Lipids and Lipoprotein Complex in Photosynthetic Tissues

Part V A Comparison of Lipid and Pigment Patterns

in Light-grown and Dark-grown Plant Leaves

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The lipids and pigments of corn and soybean leaves grown in light and in dark were examined. The green leaf contained appreciably greater amounts of the two galactolipids, sulfolipid, phosphatidyl glycerol and diphosphatidyl glycerol, while the etiolated leaf had relatively more cerebroside, sterol glucoside, phosphatidyl ethanolamine and lecithin. The prominent component of glycerides was 1,2-diglyceride in green leaf, and triglyceride in etiolated leaf. The green leaf lipids showed a remarkable specificity of component fatty acids, but those of etiolated leaf only at lower rate. From the results, possible functions of the lipids were discussed.

In previous paper,^{1,2)} some evidences suggested that all algae and green leaves performing the Hill reaction had a common pattern of composition of lipids, pigments, and quinones, which apparently differed from those of photosynthetic bacteria, non-O₂-producing photosynthetic organisms.

The present study is designed to furnish some clue to a specific function of individual components of lipids and pigments in higher plant leaves, by examining their components of light-grown and dark-grown plant leaves. Similar attempts were made on algae, and light-induced changes in the content of gross lipids or a few lipid components and in their fatty acid components were partially reported.³⁻⁶⁾ This paper describes the changes of all lipids and lipid-soluble materials of corn and soybean leaves.

MATERIALS AND METHODS

Materials and lipid extraction. Two groups of yellow dent corn seeds (*Zea mays* Linn) were germinated and grown for 20 days in the light and in the dark, separately. The green and etiolated leaves were harvested from 30 plants, respectively, and the leaves were repeatedly extracted with cold chloroform-methanol (2/1, v/v) in homogenizer. The extracts were combined, washed according to the Folch's method and evaporated *in vacuo* to appropriate concentrations. Soybean leaves and their lipid extracts were also obtained by the same techniques, except for that green plants were grown for 34 days in the light, and etiolated plants were brought up in the dark for 14 days after germination and then grown for 20 days in the light.

Separation and determination of pigments and quinones. The methods were essentially the same as described in the previous papers.^{1,2)} Lipid-soluble pigments were separated by chromatography using

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2) O. Hirayama, *Agr. Biol. Chem.*, **32**, 34 (1968).

3) A. Rosenberg, *Biochemistry*, **2**, 1148 (1963); A. Rosenberg and M. Pecker, *ibid.*, **3**, 254 (1964); A. Rosenberg, M. Pecker and E. Moschiders, *ibid.*, **4**, 680 (1964).

4) A. Rosenberg, J. Gouaux and P. Milch, *J. Lipid Res.*, **7**, 733 (1966); A. Rosenberg and J. Gouaux, *ibid.*, **8**, 80 (1966).

5) D. Hulanicka, J. Erwin and K. Bloch, *J. Biol. Chem.*, **239**, 2778 (1964).

6) B. W. Nichols, *Biochim. Biophys. Acta*, **106**, 274 (1965).

silica-gel thin-layers, then the colored bands were extracted and determined by spectral analysis. The direct spectrophotometric determination of chlorophylls a and b,⁷⁾ and of total carotenoids⁸⁾ were carried out with total lipid extracts. The quinone components were similarly separated by thin-layer chromatography, and determined by spectral analysis with borohydride reduction technique.⁹⁾ The concentrations of the various components were obtained using the following values: β -carotene, $E_{1\text{cm}}^{1\%}$ (449 m μ) 2500; lutein, $E_{1\text{cm}}^{1\%}$ (445 m μ) 2500; violaxanthin, $E_{1\text{cm}}^{1\%}$ (441 m μ) 2550; neoxanthin, $E_{1\text{cm}}^{1\%}$ (435 m μ) 2270; plastoquinone $\Delta E_{1\text{cm}}^{1\%}$ (255 m μ) (oxidized-reduced) 200.

Separation and determination of lipids. For the separation of lipids, silica-gel column chromatography was used according to the method reported in a previous paper¹⁰⁾ with a partial modification. Total lipid extracts (about 400 mg) were applied to a column (2.0 cm in diameter) containing 15 g of silica gel, and elution was carried out with the solvents as shown in Table III. The eluates were collected in 15 ml or less portions monitored by thin-layer chromatography, and combined fractions were evaporated *in vacuo* and taken up in chloroform to give known volume solutions. The lipid fractions containing more than two components were further separated by preparative thin-layer chromatography as follows. The solvents used were *n*-hexane or *n*-hexane-ether-acetic acid (70/30/1, v/v) for non-polar lipids and chloroform-methanol-acetic acid-water (70/22/2/2, v/v) for polar lipids. The chromatograms were visualized with spray of 2',7'-dichlorofluorescence, rhodamine 6G or iodine solution, and then the lipid bands were collected into a glass-filter with aspirator and extracted with methanol followed by chloroform-methanol (1/1, v/v). The individual lipids thus isolated were identified by means of thin-layer chromatography, chemical analysis and absorption spectra in ultraviolet and infrared. And their quantities were evaluated by determining ester group,¹¹⁾ cholesterol,¹²⁾ free fatty acid,¹³⁾ phosphorus,¹⁴⁾ or sugar¹⁵⁾ content.

Gas chromatography. The fatty acid methyl esters were prepared by transmethylation of lipids with 0.5% sodium methylate in methanol. The gas chromatographic separations were performed with Shimadzu GC-2P type fitted with a thermal conductivity detector, using a copper column (0.4 $\times$ 300 cm) packed with 15% diethylene glycol succinate polyester on 60-80 mesh Cerite. The instrument was operated isothermally at 200°C with helium as the carrier gas.

Determination of absorption spectra. Absorption spectra in ultraviolet and visible ranges were measured with a Shimadzu QR-50 spectrophotometer, and infrared spectra with a Shimadzu IR-27 C infrared spectrophotometer.

RESULTS

The gross lipid contents of green and etiolated leaves are given in Table I. In both cases of corn and soybean, total weight of fresh leaves per plant was higher in light-grown than in dark-grown plants. The lipid content (per g of leaf) was similarly higher in green leaf of corn, but soybean leaves showed a reversed relation.

Table II shows the concentrations of pigments and quinones of green and etiolated corn leaves. The values are given as weight per g of total lipid extracts. Etiolated leaf showed the virtual lack of chlorophylls and less contents of xanthophylls, plastoquinones and tocopherols, although it had β -carotene in

TABLE I. TOTAL LIPID CONTENTS OF GREEN AND ETIOLATED LEAVES

	Fresh leaf weight (g/plant)	Lipid content	
		(mg/plant)	(mg/g fresh leaf)
Corn leaf			
Green	3.1	26.7	8.6
Etiolated	1.5	5.7	3.8
Soybean leaf			
Green	1.2	14.5	12.1
Etiolated	0.6	17.5	29.2

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TABLE II. PIGMENT AND QUINONE COMPONENTS OF CORN LEAF

Components	Green (mg/g total lipids)	Etiolated (mg/g total lipids)
Total chlorophylls	80.8	0
Chlorophyll a	57.2	0
Chlorophyll b	23.6	0
Total carotenoids	14.5	7.5
β -Carotene	5.2	5.2
Lutein	4.5	1.6
Zeaxanthin	1.1	0.4
Violaxanthin	0.6	0.3
Neoxanthin	3.1	trace
Quinones*	3.6	2.5
Tocopherol**	###	±

* Probable identification: plastoquinone A+B, C, and D, and ubiquinone.

** The quantity was roughly estimated from its spot area on thin-layer plate.

equal proportion as that of green leaf. Both leaves gave three spots of quinones on thin-layer plates, which were presumed to be plastoquinone A (and B), plastoquinone C (and ubiquinone) and plastoquinone D. Their spot area suggested that the prominent component of green leaf was plastoquinone A and that of etiolated leaf might be ubiquinone.

Table III indicates the elution scheme for

TABLE III. ELUTION SCHEME FOR LEAF LIPIDS BY SILICA-GEL COLUMN CHROMATOGRAPHY

Solvents	Volume	Lipids eluted
Hexane	100 ml	hydrocarbon, β -carotene
Benzene	100 ml	plastoquinone, triglyceride, higher alcohol
Chloroform	100 ml	diglyceride, free fatty acid, sterol
Chloroform/acetone (4/1)	100 ml	chlorophyll, xanthophyll
Chloroform/acetone (1/2)	200 ml	xanthophyll, esterified sterol glucoside, monogalactolipid, cerebroside, sterol glucoside
Acetone/methanol (9/1)	150 ml	cerebroside, sterol glucoside, digalactolipid, sulfolipid
Methanol	250 ml	diphosphatidyl glycerol, phosphatidyl glycerol, phosphatidyl inositol, phosphatidyl ethanolamine, lecithin

leaf lipids on silica-gel column. The chromatographic technique disclosed to separate the following lipids in pure state: plastoquinone A, triglyceride, higher alcohol, monogalactosyl diglyceride, digalactosyl diglyceride, sulfoquinovosyl diglyceride, phosphatidyl ethanolamine and lecithin. Successively, di-

TABLE IV. NON-POLAR LIPIDS OF CORN LEAF

Components	Green (μ mole/g total lipids)	Etiolated (μ mole/g total lipids)
Triglyceride	29	44
Diglyceride*	56	38
Monoglyceride**	5	4
Free fatty acid	3	86
Free higher alcohol***	###	###
Sterol	40	147
Squalene***	+	+

* Only 1,2-isomers were present in both green and etiolated leaves.

** Green leaf had only 2-isomer, and etiolated leaf contained both 1- and 2-isomer.

*** The quantities were roughly estimated from their spot area on thin-layer plates.

TABLE V. POLAR LIPID COMPOSITION OF LEAVES

Compounds	Corn (μ mole/g total lipids)		Soybean (μ mole/g total lipids)	
	Green	Etiolated	Green	Etiolated
Total glycolipids	555	347	892	433
Monogalactosyl diglyceride	226	72	440	89
Digalactosyl diglyceride	122	69	197	51
Sulfoquinovosyl diglyceride	83	36	92	75
Sterol glucoside } Cerebroside }	65	148	101	163
Esterified sterol glucoside*	(59)	22	(64)	55
Total phospholipids	165	174	215	138
Phosphatidyl ethanolamine	46	53	56	57
Phosphatidyl glycerol	37	12	61	50
Lecithin	25	83		
Diphosphatidyl glycerol	30	19	78	23
Other phospholipids	27	7	20	8

* Esterified sterol glucoside isolated from green leaf was considerably contaminated with xanthophylls, and its content estimated by sugar determination seems to have a significant error.

glyceride, free fatty acid, sterol, monoglyceride, diphosphatidyl glycerol and phosphatidyl inositol were isolated from the mixed fractions by preparative thin-layer chromatography. All these lipid components obtained were chromatographically pure on thin-layer.

Table IV shows the concentrations of non-polar lipid components, which were estimated by chemical analyses (see Methods). The values are presented as μ mole per g of total lipid extracts. Green leaf was found to contain diglyceride as the prominent component, while etiolated leaf had more proportions of triglyceride, free fatty acid and sterol. Chromatography using silica-gel plate containing boric acid¹⁶⁾ further indicated that diglycerides of green and etiolated leaves were 1,2-isomer, and monoglyceride of the green leaf was 2-isomer and that of the etiolated consisted of both 1- and 2-isomer. The presences of squalene and free higher alcohol were confirmed by thin-layer chromatography and infrared absorption spectra. Particularly, the amounts of free higher alcohol appeared to be considerably greater in both green and etiolated leaves.

Polar lipid compositions of corn and soybean leaves are summarized in Table V. In both cases, green leaves had much higher contents of mono- and digalactosyl diglyceride and a higher content of sulfolipid, whereas etiolated leaves contained relatively more cerebroside and sterol glucoside. These leaves also revealed different patterns of phospholipid composition, indicating that the green leaf had more phosphatidyl glycerol and diphosphatidyl glycerol, and the etiolated more lecithin and phosphatidylethanolamine. It is also noteworthy that green soybean leaf contained diphosphatidyl glycerol in remarkably higher concentration as compared with that of corn leaf.

Tables VI and VII show that the different lipid classes possess differing and distinctive fatty acid composition. Diglyceride from green corn leaf contained much saturated acids, especially lauric acid, which was almost absent in diglyceride from etiolated leaf. Relatively larger content of lauric acid was also observed in phosphatidyl ethanolamine from green leaf. Mono- and digalactolipids of green leaf had much higher linolenic acid, while the galactolipids of the etiolated showed

TABLE VI. FATTY ACID COMPOSITION OF GREEN CORN LEAF LIPIDS

Lipid components	Fatty acids (wt. %)							
	12:0	14:0	16:0	16:1* <i>trans</i>	18:0**	18:1	18:2	18:3
Total	0.6	0.2	16.8	2.0	0.4	0.8	14.0	65.2
Triglyceride	4.2	0.3	9.3	—	0.7	5.2	37.2	42.5
Diglyceride	41.0	8.8	7.1	—	43.1	—	—	—
Monoglyceride	—	5.8	76.5	—	7.2	8.3	—	—
Free fatty acid	—	—	4.3	—	—	0.5	7.2	87.8
Monogalactosyl diglyceride	0.2	—	1.8	—	—	—	4.3	93.7
Digalactosyl diglyceride	—	0.3	11.2	—	1.8	1.2	5.5	80.0
Sulfoquinovosyl diglyceride	—	—	50.2	—	0.5	—	7.7	41.6
Phosphatidyl glycerol	—	—	31.7	20.4	—	1.3	27.0	19.6
Phosphatidyl ethanolamine	13.6	—	33.8	—	4.1	5.0	22.8	20.7
Lecithin	—	—	18.5	—	2.3	4.7	43.0	31.5

* The presence of *trans*-hexadecenoic acid was confirmed by retention time on gas chromatogram, infrared absorption spectra and thin-layer of silica-gel containing silver nitrate.

** If hexadecatrienoic acid were present, the acid would overlap on the peak of stearic acid on gas chromatogram. However, no detailed investigation was performed.

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TABLE VII. FATTY ACID COMPOSITION OF ETIOLATED CORN LEAF LIPIDS

Lipid components	Fatty acids (wt. %)							
	12:0	14:0	16:0	16:1*	18:0**	18:1	18:2	18:3
Total	0.1	—	18.8	0.5	1.2	3.3	38.5	37.6
Triglyceride	0.5	0.7	8.8	1.2	0.2	1.8	49.7	37.1
Diglyceride	0.9	1.2	27.1	—	—	5.2	54.2	11.4
Free fatty acid***	2.9	0.7	12.2	0.9	0.7	0.7	5.3	76.1
Monogalactosyl diglyceride	—	—	5.3	—	0.1	1.2	11.0	82.4
Digalactosyl diglyceride	—	—	14.4	—	0.1	0.3	19.0	66.2
Sulfoquinovosyl diglyceride	—	—	27.7	—	0.6	0.4	6.1	65.2
Phosphatidyl glycerol	—	—	28.2	—	0.7	2.0	50.5	18.6
Phosphatidyl ethanolamine	—	—	24.2	—	0.1	1.0	63.0	11.7
Lecithin	—	0.3	22.0	—	1.2	3.1	54.1	19.3

* No *trans* acid was detected.

** No investigation was performed on the presence of hexadecatrienoic acid.

*** The fraction contained 0.5% of 15:0 acid except the acids described above.

less content of the acid, replaced by linoleic acid. The sulfolipid of green leaf gave equal amounts of saturated and unsaturated acids, and the lipid of the etiolated decreased in saturated acid content. Analyses by infrared absorption spectra, gas chromatography and silica-gel thin-layer containing silver nitrate revealed that phosphatidyl glycerol of the green leaf had a high proportion of *trans*-hexadecenoic acid which was absent in other lipid classes. This *trans* acid, however, could not be found in the phosphatidyl glycerol of the etiolated leaf, coinciding with the results of Nichols *et al.*¹⁷⁾ Still, it is of interest that phospholipids had much linoleic acid and glycolipids possessed much linolenic acid in both cases of green and etiolated leaves.

DISCUSSION

As shown above, growth condition of higher plants profoundly affects the relative amounts of the various lipid classes and their fatty acid composition, as well as the pigment composition in leaves. In light-grown corn leaf, polar lipids accounted for about 80% of total lipids, while those of the etiolated were about 60%. Prominent polar lipids of the green leaf (common to corn and soybean) were monogalactosyl

diglyceride, digalactosyl diglyceride, sulfoquinovosyl diglyceride, phosphatidyl phosphatidyl glycerol and diphosphatidyl glycerol. These results are nearly in accordance with those of Wintermans¹⁸⁾ on spinach between green and yellow leaves. In conjunction with lipid localization in chloroplasts,^{1,18,19)} it seems that the above-mentioned lipids with light-induced increase are specifically connected with the photosynthetic apparatus. All these lipids components, however, are similarly present in the etiolated leaves in significant amounts. Thus, it is conceivable that the lipids can display their functions in photosynthetic tissues only when the lipids are made up of definite fatty acid components as shown in Table VI. On the other hand, lecithin, phosphatidyl ethanolamine, sterol glucoside and cerebroside appear to be the lipids required to nonphotosynthesizing tissues, as do sterol, triglyceride and squalene.

Recently, Rosenberg *et al.*³⁾ reported that wax esters containing saturated shorter chain acids were present in the etiolated cells of *Euglena* and that they decreased rapidly during

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illumination of the etiolated cells. In corn leaf, however, a major part of lower saturated acids (C_{12} and C_{14}) was distributed rather to diglyceride, and their concentrations increased in green leaf, differing from the finding on *Euglena* by Rosenberg *et al.* The distribution of lauric and myristic acids among lipid classes will rouse our interest, since either of them may very well be the precursor of oleic, linoleic and linolenic acids in chloroplasts.²⁰⁾

The major surfactant lipids of the green corn leaves showed a remarkable specificity in their component fatty acid pattern; the two galactolipids had much higher linolenic acid (probably α -linolenic acid⁶⁾), the sulfolipid contained equal amounts of saturated and unsaturated acids, and the phosphatidyl glycerol had *trans*-hexadecenoic acid to give equal saturated and unsaturated acids when the *trans* acid was estimated as saturated acid. These patterns resemble those of spinach leaf lipids.²¹⁾ On the other hand, the lipids of the etiolated leaf indicated the specificity only at lower rate. In consideration of a hydrophobic association theory for chloroplast membranes proposed by Benson,²²⁾ it appears likely

that the specific component fatty acids such as α -linolenic and *trans*-hexadecenoic acids in the surfactant lipids closely associate with spacial alignment of structural protein, pigments and enzymes during development of chloroplasts.

It has been shown that, during biosynthesis of carotenoids, oxygen of hydroxyl groups of xanthophylls is derived from atmospheric oxygen, while that of epoxide groups is derived from water.^{23,24)} Moreover, it is postulated that xanthophylls containing epoxy and hydroxyl groups are involved in transport of oxygen originated from water during photosynthesis.²⁵⁾ If the hypothesis is true in corn leaf, it is reasonable that xanthophylls such as neoxanthin, violaxanthin, zeaxanthin and lutein showed less concentrations in the etiolated leaf, although β -carotene was present in equal proportion between green and etiolated leaves.

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