (*b*) 0.5-cm long leaf slices were floated on the acetate

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TABLE 5 Fatty Acid Composition of Lipid Classes A to D from Leaf Slices of Dark-Grown Barley Seedlings Greened for Different Lengths of Time and from Leaf Slices of Light-Grown Seedlings ("190" Hr)

Lipid Class	Time of Greening Prior to Incubation	Fatty Acids					
		16:0	16:u*	18:0	18:1	18:2	18:3
	hr			%			
A. Phospholipids	3	22	2	6	10	37	23
	9	28	tr.	7	9	34	22
	18	28	tr.	5	11	31	25
	"190"	25	tr.	10	16	25	20
B. Phospholipid + sulfolipid	3	33	4	4	6	26	26
	9	25	3	5	7	28	31
	18	24	3	14	16	16	28
	"190"	28	7	6	7	11	37
C. Digalactosyl diglyceride	3	20	tr.	8	7	11	50
	9	24	tr.	6	8	13	49
	18	27	tr.	8	10	9	45
	"190"	18	tr.	12	11	5	52
D. Monogalactosyl diglyceride	3	17	6	12	10	3	50
	9	15	3	3	6	7	65
	18	18	2	7	9	4	59
	"190"	10	tr.	7	6	4	71

Incubation: 3 hr in light at 30°C. Small amounts of 14:0 are included in the calculation, but not listed in the table.
* 16:u, C_{16} unsaturated fatty acids.

TABLE 6 Distribution of ^{14}C Label in Fatty Acids of Lipid Classes A to D Synthesized by Leaf Slices of Dark-Grown Barley Seedlings Greened for Different Lengths of Time and by Leaf Slices of Light-Grown Seedlings ("190" hr)

Lipid Class	Time of Greening Prior to Incubation	Label in Fatty Acids					
		16:0	16:u*	18:0	18:1	18:2	18:3
	hr			%			
A. Phospholipids	3	23	3	10	25	31	4
	9	13	tr.	tr.	30	53	4
	18	10	tr.	tr.	48	41	1
	"190"	19	0	3	36	39	2
B. Phospholipid + sulfolipid	3	27	8	5	14	31	8
	9	26	4	1	20	37	12
	18	31	2	1	31	29	6
	"190"	30	tr.	tr.	29	34	8
C. Digalactosyl diglyceride	3	31	7	7	16	27	13
	9	16	tr.	4	29	40	10
	18	20	0	3	44	33	tr.
	"190"	34	tr.	0	28	26	12
D. Monogalactosyl diglyceride	3	16	tr.	6	18	28	32
	9	14	tr.	2	19	32	32
	18	15	tr.	2	29	32	22
	"190"	14	3	0	24	40	20

Incubation: 3 hr in light at 30°C. Small amounts of C_{14} and C_{22} fatty acids are not considered in the table.
* 16:u = C_{16} unsaturated fatty acids.

solution; (*c*) 15-cm long leaf pieces were dipped momentarily into acetate solution every 15 minutes and were then allowed to stand in the air with their bases immersed in water between each dip.

The leaf slices in treatment (*b*) did not continue to synthesize chlorophyll (Table 7). As found in the previous experiment these slices lost pigment and showed the same high acetate incorporation into lipids, of which 36% went into phospho-, sulfo-, and galactolipids.

The 8-cm leaf pieces in treatment (*a*) continued to synthesize chlorophyll—possibly at a somewhat reduced rate—during the incubation. Acetate incorporation into the total lipids (Table 7) was about 20 times higher than that observed with whole leaves standing with their bases in the acetate solution (Table 1). 26% of the incorporated acetate went into the fatty acids of phospho-, sulfo-, and galactolipids.

The intermittent submersion of the whole leaves in

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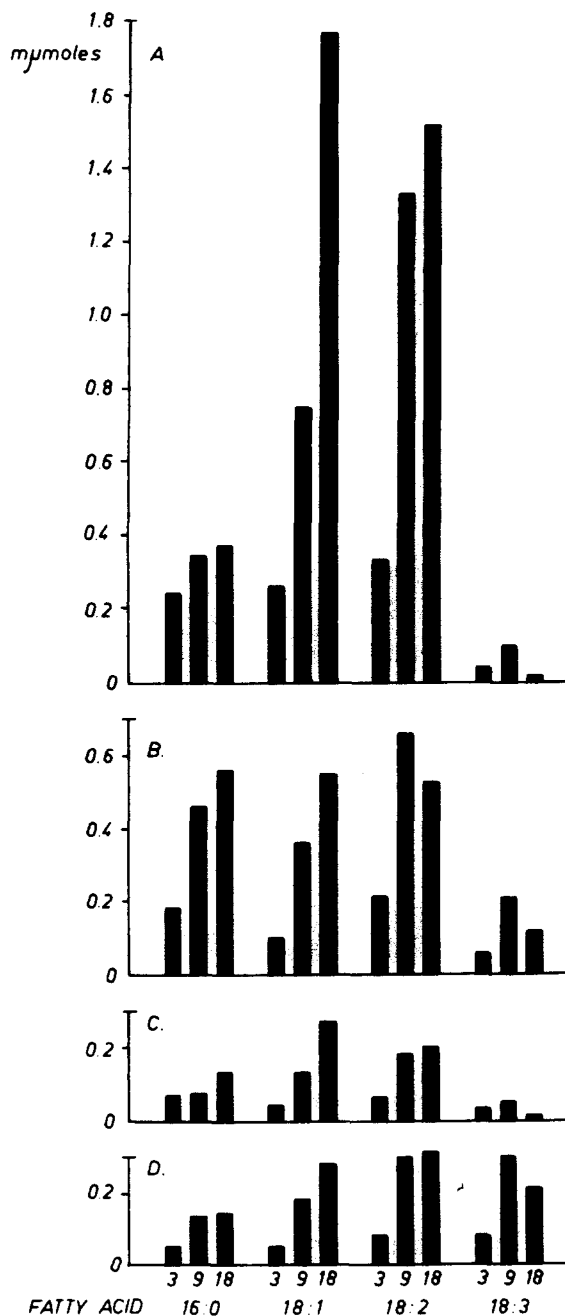

FIG. 3. Incorporation of sodium acetate-^{14}C into individual fatty acids of lipid classes *A–D* synthesized by leaf slices of dark-grown barley seedlings greened for different lengths of time (given in hours on abscissa). Incubation: 3 hr in light at 30°C. Minor amounts of labeled 14:0, unsaturated C_{16}, and 18:0 fatty acids have not been considered in the graph. *A*, phospholipids; *B*, phospholipids + sulfolipids; *C*, digalactosyl diglyceride; *D*, monogalactosyl diglyceride.

treatment (*c*) allowed chlorophyll synthesis to continue at a normal rate. An acetate incorporation into lipids approaching that of leaf slices is achieved (Table 7), with 23% of the acetate being incorporated into the phospho-, sulfo-, and galactolipids. Thus a high acetate incorporation into leaf lipids can be obtained while normal greening and chloroplast development is proceeding.

The amounts of acetate incorporated into the six lipid classes are shown in Fig. 4. The leaf slices behaved in the same way as those studied in the previous experiment with the Warburg incubator, resulting in a high incorporation into the steroid (*E*), triglyceride (*F*), and phospholipid (*A*) fractions. Of the ^{14}C label found in the phospho-, sulfo-, and galactolipids, a higher percentage is incorporated into the mono- and digalactosyl diglycerides by the whole leaves, either floated or dipped, than by the leaf slices. The leaf slices with their blocked chlorophyll synthesis incorporate rather considerable amounts of label into the chloroplast nonspecific phospholipid *A* fraction. The whole dipped and whole floated leaves incorporated proportionally more label into steroids than did the leaf slices.

The distribution of labeled fatty acids within the four lipid classes (Fig. 5 and Table 8) is on the whole similar to that observed in the previous experiment with leaf

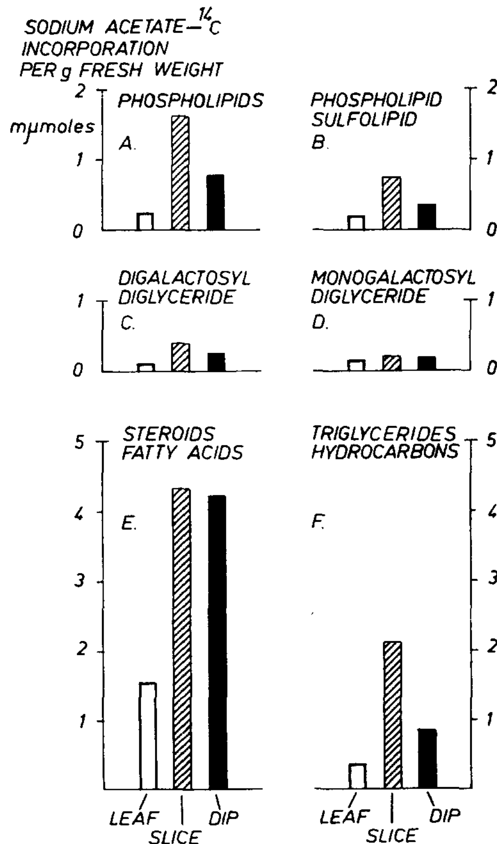

FIG. 4. Incorporation of sodium acetate-^{14}C into various lipid classes by dark-grown barley leaves greened for 5 hr. Different techniques of acetate feeding during a 3 hr incubation in light at 20°C are compared: *leaf*, whole leaves floated; *slice*, 0.5 cm slices floated; *dip*, whole leaves dipped every 15 min.

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TABLE 7 Sodium Acetate-^{14}C Incorporation into Lipids by Detached Barley Leaves Fed Acetate by Different Techniques

Technique of Acetate Feeding	Time of Illumination Prior to Incubation	Chlorophyll Content		Acetate Incorporation into		
		Prior to Incubation	After Incubation	Total Lipids	Phospho-, Sulfo-, and Galactolipids	
	hr	*mg/g fresh weight*		*mμmoles/g fresh weight*		*(% of total)*
Whole leaves floated	5	0.05	0.07	2.54	0.65	(25.7)
0.5 cm slices floated	5	0.05	0.03	9.33	3.00	(36.2)
Whole leaves dipped every 15 min	5	0.05	0.09	6.57	1.51	(23.0)
Control, whole leaves standing in H_2O	5	0.05	0.08	—	—	(—)

Incubation: 3 hr in light at 20°C.

TABLE 8 Distribution of ^{14}C Label in Fatty Acids of Lipid Classes A to D Synthesized by Detached Barley Leaves Fed Acetate by Different Techniques

Lipid Class	Technique of Acetate Feeding	Label in Fatty Acids					
		16:0	16:u*	18:0	18:1	18:2	18:3
					%		
A. Phospholipids	Whole leaves floated	17	0	11	40	31	0
	0.5 cm slices floated	21	0	12	36	26	2
	Whole leaves dipped	13	0	6	22	53	6
B. Phospholipid + sulfolipid	Whole leaves floated	28	14	0	40	17	0
	0.5 cm slices floated	29	10	7	22	17	9
	Whole leaves dipped	34	13	0	23	23	7
C. Digalactosyl diglyceride	Whole leaves floated	27	0	0	42	31	0
	0.5 cm slices floated	18	0	18	41	23	tr.
	Whole leaves dipped	16	0	tr.	25	59	0
D. Monogalactosyl diglyceride	Whole leaves floated	18	0	0	37	23	22
	0.5 cm slices floated	13	0	0	19	38	36
	Whole leaves dipped	9	0	0	15	44	33

Dark-grown leaves greened for 5 hr prior to incubation for 3 hr in light at 20°C. Small amounts of C_{14} and C_{22} fatty acids are considered in the calculations, but not listed in the table.
* 16:u = C_{16} unsaturated fatty acids.

slices incubated in the Warburg. The relatively small amount of labeled linolenic acid in all four classes is also apparent in treatments (*a*) and (*c*) which allowed chlorophyll synthesis to proceed. The leaves dipped into the acetate solution (*c*) incorporated proportionally more label into linoleic acid of phospholipids and di- and monogalactosyl diglycerides than did those in treatment (*a*) and treatment (*b*). These results show that, in the greening leaves, chlorophyll synthesis and a parallel high incorporation of acetate are not sufficient conditions to label the most prominent lipids normally synthesized during chloroplast development. Even if the dipping treatment channels more label into the galactolipids, which are richer in the linoleic acid, the labeled lipids cannot be considered as the typical lamellar lipids of the chloroplast.

DISCUSSION

The increase in linolenic acid content of the lipids observed in greening barley leaves (Table 2) agrees with what is known from other species of higher plants (1–4). Where isolated plastid fractions have been analyzed, the difference in linolenic acid content between the dark- and light-grown leaves is even more pronounced (3, 4) because of the small amount of linolenic acid recovered from the proplastid fractions. The enrichment of this fatty acid in the monogalactosyl diglycerides and sulfolipids reported for greening *Ricinus* seedlings (2) is also apparent for barley (Table 5). We, however, did not find an enrichment of this fatty acid in the digalactosyl diglyceride fraction. The only other compositional change accompanying greening in *Ricinus* is the appearance of *trans*-3-hexadecenoic acid in the phosphatidyl glycerol

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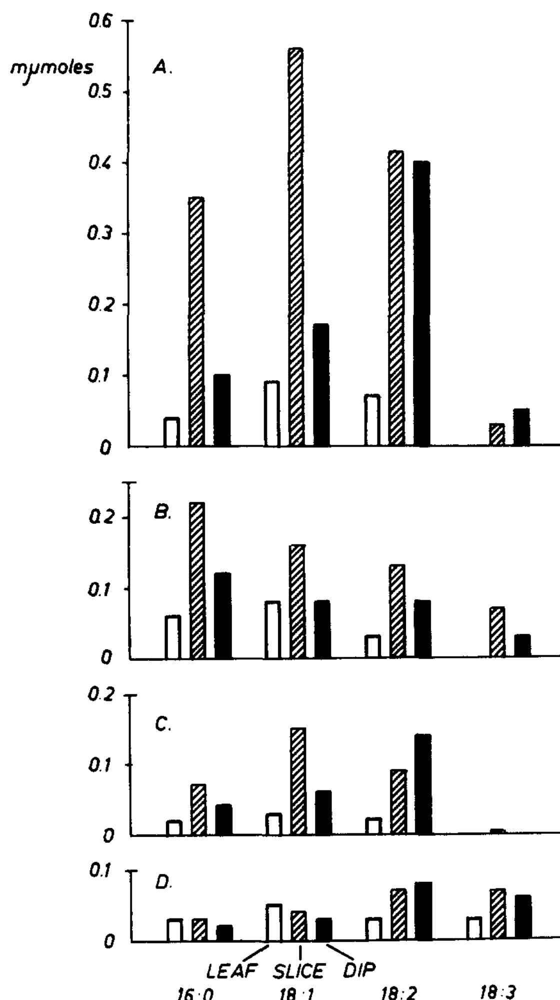

FIG. 5. Incorporation of sodium acetate-^{14}C into individual fatty acids of lipid classes *A–D* synthesized by dark-grown barley leaves greened for 5 hr. Different techniques of acetate feeding during a 3 hr incubation in light at 20°C are compared. Minor amounts of labeled 14:0, unsaturated C_{16}, 18:0, and C_{22} fatty acids have not been considered in the graph. *Leaf*, whole leaves floated; *slice*, 0.5 cm slices floated; *dip*, whole leaves dipped every 15 min.

fraction (2). Small mass peaks corresponding to *trans*-3-hexadecenoic acid were present in the phospholipid *B* fraction of barley at all stages of greening.

In our experiments, the increase in linolenic acid content of the total lipids and the enrichment of the monogalactosyl diglyceride fraction with this fatty acid were found to occur in the first 15 hr of greening, with little change thereafter. This coincides with the early portion of the exponential phase of chlorophyll synthesis and formation of the chloroplast lamellar system.

An increase of the linolenic acid content in the total lipids and an enrichment of this fatty acid in the galactolipids also occurs during induced chloroplast development in dark-grown *Euglena gracilis*, strain Z (22–25); in the y^2 mutant of *Chlamydomonas reinhardi*, grown in the dark (22); and in heterotrophically grown *Chlorella vulgaris* (26). In *Euglena* and *Chlorella*, a net synthesis of monogalactosyl diglycerides accompanies chlorophyll synthesis and chloroplast development (23, 25). In glucose-bleached *Chlorella protothecoides* cells, sulfolipid synthesis is parallel with the formation of chloroplast lamellar structures during greening of these cells on inorganic medium (27).

The data obtained so far on changes in lipid composition in greening leaves are compatible with the view that formation of grana structures in the developing chloroplast is associated with a synthesis of monogalactosyl diglycerides and sulfolipids in these organelles. It is not known whether the considerable amounts of galactolipids (2) and sulfolipids (28) present in dark-grown leaves are constituents of the proplastids, the cytoplasm, and(or) other cell organelles, e.g. mitochondria (29, 30). To what extent these endogenous lipids are used for the construction of the lamellar system in developing chloroplasts during greening of dark-grown leaves will have to be determined.

In previous investigations, at best only small amounts of acetate-^{14}C were found to be incorporated into the linolenic acid of the leaf lipids even during extended periods of incubation (31–33). On exposure of heterotrophically grown *Chlorella vulgaris* cells to light labeling of linolenic acid from exogenous acetate is reported (33). Erwin and Bloch (22) achieved a considerable incorporation of acetate-^{14}C into linolenic acid of light-grown *Euglena* cells.

In barley leaves about 16% of the total fatty acid label resides in linolenic acid at the later stages of greening after a 3 hr incubation period (Table 3). A subsequent, 3 hr treatment with unlabeled acetate results in an increased percentage of labeled linolenic acid. The aforementioned increase in the linolenic acid content during the first 15 hr of greening appears to be paralleled by an increase of the percentage of label found in linolenic acid (Table 3). The experiment with barley leaf slices reveals that the amount of labeling of the linolenic acid in the lipid classes studied differs markedly (Fig. 3 and Table 6), being highest in the monogalactosyl diglyceride fraction. Similar differences in the labeling pattern of individual fatty acids in the various lipids are found in dark-grown *Chlorella* cells exposed to light (33). In these cells the linolenic acid of the monogalactosyl diglycerides is labeled faster than that of the other lipids. Only after prolonged incubation, over a period of one to two cell generations, is a large amount of labeled linolenic acid found in the digalactosyl diglycerides.

Thus the synthesis of a linolenic acid intermediate (CoA or ACP derivative) that is utilized in the assembly

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of complex lipids need not be as slow a process as has been assumed earlier (31). The different labeling patterns for fatty acids of the two galactolipids add to the notion that the digalactosyl diglycerides are not made from monogalactosyl diglycerides by direct glycosylation (23, 34) as has been suggested (35, 36).

Our experiments with whole leaves and leaf slices demonstrate the gradual development of the capacity to synthesize fatty acids and to insert them into phospho-, sulfo-, and galactolipids in the light as a result of chloroplast differentiation. This implies that one or several of the enzymes necessary for the conversion of acetyl CoA to malonyl CoA and for subsequent steps in fatty acid synthesis are either formed or activated during chloroplast development. On the other hand, slices of barley leaves greened for 3 hr show a high capacity to synthesize acetyl CoA from acetate and to utilize this acetyl CoA for synthesis of polyisoprenoids (Figs. 2, 4). The decrease in the amount of β-amyrin synthesized by leaf slices greened for progressively longer periods of time might be ascribed either to the development of a light-controlled inhibition of polyisoprenoid synthesis or to the possibility that acetyl CoA carboxylase competes more favorably with the mevalonic acid pathway for the acetyl CoA as the chloroplasts develop. We have found that mevalonic acid incorporation into lipids by green barley leaf slices appears not to be inhibited by light. Also, mevalonate incorporation into the unsaponifiable lipids of maize seedlings is known to increase during greening (37). Therefore if this pathway can be inhibited by light, it has to involve a step prior to mevalonic acid.

In the experiments in which we varied the physiological conditions of the tissue during acetate feeding (Figs. 4 and 5, Tables 7 and 8), we observed that a larger portion of labeled acetate was incorporated into the phospholipid *A* fraction, which consists of phosphatidyl inositol and phosphatidyl choline, in tissue slices where chlorophyll synthesis is blocked. Relatively more monogalactosyl diglycerides are labeled under conditions which allow greening to proceed. This demonstrates that the incorporation of labeled acetate into the various phospho- and galactolipids can be channeled simply by the use of different physiological conditions of the tissue during incubation. The implications of these findings merit further studies and suggest that the mode of the substrate feeding can greatly alter the pattern of incorporation of the substrate.

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