Methodology for the Separation o Plant Lipids and Application to Spinach Leaf and Chloroplast Lamellae[1]

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Abstract

Procedures for separation of complex plant lipids and results obtained are reviewed.

Procedures based on DEAE cellulose and silicic acid chromatography, which may be preceded by countercurrent distribution, are presented for separation of the individual glycerol- and sphingolipid classes of spinach leaf and chloroplast lamellae. These procedures appear to be generally applicable to photosynthetic tissue of plants and algae.

The separation and infrared spectra of mono- and digalactosyl diglycerides, lecithin, phosphatidyl ethanolamine, phosphatidyl glycerol, phosphatidyl inositol, plant sulfolipid, cerebroside, and sterol glycosides from spinach are recorded. Chloroplast lamellae lipids are in the molar ratio monogalactosyl diglyceride (14.0), digalactosyl diglyceride (8.0), phosphatidyl glycerol (5.5), sulfolipid (3.9), lecithin (2.0), phosphatidyl inositol (1.0). Phosphatidyl ethanolamine, cerebrosides, and sterol glycosides were not detected in chloroplast lamellae. Fatty acid composition of individual lamellae lipids have been determined: The galactosyl lipids contain more than 90% trienoic acids. *Trans*-3- hexadecenoic acid is restricted almost exclusively to phosphatidyl glycerol.

In this report techniques which have been applied to the isolation of plant glycero- and sphingolipids are reviewed and a new scheme presented for the separation of several of the plant lipid classes. Results obtained with spinach leaf and its photosynthetic apparatus are presented and discussed.

I. Review of the Literature

Lipid Composition of Photosynthetic Tissue

Spinach chloroplasts contain a lamellar structure wherein the light reactions and electron transport operations leading to oxygen evolution in photosynthesis take place. Park and co-workers (1,2) have calculated and tabulated a representative quantitive analysis of spinach lamellae based on their work with lyophilized lamellae preparations and the work of others with chloroplast preparations. The lamellae contain nearly equal amounts of protein and lipid (including pigments, quinones, and tocopherols). Eighteen per cent of the lipid was classified as unidentified, but this must be regarded as a rough approximation based on partial analyses of chloroplasts isolated from different material in several laboratories. Glycerolipid compositions were based on the work of Wintermans (3,4) who separated deacylated lipids from beet and spinach chloroplasts and leaves, bean seedlings, and elder leaves (green and *aurea* forms) by two-dimensional paper chromatography and calculated the lipid composition of the plant tissue from phosphorous and sugar determinations of eluted spots. Phosphorus recovery was 60–90% of that applied, and sugar determinations were complicated by the possible contamination of eluted spots with cellulose fiber (4). Based on these analyses of spinach chloroplast lipids the relative concentration in micromoles per gram of packed tissue were: monogalactosyl diglyceride (18), digalactosyl diglyceride (7.5), phospholipid (4.5), sulfolipid (2.25). Relative molar concentration of phospholipids were reported to be phosphatidyl choline (39%), phosphatidyl inositol (13%), phosphatidyl glycerol (46%), phosphatidyl ethanolamine (2%). Phosphatidic acid was found in trace amounts, but is considered to be an artifact formed by enzymatic degradation of phospholipids during isolation of the chloroplasts. Small amounts of other phospholipids may be present. Comparison of these analyses with whole leaf lipid analyses indicated that more phosphatidyl ethanolamine and lecithin are outside the chloroplast than within, and that galactolipids may be confined to the chloroplasts but the sulfolipid probably is not.

Benson and co-workers (5) combined paper chromatography and quantitative radiochemistry of neutron-activated phospholipids to make similar analyses of *Scenedesmus, Rhodospirillum rubum* chromatophores, New Zealand spinach *(Tetragonia expansa)* leaf and chloroplasts, tobacco leaf, sweet clover, barley leaf and tomato leaf. In *Chlorella* glycolipids were also determined (6). Since the photosynthetic tissues investigated in Benson's and Wintermans' laboratories differed, direct comparison of the two analytical techniques is precluded. The accuracy of neither technique is known, but the phospholipid compositions reported are qualitatively similar. Phosphatidyl choline, glycerol, inositol and ethanolamine were the major phospholipids in all cases. Phosphatidyl serine was present in *Scenedesmus* (1%), and sweet clover (6.3%) only. Ferrari and Benson (6) have calculated the molar concentration of lipids in *Chlorella* grown on $C^{14}O_2$ from a consideration of the C^{14} in deacylated lipids, and neutron activation chromatography of the phospholipids: phosphatidyl glycerol (8.6×10^{-3}M), phosphatidyl inositol (2.0×10^{-3}M), phosphatidyl choline (3.9×10^{-3}M), phosphatidyl ethanolamine (1.7×10^{-3}M), galactosyl diglyceride (25×10^{-3}M), digalactosyl diglyceride (10×10^{-3}M), and sulfolipid (2.5×10^{-3}M). High concentrations of the galactosyl lipids, the sulfolipid and a mixture of phospholipids appear to be characteristic of photosynthetic tissues of plants and algae.

Quantitative lipid composition varies with illumination and nutrition: large variations in glycolipid concentration (but less in phospholipid concentration) occur in bean seedlings (3) and *Euglena* (7) as the period of illumination is varied. Diphosphatidyl glycerol concentration in *Chlorella* increased markedly in a phosphate deficient growth medium (8).

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Kates and co-workers (9–13) have determined the lipid components of runner bean leaves. In addition to the major lipids mentioned above, cerebrosides and sterol glycosides were identified as minor components and other unidentified minor lipids are present. Zill and Harmon (14) used silicic acid columns to fractionate spinach leaf and chloroplast lipids. Unidentified glycosides were detected through their hydrolysis products (galactose, arabinose, possible glucose). A proteolipid easily decomposed by evaporation of its solvent has also been isolated from spinach chloroplasts (15). Thin-layer chromatography (TLC) (16,17), silicic acid impregnated paper (6,18) and aminoethyl cellulose ion exchange paper (19) have also been used for qualitative separation of lipids of green plant tissue. Not all the minor lipids detected by these techniques have been identified. The possible significance of lipids in plant membranes has been reviewed by Benson (20).

Procedures for Separation of Plant Lipid Mixtures

Solvent fractionation alone is generally inadequate for the isolation of pure components of naturally occurring lipid mixtures, but can be of tremendous help in preliminary separations of large quantities of material. Among the more effective applications to complex plant lipid mixtures are separations of seed phytoglycolipid (a mixture) (21,22) with a deacylation step to separate sphingolipids from glycerolipids, and procedures for preparation of phosphatidyl inositol and its salts from peas (23) or wheat germ (24), utilizing the strongly acidic character of phosphatidyl inositol. The preparations appear to be better than 95% pure. More typically, impure lipids are obtained [e.g., phosphatidyl ethanolamine containing about 15% impurities was isolated from soybean (25)]. Solvent fractionation is generally most effective when applied to simple mixtures already partially separated by other techniques.

Limitations of the solvent fractionation technique are well known. The fatty acid compositions of individual lipid classes in the plant may be markedly different from that of the isolated material if recovery is not quantitative. Furthermore, the composition of the lipid mixture may have a profound effect on the solubility of its components as a result of lipid-lipid interaction.

Countercurrent Distribution. The application of countercurrent distribution to fractionation of lipid mixtures has been discussed by Scholfield (21) and Therriault (34). Application to polar plant lipid mixtures has been primarily with seed lipids as indicated below. Varying degrees of fractionation of these complex mixtures were accomplished, but individual lipid classes have rarely been separated. The distribution of lipids was analyzed by gross elemental or hydrolysis product analysis (inositol, choline, sugar, ethanolamine, etc.). Such methods generally do not permit identification of the individual lipid components and are thus inadequate with such complex mixtures.

Phospholipids of linseed (26), soybean (28,30), corn (32), and peas (33) can be separated into choline-rich ethanol soluble and inositol-rich ethanol insoluble fractions. Countercurrent distribution of the choline-rich material in a hexane-90% methanol system gave little further separation, but the inositol-rich fraction could be resolved into two inositol-containing peaks by a relatively small number of transfers in a hexane-90% ethanol system. The system carbon tetrachloride-methanol-water (62/35/3) resolved soybean phospholipids into four major peaks (27), each containing a mixture of lipid classes. A similar system with chloroform and methylene chloride as additional components was used to fractionate soybean lipids extractable with a hexane-ethanol mixture but not with hexane (29). Again several peaks of mixed lipids were obtained. A heptane-butanol-water-methanol system which does not readily form emulsions was introduced from Carter's laboratory, and applied to a corn inositol lipid fraction prepared by solvent fractionation of commercial corn phosphatides (22). A 200 tube apparatus was used for a 400 transfer separation; lipid was distributed into a peak composed primarily of phosphatidyl inositol, three partially resolved peaks rich in phytosphingolipids, and a peak traveling rapidly with the heptane phase which was rich in lipophytin. An 800 transfer separation (31) of lipids from two varieties of wheat in the above system again separated the material into a series of peaks containing mixtures, but nearly pure digalactosyl diglyceride formed a major peak.

The main utility of countercurrent distribution of polar lipids appears to be for preliminary separation into simpler mixtures rather than isolation of individual lipid classes free of contaminants.

Column Chromatography. Silicic acid has proved to be a more useful adsorbent than aluminum oxide. The Hirsch and Ahrens (35) elution scheme for blood lipids using mixtures of petroleum ether, diethyl ether, and methanol was applied for separation of total spinach leaf and chloroplast lipids on a silicic acid column (14) but was of limited value. Relatively nonpolar lipids (hydrocarbons, waxes, β-carotene, n-hexacosanol) are separated, but the more polar glyco- and phospholipids are eluted as complex mixtures. Chlorophyll contamination of polar fractions was a serious unsolved problem. Kates and co-workers used repeated silicic acid chromatography, ion exchange chromatography on Amberlite MB-3 (to remove phospholipids), aluminum oxide columns, and solvent precipitation to separate runner bean leaf lipids including lecithin and the two galactolipids (11) and cerebrosides (12). Pure lipids were obtained, but recovery was far from quantitative. In Carter's laboratory silicic acid columns were used (36) as a step in the purification of wheat flour cerebrosides and galactolipids obtained by solvent fractionation. Pure monogalactosyl diglyceride was obtained directly, but digalactosyl diglyceride and cerebroside required further purification by other means. Haverkate and van Deenen (37) obtained uncontaminated phosphatidyl glycerol (from spinach leaves) by silicic acid chromatography of material previously enriched as the magnesium salt by acetone precipitation.

Ion exchange resin columns can be used to isolate individual acidic deacylation products, as illustrated (38) by the clean separation of glycerophosphoryl inositol, glycerophosphoryl glycerol, glycero-6-sulfoquinovose, and glycerophosphate on Dowex-2 columns (acetate form). For intact lipids diethylaminoethyl cellulose columns which combine ion exchange and partitioning qualities are effective for separation of polar lipids as demonstrated by Rouser and co-workers (39,58) primarily with lipids derived from the animal kingdom. Application to spinach leaf lipid extracts (40) permitted isolation of the major lipids as reported here. The formation of polyvalent metal salts of some lipids such as phosphatidyl inositols

may alter the relative order of elution from DEAE cellulose columns (42).

Magnesium silicate columns will retain phospholipids, while allowing sulfatides to pass (39,43). In an interesting application of the multicolumn techniques devised for animal brain cerebrosides and sulfatides (39), alfalfa and *Chlorella* lipid extracts were freed of phospholipids with a Florisil (magnesium silicate) column, sulfolipid was retained on a diethylaminoethyl cellulose column (and later recovered), and the galactolipids which passed through both columns were separated and freed of pigment on a silicic acid column (41). Most of the chloroplast pigment was retained on the Florisil.

TLC on silicic acid, like paper chromatography, is generally a better tool for analysis than for isolation of more than a few milligrams of lipid. However, the high resolution possible with the technique makes it very useful for isolation of small amounts of mixtures which would be difficult to isolate from columns. Useful developing mixtures for polar lipids are chloroform/methanol/water 65/25/4 and diisobutyl ketone/acetic acid/water about 8/5/1. Neither mixture will resolve all components of common polar plant lipid mixtures, but application of both systems improves resolution. Lepage applied these solvents for two-dimensional TLC separation of alfalfa leaves, potato leaves and tubers, and *Chlorella* (17). Most of the polar lipid classes were cleanly separated. Not all of the lipids were identified. The same technique has been applied with similar results to wheat endosperm lipids (44). Lipids of lettuce leaf and chloroplasts, and of cabbage leaf, have been investigated by one-dimensional TLC using the same solvent systems (16). For some separations, it was convenient to make a preliminary separation of neutral and pigmented material on a silicic acid column. Alfalfa lipids have also been separated into classes by a one-dimensional technique (45).

Resolution by silicic acid impregnated paper chromatography is generally inferior to that obtained by TLC. However, application of two-dimensional development on a commercial paper resolved sugar beet leaf lipids into 23 spots corresponding to the expected plant lipids, but again including several unidentified materials (18). Complex solvent systems were used. Silicic acid paper chromatography with the diisobutyl ketone-acetic acid/water solvent system and detection of lipids by radioautography was used to separate and locate C^{14} and P^{32} labelled plant lipids (6). Appreciable tailing and some overlapping of spots was observed. R_f values were given for several common plant lipids. Double development of runner bean leaf lipids in one direction with the same solvent improved the separation of lipids with low R_f values not adequately resolved with a single development (10).

Paper chromatography of deacylated plant lipids has been applied more effectively. Resolution of lipid spots by paper chromatography is generally unsatisfactory before deacylation. Deacylated lipids of photosynthetic tissue appear to be adequately separated for quantitative analysis by two-dimensional development in phenol/water, and propionic acid/butanol/water (3,5,46,47). These products can be detected in the microgram range by radioautography if radioisotopes of phosphorus, carbon, or sulfur have been used as labels, or by neutron activation of phosphorus (5). An alternative periodate-Schiff spray is not as sensitive. The intact phospholipids overlap more in this system (47) and in other solvent systems (48).

A formaldehyde treated paper (49) has been developed which gives good separation of phosphatidyl serine and mono- and diphosphoinositides, although phosphatidyl choline and phosphatidyl ethanolamine are poorly separated. Common plant lipids (of wheat and algae) were resolved on an aminoethyl cellulose paper (19) using diisobutyl ketone/acetic acid/water, but phosphatidyl inositol and sulfolipid had very similar R_f values. Neither of these paper systems give the well-defined sharply separated lipid spots usually attained with silicic acid TLC.

II. New Observations

Separation of Lipids from Photosynthetic Tissue

Column chromatography on DEAE cellulose followed by silicic acid column chromatography as originally devised for animal lipids (39,57) provides the basis for a separation scheme applied to spinach leaf and chloroplast lamellae lipids. It should be generally useful with green plant tissue as judged by its successful application (unpublished) to lipids of *Chlorella, Euglena,* and *Anacystis nidulans.* Countercurrent distribution prior to column chromatography possesses some advantages.

Experimental

In all operations lipid contact with oxygen was minimized by use of nitrogen or carbon dioxide atmosphere and solvents were deoxygenated with nitrogen. Solvent compositions are based on volume (v/v). All organic solvents were distilled before use.

Spinach Leaf Lipids. An extraction technique based on the method of Bligh and Dyer (50) was used in preference to chloroform/methanol 2/1 extraction used previously (40). Five hundred grams of fresh spinach leaves was blended in batches under carbon dioxide with 3 liters of ice-cold chloroform/methanol 1/2 for about one minute. The homogenate, which contained only one liquid phase, could be filtered rapidly through a large sintered glass funnel, again under carbon dioxide. An additional 300 ml of chloroform/methanol 1/2 was allowed to filter through the undisturbed filter cake, and this was followed by 1100 ml of chloroform. The insoluble residue was completely free of pigments at this point. Water (1100 ml) was stirred into the combined extracts, the chloroform layer was separated, and the methanol/water phase was reextracted with about 500 ml of chloroform. To check for lipid remaining in the aqueous phase, 10 ml was evaporated to dryness and the residue thoroughly extracted with chloroform/methanol. All of the material extracted was applied as one spot for TLC. No lipids were found, although a trace of material remained at the origin.

The combined chloroform extracts were distilled under vacuum in a rotary evaporator (below 35C). The residue was taken up in chloroform. A small amount of insoluble material was removed by filtration and washed with chloroform and chloroform/methanol 2/1. The combined chloroform/methanol and chloroform extracts were again evaporated: 4.4 g of lipid mixture was obtained, which was completely soluble in hexane.

Lamellae Lipids. Chloroplast lamellae from summer spinach *(Spinacea oleracea)* grown in Oxnard, California, were isolated by the procedure of Park and Pon (51). Lipids were extracted three times with chloroform/methanol 1/1 and the solution worked

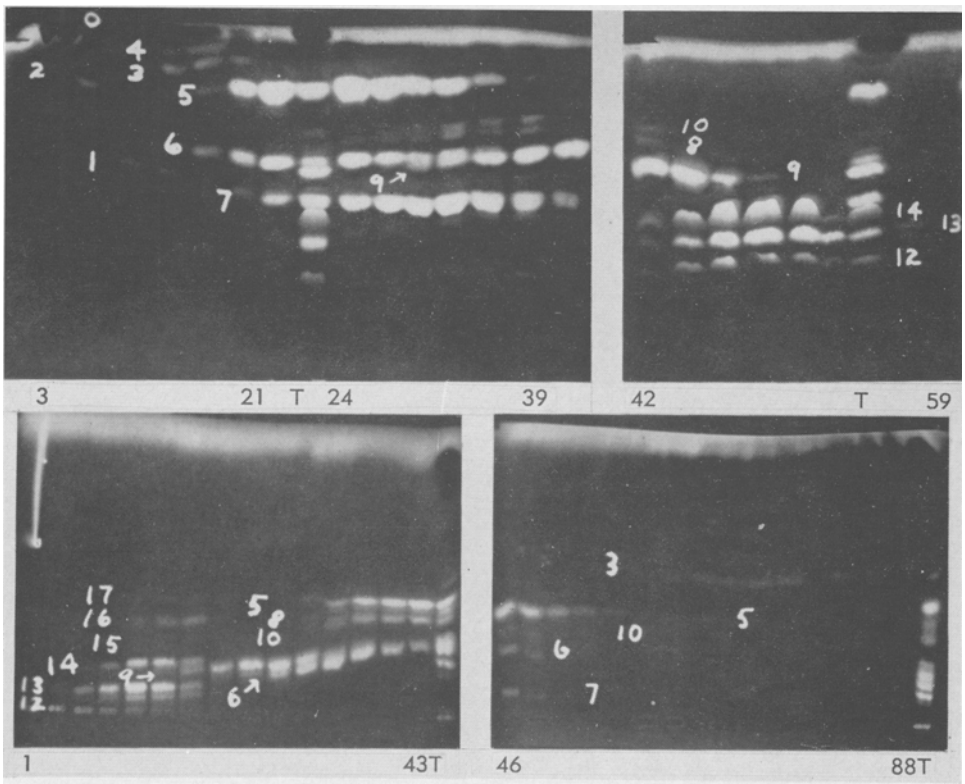

FIG. 1. Separation of spinach leaf lipids by countercurrent distribution in a 60 tube apparatus. Every third tube was spotted for TLC. Lipids were localized with the alkaline rhodamine 6G spray. (*a*) 60 transfers in the carbon tetrachloride/methanol/water system. TLC with chloroform/methanol/water 65/25/4. (*b*) 120 distributions in the butanol/hexane/methanol/water system. TLC with diisobutyl ketone/acetic acid/water 8/5/1. The tubes above 88 are not included since they contained little lipid. Lipids are identified in Table I using the numbering system of a previous communication (40)
T = total mixture.

up as described above for spinach leaf. The protein residue was free of lipid pigments. In one run lamellae isolated from 185 g of leaves yielded 88.2 mg of lipid and 93.6 mg of insoluble dry tan residue.

Diethylaminoethyl Cellulose (DEAE) Chromatography. The column preparation procedure utilized is essentially that of Rouser et al. (39,57). DEAE (Selectacel DEAE Type 20) was washed in sequence with water, 1 M HCl, water, 1 M NaOH and water (until neutral). Large batches were stored dry, and soaked in acetic acid overnight before use. Columns 2.5 cm (I.D.) were slurry packed with 15 g of DEAE under 10 lb of nitrogen pressure to a height of about 18 cm. The DEAE was added in about five portions and gently compressed with a tamper after each addition. Uniformity of packing was tested at this point with a few milligrams of azobenzene, which should move down the column in an even band. Typically at least 75 ml of colorless eluate could be collected before the azobenzene began to emerge and elution of the band was complete with less than 15 ml of additional solvent. Acetic acid was washed from the column with methanol (about three column volumes). About one-half column volume of methanol was left above the packing and mixed with an equal volume of chloroform. Half of this mixture was allowed to drain into the column whereupon an equal volume of chloroform was again mixed in. This process was repeated until the concentration of chloroform/methanol was close to the desired range. The 1–3 column volumes of the initial eluting solvent was passed through the column. At this point the azobenzene packing test was repeated. We have not been successful in completely removing the methanol with this procedure without impairing the performance of the column by channeling as indicated by the azobenzene test, although chloroform/methanol 49/1 has been used successfully. During chromatography a flow rate of about 3 ml/min was normally used and 15 ml fractions were collected. A 500 mg load gave satisfactory resolution of plant lipid extracts rich in chlorophyll, pigments and other rapidly eluted material. Lower loading (ca. 300 mg) was used for separation of mixtures relatively free of the pigments. The columns could generally be reused several times without repacking, but increasing nitrogen pressure (to about 5 lb) was sometimes necessary to maintain the indicated flow rates.

TABLE I

Spinach Leaf Lipids

Spot	Identity	Spot	Identity
0	Carotene and chlorophyll	9	Digalactosyl diglyceride (DG)
1	Unknown		
2	Unknown	10	Cerebroside (Cer)
3	Unknown	11	Unknown
4	Unknown	12	Phosphatidyl inositol (PI)
5	Monogalactosyl diglyceride (MG)	13	Sulfolipid (SL)
		14	Phosphatidyl glycerol (PG)
6	Phosphatidyl ethanolamine (PE)	15	Unknown
		16	Unknown
7	Lecithin (PC)	17	Unknown
8	Sterol glycoside (SG)		

This list of lipids refers to materials detected in a countercurrent distribution reported earlier (40). The numbering is retained here for convenience. The presence of the unidentified minor lipids is variable: some are not always found in the preparations of spinach leaf lipid, and other trace components are sometimes present. Differences of this sort have been found in plants purchased at different times of the year, and are probably not artifacts.

Silicic Acid Chromatography. Silicic acid (0.08 to 0.2 mm manufactured by Merck, Darmstadt) was deoxygenated and dehydrated by distilling chloroform from a stirred chloroform slurry. Nitrogen was bubbled through the mixture as it cooled; columns were slurry packed: 30–60 g of silicic acid in a 2.5

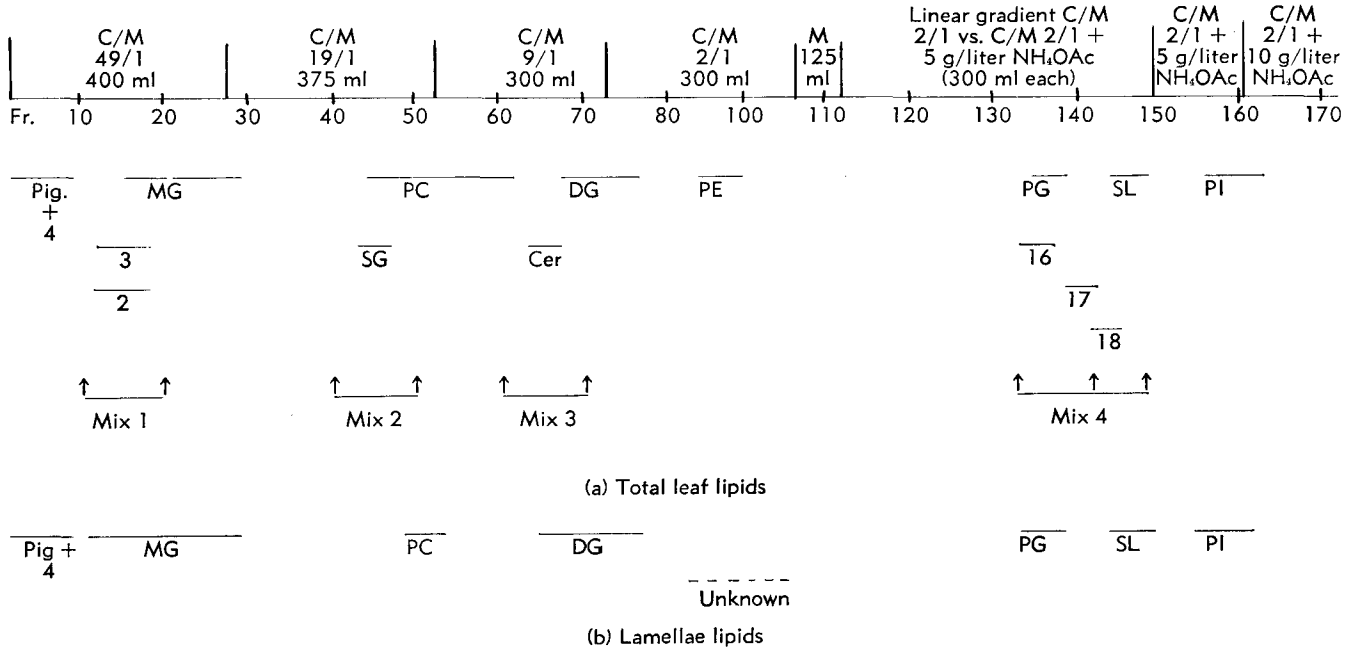

FIG. 2. (*a*) Elution scheme for the separation of 500 mg of total spinach leaf lipid on a 2.5 cm I.D. x 15 cm DEAE column containing 15 g of DEAE. Each division represents 150 ml of eluate (ten 15 ml fractions). Lipids are abbreviated as in Table I. Mixtures were further separated on silicic acid column as indicated in Figure 3. Lipid 18 is a component not listed in Table I. It moves just under PI in the diisobutyl ketone system, and with PI in the C/M/W TLC system. The relative concentration and the presence of minor lipids varies with the age and time of harvest of the spinach as well as with the tissue examined.

(*b*) Similar elution scheme with 210 mg of lamellae lipids. The mixture is much simpler than total leaf lipid mixtures. The "Unknown" lipid (4 mg in Table II) is free of P and S. In the diisobutyl ketone system it moves below PI, and near SL in the C/M/W system.

cm I.D. column permitted a very rapid flow rate, but the eluate was usually collected at about 3 ml/min in 15 ml fractions.

Countercurrent distribution was carried out in a Craig-Post 60-tube glass apparatus (each phase 10 ml). Two-phase systems prepared from carbon tetrachloride/methanol/water 62/35/4 (52), or water saturated n-butanol/95% aqueous methanol/hexane 2/3/1 (53) were used. With the carbon tetrachloride system up to 1 g of lipid mixture was introduced into each of the first four tubes. With the hexane system 0.5 g of lipid per tube was used. When more than 60 distributions were carried out, the upper phase was collected in individual vials as it emerged from the apparatus.

Liquid-Liquid Partition Columns. Acid-washed firebrick (80–100 mesh; Matheson, Coleman and Bell) was used as a stationary phase. Before use, it was washed with water until the washings were neutral, and dried at 110C. The dried firebrick (300 ml) thoroughly mixed with 85 ml of the upper (aqueous) phase of the carbon tetrachloride/methanol/water 62/35/4 system in a closed container formed a damp, free-flowing powder. This was packed into a 2 cm I.D. column, and gentle tapping was used to settle the material (packed length 83 cm). A 1 cm layer of sand was placed on top of the packing. Lower phase was introduced and slowly run through the column under pressure to remove gas bubbles (54). Spinach lipid could be applied in the lower phase at a concentration of 5% or less. No bleeding of the upper phase from the stationary support was observed when 250 ml of lipid mixture was applied. With the smaller lipid samples separations comparable with the countercurent distribution system were obtained. Chlorophyll and other nonpolar materials were eluted with the lower phase, and polar lipids retained on the column were washed out with additional lower phase.

Thin-Layer Chromatography. Silicic acid thin-layer plates were prepared with Silica Gel G (Merck, Darmstadt). Lipid solutions were applied in bars approximately 1 cm long from open melting point capillaries (1 mm I.D.). Plates were commonly developed in diisobutyl ketone/acetic acid/water 40/25/4 or chlo-

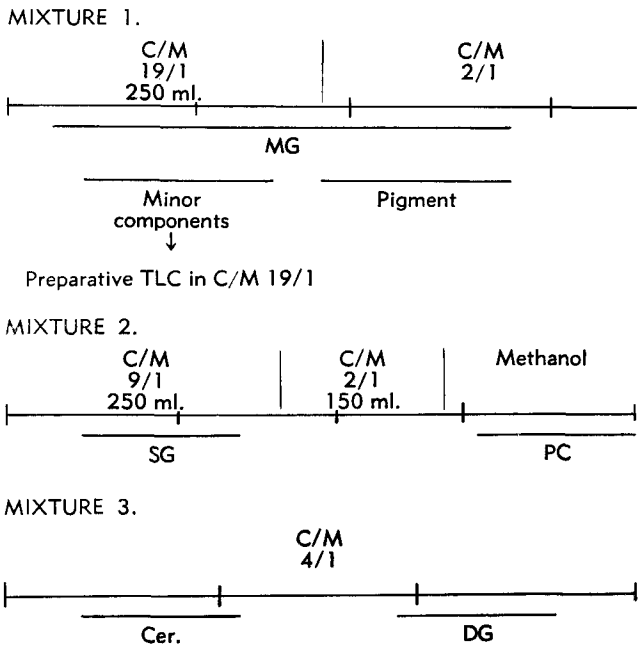

FIG. 3. Further separation of lipid fractions from DEAE columns (see Fig. 2) by silicic acid column chromatography (see text). Each division represents 150 ml of eluate. Silicic acid columns are not adequate for complete separation of the minor lipids associated with phosphatidyl glycerol (Mixture 4) but preparative TLC yields chromatographically homogeneous samples. The quantity of these minor lipids in spinach leaf is variable.

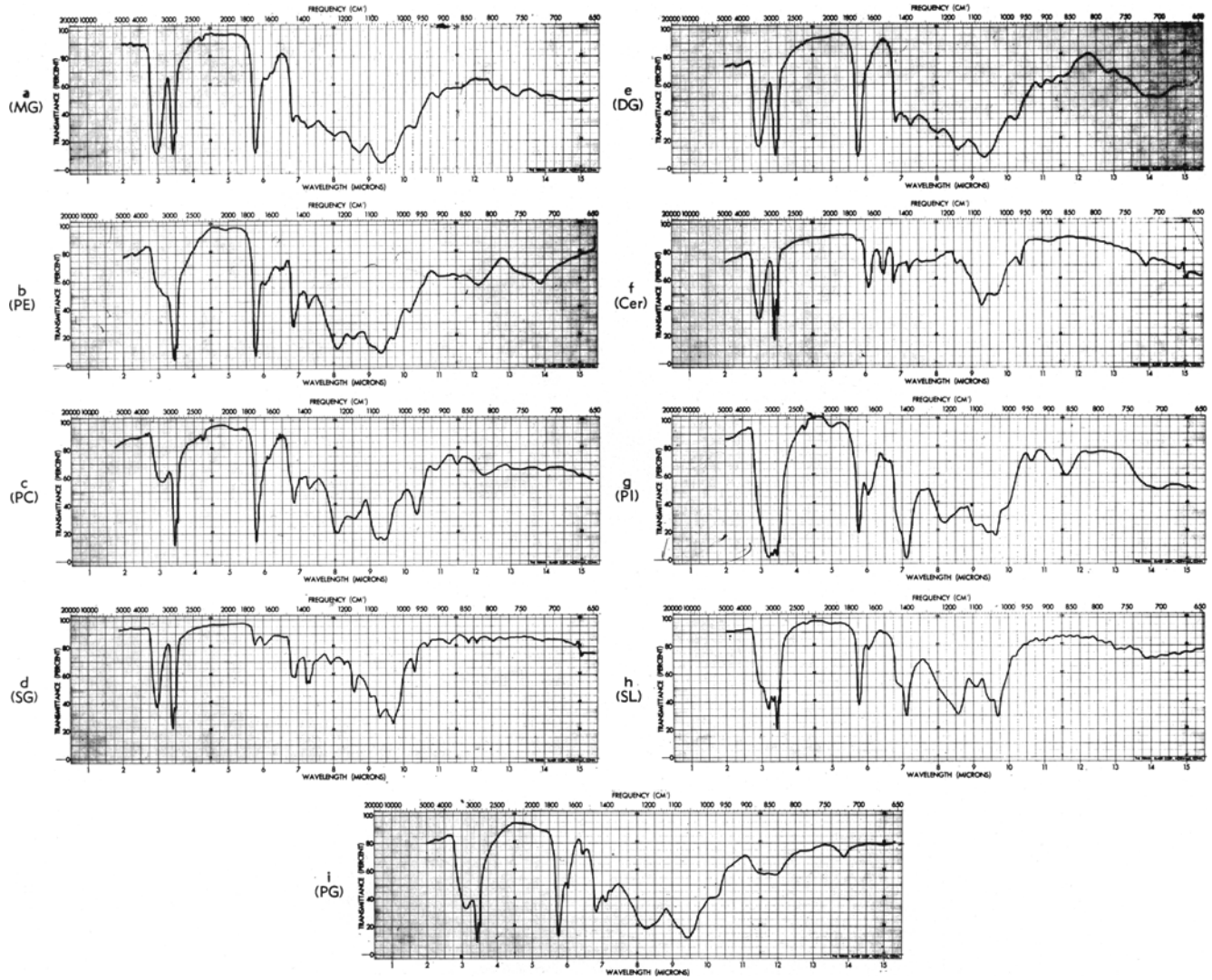

FIG. 4. Infrared spectra (KBr pellets) of spinach lipids obtained from DEAE columns (additional purification steps are indicated): (*a*) monogalactosyl diglyceride freed of contaminating pigments by elution with chloroform/methanol 9/1 from a silicic acid column; (*b*) phosphatidyl ethanolamine from a chloroform/methanol 2/1 eluate; (*c*) lecithin from a chloroform/methanol 19 eluate; (*d*) sterol glycoside (a mixture) separated from lecithin by chromatography on silicic acid; (*e*) digalactosyl diglyceride from a chloroform/methanol 9/1 eluate; (*f*) cerebroside separated from a digalactosyl diglyceride fraction by elution from a silicic acid column with chloroform/methanol 4/1; (*g*) phosphatidyl inositol obtained by evaporation and lyophilization of a chloroform/methanol 2/1 + ammonium acetate eluate; (*h*) sulfolipid obtained as for *g*; (*i*) phosphatidyl glycerol obtained as for *g*. The last three lipids are probably largely in the ammonium salt form.

roform/methanol/water 65/25/4 for polar lipids, and chloroform or chloroform/methanol 9/1 for less polar materials in chambers lined with Whatman 3 MM paper. Lipid spots were visualized under ultraviolet light with a 0.003% solution of rhodamine 6G in 1M NaOH prepared just before use by mixing equal volumes of 0.006% aqueous rhodamine 6G and 2M NaOH stock solutions (55). The background was made a deep uniform violet by briefly drying the plates with a hair dryer (overdrying will cause the background to brighten). The yellow-orange lipid spots were photographed with Ansco Versapan (1–5 min exposure) or Kodak Royal X-Pan (approximately 10 sec exposure) through an orange filter. As little as 0.1 μg of lipid was detectable. Plates run in chloroform systems required no special drying before spraying, but those run in the diisobutyl ketone system gave a darker background when they were heated at 180C for about 20 min and cooled before spraying.

Two-dimensional TLC with chloroform/methanol/water followed by diisobutyl ketone/acetic acid/water (17) gave better resolution than either system used alone, but resolution of certain critical pairs of lipids was not consistent.

Fatty Acid Analysis. Lipids were transesterified with 5% methanolic sulfuric acid at 70C overnight (56), the reaction mixture diluted with water, and the methyl esters extracted with hexane. The concentrated hexane extracts were chromatographed on a 5 ft x ⅛ in. O.D. column of Reoplex-400 (15% on Chromosorb W) at about 190C under 40 lb helium pressure at a flow rate of 33 ml/min. A similar column of diethylene glycol succinate polymer at 175C was used as a check for possible peak overlap, but not for quantitative analysis since methyl oleate and methyl hexadecatrienoate emerge as 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.

Results and Discussion

Figure 1 illustrates the components of spinach leaf lipids separated in a 60-tube countercurrent distribu-

tion apparatus. The spots are identified in Table I. The system carbon tetrachloride/methanol/water retains chlorophyll and other relatively nonpolar pigments in the tubes near the origin and permits their continued distribution, whereas the polar lipids move rapidly through the apparatus. The acidic lipids (phosphatidyl inositol, sulfolipid, phosphatidyl glycerol) emerge in the first tubes withdrawn if more than 60 transfers are made, and thus no further distributions can be made. The behavior of the hexane-containing system permits continued separation of the highly polar lipids and consequently improvement in their separation. Isolation of individual lipids with these systems is not possible, even for a large apparatus, but preliminary separation into broad classes is easily accomplished. Chlorophylls (and other lipids of low polarity) are retained in the early tubes and are cleanly separated from monogalactosyl diglyceride and more polar lipids after about 100 transfers. Other pigments (orange) distribute with the lipids of intermediate polarity.

Countercurrent distribution preceding DEAE chromatography permits superior fractionation of the nonpolar lipids and the occasional separation of minor lipids not readily separated on the column, but these advantages are not sufficient to recommend its routine use prior to chromatography.

It was hoped that firebrick partitioning columns (54) with the carbon tetrachloride system could be used to duplicate or improve upon the results with the Craig apparatus. However, we were unable to separate chlorophyll from monogalactosyl diglycerides and other lipids of moderate polarity.

Diethylaminoethyl Cellulose Columns. The dual partioning and ion exchange action of these columns is adequate to cleanly separate the major plant lipids. Figure 2 presents the elution scheme which has been useful with spinach leaf lipids and with the lamellae lipids. The polar lipid components of lamellae are effectively separated without further chromatography since several of the interfering minor lipids such as sterol glycosides and cerebrosides which are present in whole leaf extract are absent. Much of the chlorophyll and other relatively nonpolar lipids can be eluted with chloroform/methanol 49/1 before monogalactosyl diglyceride emerges, but the small quantities of neutral and acidic pigments retained can be troublesome when uncontaminated lipids are desired. Traces of pigment appear in the neutral lipid and phosphatidyl glycerol fractions.

TLC of eluates has the dual advantage of monitoring the separation and indicating the separability of eluted mixtures on silicic acid columns or by preparative TLC. It has been advantageous to use chloroform/methanol/water and diisobutyl ketone systems for TLC (Fig. 5).

Spinach cerebroside, sterol glycoside and other minor lipids as yet uncharacterized have been separated on silicic acid columns or by preparative TLC as outlined in Figure 3. These techniques are also useful for removing the small amounts of pigment remaining in lipids after DEAE chromatography, but are ineffective if large amounts of chlorophyll must be separated.

Fractional crystallization of the sterol glycoside indicates that it is not homogeneous, although it moves as one spot by TLC with chloroform/methanol, chloroform/methanol/water, and diisobutyl ketone/acetic acid/water systems.

It is interesting that while brain cerebroside is distinctly separated into two spots corresponding to species containing hydroxy and nonhydroxy acids, the plant cerebroside forms only one spot moving with the same R_f as the hydroxy acid cerebroside of beef brain (Fig. 5 and 6).

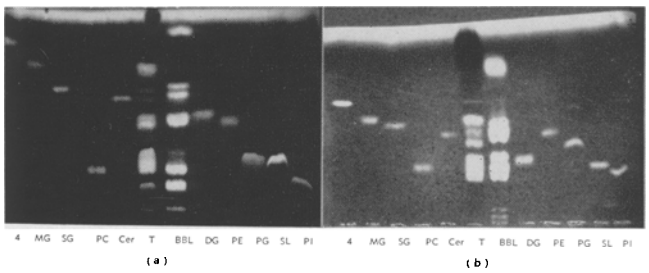

FIG. 5. TLC of purified lipids used for IR spectroscopic examination. From left to right, minor 4, MG, SG, PC, Cer, total spinach leaf lipid, total beef brain lipid, DG, PE, PG, SL, PI. (a) in chloroform/methanol/water 65/25/4; (b) in diisobutyl ketone/acetic acid/water 8/5/1. Comparison of R_f values of lipids in chromatograms reproduced here and in an earlier report (40) indicates the considerable variation we have found using the same batch of silicic acid with fresh solvent mixtures in paper lined chambers shaken to saturate the chamber just before use.

Infrared spectra of the major polar lipids of spinach are presented in Figure 4. The main features of these spectra are relatively insensitive to changes in the fatty acid composition and this has made them useful in identification of lipids from other sources [e.g., *Anacystis nidulans* which contains no polyunsaturated acids (57)]. Comparable spectra of many lipid classes isolated from other sources have been published by Rouser et al. (61).

Each lipid isolated by the techniques described and

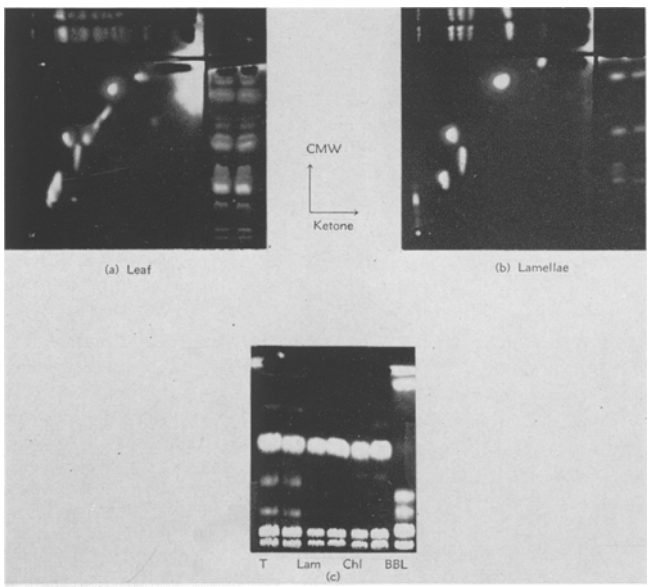

FIG. 6. (*a*) and (*b*): Two-dimensional TLC of spinach leaf lipid and spinach chloroplast lamellae lipid respectively. Both were developed first in chlorofrom/methanol/water 65/25/4, dried briefly, and rerun in diisobutyl ketone/acetic acid/water 8/5/1. Duplicate one dimensional chromatograms at the top (ketone) and to the right (C/M/W) are of the same mixtures run in two dimensions. The relatively simplicity of the lamellae lipid mixture is readily apparent. (*c*) TLC with chloroform/methanol 9/1. From left to right: total spinach leaf lipid (2 places), lamellae lipid (2 places), chloroplast lipid (2 places), and a DEAE beef brain fraction containing (top down) cholesterol, cerebroside (double spot), PE, and (at the origin) lecithin, sphingomyelin and other lipids. The spots visible in the spinach leaf lipid are (top down) minor 4 (just under dark chlorophyll spot), minor 3, MG, SG, Cer, PE = DG, and more polar lipids (at the origin).

TABLE II

Lipids of Spinach Lamellae

Lipid[b]	Wt (mg)[c]	Molar ratio	Fatty acid composition (% by weight)[a]										
			14-0	14-3	15-0	16-0	Δ^3-*trans* 16-1	16-2	16-3	18-0	18-1	18-2	18-3
Monogalactosyl diglyceride	47	14.0	Tr	0.1		0.4		0.5	25.0		0.6	1.6	71.9
Digalactosyl diglyceride	32	8.0	Tr	0.6	Tr	3.4		Tr	5.4		1.8	1.7	86.7
Phosphatidyl glycerol	17	5.5	1.1	0.2	0.1	11.0	32.3		2.0	0.4	2.2	3.6	47.0
Sulfolipid	14	3.9	0.2	Tr	0.1	38.5	0.3	0.1	0.6	0.5	0.8	6.5	52.5
Lecithin	6	2.0	0.3	0.1	0.1	12.4		0.2	3.6		8.7	16.3	58.2
Unknown[d]	4		0.9	0.5	0.4	9.3		1.4	14.6	0.2	1.0	0.9	70.0
Phosphatidyl inositol	3	1.0	3.7	0.6	1.2	33.5	1.0	6.0	2.5	2.3	6.8	15.1	26.7
Nonpolar[e]	74	....											
Total			0.1	0.4	0.2	13.5	1.8	Tr	10.6	0.1	4.2	9.4	55.8
Other (largely nonlipid)	8												

[a] Acids present to an extent of less than 0.1% in any fraction are not included.
[b] A trace of unidentified lipid was eluted between monogalactosyl diglyceride and lecithin (estimated 0.5 mg), and a barely detectable trace of another (phosphatidic acid?) with phosphatidyl glycerol.
[c] Weight of residue after evaporating appropriate DEAE column fractions. Of 210 mg of material applied to the column, 205 mg was recovered.
[d] A lipid eluted with C/M 2/1 moving below PI in the diisobutyl ketone TLC system, and near lecithin the chloroform/methanol/water system.
[e] Includes several mg (estimated) of a lipid fluorescing with Rhodamine 6G in ultraviolet light. Possibly lipid 3 in Table I.

used for infrared spectra gave only one spot by TLC in the chloroform/methanol/water and diisobutyl ketone/acetic acid/water systems (Fig. 5). Two-dimensional chromatography (3,5,46) of deacylated glycerol lipids in phenol/water and butanol/propionic acid/water (detection with periodate-Schiff spray) confirmed the identity and purity of the lipids.

Spinach Chloroplast Lamellae Lipids. The simplicity of the lamellae lipid mixture relative to whole leaf lipids is illustrated by two-dimensional chromatograms in Figure 6. Phosphatidyl ethanolamine, sterol glycosides, cerebrosides, and other minor lipids were not detected, and are thus unlikely to comprise more than 0.1% of the lipid. This permits a nearly quantitative separation of the polar components on a diethylaminoethyl (DEAE) cellulose column without subsequent silicic acid chromatography (Fig. 2). The results of such an analysis are shown in Table II together with the fatty acid composition of each lipid. Retention times of fatty acid methyl esters relative to methyl palmitate are given in Table III. More than 90% of the acyl residues in the galactosyl lipids are trienoic (16:3 and 18:3). As with whole leaf of plants grown the previous year, the concentration of hexadecatrienoate in the monogalactosyl diglyceride is much higher than in the digalactosyl lipids, and *trans*-3-hexadecenoic acid makes up approximately one-third of the acyl groups in phosphatidyl glycerol. Only this one *trans* fatty acid is present in spinach and it is found almost exclusively in this one lipid. Recently Weenink and Shorland (59) also reported concentration of the *trans*-3-hexadecenoic acid in phosphatidyl glycerol of red clover, and found the acid to be present in a phospholipid fraction of the pea and of rye grass. It may well be a common constituent of the photosynthetic apparatus of higher plants.

Our data indicate that 48.5% of the dry weight of lamellae is lipid. Park and Pon (51) reported a somewhat larger lipid fraction (55%) on the basis of total material extracted from about 15 mg of lyophilized lamellae with hexane, acetone, and methanol. The solvents probably extracted some protein and other nonlipid material that accounts for the higher value.

In our analysis by DEAE and silicic acid column chromatography (Table IV), phospholipid and sulfolipid comprise a larger mole fraction of the total lamellae lipid than had been reported by Wintermans (3,4) for whole chloroplasts as indicated in the accompanying compilation (PI taken as 1.0).

Appreciable differences are to be expected in these analyses carried out by different methods with different starting material. Galactolipids of bean seedlings grown with differing illumination varied, galactolipid concentration increasing with illumination (3). The twofold difference in galactolipid concentration may be significant and suggests a higher ratio of galactolipids than the other lipids may exist in that portion of the chloroplast surrounding the lamellae.

TABLE III

Retention Times of Methyl Esters
(Relative to Methyl Palmitate)

	Reoplex 400	DEGS
Tetradecanoate (14:0)[a]	0.54	0.54
Tetradecatrienoate (14:3)	0.84	1.26
Pentadecanoate (15:0)	0.74	0.73
Hexadecanoate (16:0)[a]	1.00	1.00
trans-3-Hexadecenoate (16:1)[a]	1.19	1.32
Hexadecadienoate (16:2)	1.36	1.66
Hexadecatrienoate (16:3)[a]	1.66	2.30
Octadecanoate (18:0)[a]	1.84	1.84
Octadecenoate (18:1)[a]	2.06	2.30
Octadecadienoate (18:2)[a]	2.42	2.98
Octadecatrienoate (18:3)[a]	3.02	4.17

[a] Retention times checked against standards. Others identified by their carbon numbers.

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TABLE IV

	Lamellae	Chloroplast (3)	Chlorella (6)
MG	14.0	30	12
DG	8.0	12.7	5.0
PG	5.5	3.5	4.3
PC	2.0	3.0	1.9
PI	1.0	1.0	1.0
PE	....	0.1	0.8
SL	3.9	3.8	1.3

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