PLANT AND CHLOROPLAST LIPIDS
I. SEPARATION AND COMPOSITION OF MAJOR SPINACH LIPIDS*

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The major glycerol lipids of photosynthetic tissue are monogalactosyl diglyceride, digalactosyl diglyceride, phosphatidyl glycerol, phosphatidyl choline, (6-sulfoquinovosyl)-1-diglyceride, phosphatidyl inositol, and phosphatidyl ethanolamine (review by Lichtenthaler and Park 1963). These predominate in the total lipids of spinach, Chlorella, Scenedesmus, and other green plants and algae (Benson, et al. 1959; Ferrari and Benson 1961; Kates 1960; Nichols 1963; Wintermans 1960; Zill and Harmon 1962). Several minor lipids, some uncharacterized, are also present.

Separation of such mixtures on silicic acid columns (Zill and Harmon 1962) gave partial resolution of the major lipids, but contamination with chlorophylls was a major problem. Combination with other techniques (solvent fractionation, ion exchange) improves the separation of certain lipids (Wheeldon 1960; Haverkate and van Deenen 1963; Sastry and Kates 1963). Better separation has been achieved on silicic acid impregnated paper (Kates 1960) and on silicic acid thin layers (Nichols 1963), but these techniques impose severe limitations on quantities, and oxidation becomes a serious problem. The sulfolipid and galactosyl lipids have been isolated by O'Brien and Benson (in press) on diethylaminoethyl (DEAE) cellulose and Florisil.

We wish to report the separation of each of these lipids from spinach on a DEAE cellulose column. Prior countercurrent distribution is a convenient way to remove chlorophyll and effect a preliminary separation

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of lipids by hydrophilic character. Minor lipids are eluted from DEAE cellulose with certain of the major lipids, but these can be separated on silicic acid columns.

As was found to be the case with lipids of other plants, (Sastry and Kates 1963; O'Brien and Benson in press), fatty acid compositions of the glycolipids are unique. Surprisingly, the hexadecenoic and hexadecatrienoic acids reported by Debuch (1962) in spinach and by Klenk and Knipprath (1961 and 1959) in Scenedesmus obliquus are concentrated, respectively, in phosphatidyl glycerol and monogalactosyl diglyceride.

MATERIALS AND METHODS

Plant Lipids. Healthy unbroken leaves of fresh spinach plants were trimmed, washed, and extracted under nitrogen in a blendor with C/M mixtures (successively 2/1, 4/1, pure chloroform)[1] until the residue of insoluble material was colorless. Solvent was removed under vacuum from the pooled fractions in a rotary evaporator at 35°C or below. Lipid residue was redissolved in C/M 2/1, and the small amount of insoluble material washed free of lipid and discarded. One kilogram of damp leaves yielded 7.8 g of the lipid-pigment extract and 55 g of air-dried insoluble solids.

Countercurrent Distribution was carried out in a Post 60-tube glass apparatus (10 ml in each phase) with carbon tetrachloride/methanol/water 62/35/4 (Therriault, et al. 1958). About one gram of lipid mixture was introduced into each of the first one to four tubes and carried through 120 distributions. The lipid content of each fraction was monitored by chromatography on silicic acid plates. Lipid spots were identified by fluorescence under ultraviolet light after spraying the plates with 0.003% Rhodamine 6G in 1N sodium hydroxide, and were photographed on Versapan through an orange filter and developed in D-19. This technique (Allen 1962) is sensitive to a tenth microgram of lipid or less.

Diethyaminoethyl Cellulose columns were prepared from 15 g of Selectacel DEAE-20 and packed into a 2.5 cm I.D. column in acetic acid by

[1] Chloroform/methanol is abbreviated C/M. All liquid compositions are v/v. All operations involving lipids were carried out in a nitrogen atmosphere with deoxygenated solvents.

the technique of Rouser (1963). Acetic acid was completely washed from the column with methanol, which was replaced with the solvent with which elution was to begin. Column effluent was collected in 15 ml fractions, which were monitored on silicic acid plates. Appropriate fractions were combined and rotary-evaporated.

Silicic acid columns were Slurry-packed in chloroform into a 2.5 cm I.D. tube using 30 g of silica gel (Merck, Darmstadt; 0.05 to 0.20 mm) which had been deoxygenated with a nitrogen stream in boiling chloroform. Again 15 ml fractions were collected and monitored by thin layer chromatography.

Infrared Spectra were run in potassium bromide micropellets.

Lipid Analysis. Lipids were checked for homogeneity by thin layer chromatography on silicic acid using C/M/water 65/25/4 and diisobutyl ketone/acetic acid/water 40/25/3.7 (Nichols 1963). Visualization was achieved by the Rhodamine 6G-sodium hydroxide spray, or with a light spray of 80% sulfuric acid saturated with sodium dichromate and heating to 180°C (Privett and Blank 1962). The latter spray served as a check for non-lipid organic contamination.

Lipids were identified by paper chromatographic analysis of their products of deacylation and acid hydrolysis and by comparison with authentic samples of beef brain lecithin, phosphatidyl ethanolamine, and phosphatidyl inositol by infrared spectroscopy and R_f values. 6-Sulfoquinovose was compared with a sample graciously supplied by A. A. Benson. Phosphorous and sulfur content was determined by X-ray fluorescence spectroscopy (Allen, unpublished method).

Fatty Acid Analysis. Lipids were transesterified with 5% methanolic sulfuric acid at 70° overnight, the reaction mixture diluted with water, and the methyl esters extracted with hexane (Feldman, et al. 1962). The concentrated hexane extracts were chromatographed on a 5 ft. X 1/8 in. column of Reoplex-400 (15% on chromosorb W) at about 190°C. A similar column of diethylene glycol succinate polymer at 175°C was used as a check for possible peak overlap, but not for quantitative analysis since methyl oleate and methyl hexadecatrienoate emerge in one peak from this column. Quantitative results were checked with National Heart Institute methyl ester standards C and D and agreed with the stated composition data with a relative error of less than 5% in the 14 to 18 carbon acid range.

DISCUSSION

Countercurrent distribution of the spinach lipid extract separated the mixture into three more easily manageable fractions containing respectively: (1) chlorophyll and several relatively non-polar lipids, (2) more polar electrically neutral lipids (mono- and digalactosyl diglycerides, phosphatidyl ethanolamine, lecithin, and several minor lipids) and (3) highly polar and acidic lipids (digalactosyl diglyceride, phosphatidyl glycerol, 6-quinovosyl diglyceride, phosphatidyl inositol, and several minor lipids). At least seventeen lipids are apparent in these fractions exclusive of pigments and materials moving near the solvent front in C/M 9/1 on silicic acid plates. Orange pigments are present in varying concentrations in nearly all tubes. The distribution is summarized in Table I and Figure I.

The major neutral and zwitterionic lipids (pooled tubes 30-102), already freed of chlorophylls, are easily separated on a DEAE cellulose column. Elution is begun with 500 ml of C/M 19/1 which removes in sequence pigments, monogalactosyl diglyceride, and lecithin plus a minor lipid. Continuation with C/M 9/1 (400 ml) removes digalactosyl diglyceride partially overlapping a minor lipid, and C/M 2/1 (400 ml) elutes phosphatidyl ethanolamine. Some pigment is eluted with the first monogalactosyl diglyceride but can be removed on a silicic acid column by elution with C/M 9/1. The lecithin and digalactosyl diglyceride can be separated from their minor lipids on a similar column with C/M 9/1 and 4/1.

The major acidic lipids (pooled tubes 103-120) can also be separated on DEAE cellulose. Neutral lipids (mainly digalactosyl diglyceride and non-lipid material) are flushed from the column with methanol, and elution of the acidic lipids begun with a linear gradient of C/M 2/1 (300 ml) vs. 300 ml C/M 2/1 containing 1.5 g ammonium acetate and finished with the latter solution alone. Phosphatidyl glycerol and two minor lipids appear in the eluate after about 400 ml, followed by sulfolipid, and finally phosphatidyl inositol. Most of the phosphatidyl glycerol can be freed of the minor lipids by use of a shallower gradient such as C/M 9/1 vs. C/M 9/1 + 2 g/l ammonium acetate, or similar chloroform/acetic acid/ammonium acetate mixtures.

Since column loading of a quarter to half a gram is reasonable, appreciable amounts of these lipids can be isolated in a single run on columns of this size. The above techniques will probably be equally ef-

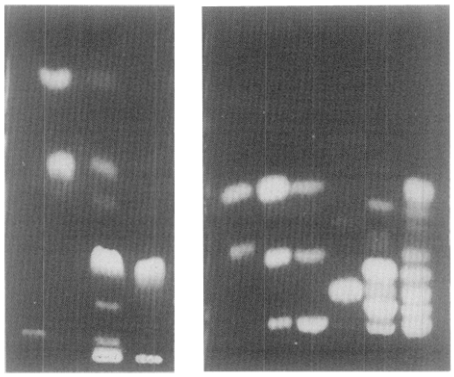
fective with lipids of most photosynthetic plant tissue. Specifically, they have proven so with lipids of Chlorella pyrenoidosa, Scenedesmus obliquus, and Euglena gracilis.

TABLE I

Distribution of Lipids

Lipid	Identity	Tubes (approx.)
	Carotene	0-6
	Chlorophyll	2-27
1		1-18
2		12-26
3		16-32
4		12-36
5	Monogalactosyl diglyceride	34-84
6	Phosphatidyl ethanolamine	30-90
7	Lecithin	40-100
8		50-70
9	Digalactosyl diglyceride	76-110
10		74-98
11		82-102
12	Phosphatidyl inositol	103-115
13	Sulfolipid	104-116
14	Phosphatidyl glycerol	103-116
15		103-108
16		102-110
17		104-110

FIGURE I



A B T C C/M 9/1

C D E F G T Ketone/HAc/H_2O 40/25/4

Thin layer chromatograms of lipids from countercurrent distribution T = total spinach lipid. Lipids listed by number from bottom upward.

A (Tube 10) : 1
B (Tube 22) : 2,3,4
C (Tube 40) : 7,6,5
D (Tube 54) : 7,6,8,5
E (Tube 68) : 7,6,8,5
F (Tube 94) : 7,9,10,11 (faint; next to spot 5)
G (Tube 108) : 12,13,9,14,15,16,17

If the countercurrent distribution step is omitted, most of the chlorophyll can be rapidly eluted from DEAE cellulose with chloroform, but traces of green material appear in several of the neutral lipid fractions, and the acidic lipid fractions contain a more complex array of minor lipids. Most lipids can, nevertheless, be isolated free of contaminants by further silicic acid chromatography.

The fatty acid composition of the major lipids is summarized below. The composition of the total lipid extract is quite similar to the analysis reported by Debuch (1962).

Fatty Acid Content of Spinach Lipids (to nearest percent)*

	Myristic	Palmatic	trans-3-Hexadecenoic	Hexadecatrienoic	Stearic	Oleic	Linoleic	Linolenic
Monogalactosyl diglyceride		Tr	Tr	30		1	1	67
Digalactosyl diglyceride		6		3	1	4	3	84
6-Sulfoquinovosyl diglyceride		27				6	39	28
Phosphatidyl glycerol		22	35		Tr	2	5	36
Lecithin		20	Tr	Tr		11	30	40
Phosphatidyl inositol	Tr	41			1	6	25	27
Phosphatidyl ethanolamine	Tr	46		2	1	2	7	43
Total lipid extract	Tr	12	3	10	Tr	4	13	57

*(Other acids present in trace amounts -- less than 1% -- are omitted.)

The digalactosyl diglyceride contains linolenic acid predominantly, as it does in runner bean leaves (Sastry and Kates 1963), and alfalfa (O'Brien and Benson, in press). The monogalactosyl diglyceride has much the same composition as the digalactosyl lipid in these plants. In spinach, however, it is rich in hexadecatrienoic acid as well as linolenic acid. In fact, nearly all of this C_{16} acid is concentrated in this one lipid. If monogalactosyl diglyceride is the metabolic precursor of the digalactosyl lipid as Ferrari and Benson (1961) propose, there must be a rapid and selective metabolism of the sixteen-carbon acid.

Concentration of trans-3-hexadecenoic acid in phosphatidyl glycerol presents an intriguing metabolic problem. The shape of this acid is much the same as palmitic acid. This suggests that the unsaturated acid plays primarily a metabolic rather than a structural role.

The fatty acid composition of the other lipids shows considerable variation, perhaps as a consequence of their wider distribution (except the sulfolipid) in plant tissues. The phosphatidyl glycerol (ammonium salt) had $[\alpha]_D^{25^\circ} = +1.0 \pm 0.5^\circ$ (95% confidence limits) (C = 5 mg/ml in $CHCl_3$). This activity, coupled with the observations that the glycerol residues have opposite configurations (Benson and Miyano 1961) and that the diglyceride unit has an L-α-configuration (Haverkate, et al. 1962), is in harmony with a (diacyl-L-α-glycerophosphoryl)-D-α-glycerol structure.

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