Isolation and fatty acid composition of the plant sulfolipid and galactolipids*

JOHN S. O'BRIEN† and A. A. BENSON

Department of Marine Biology, Scripps Institution of Oceanography, University of California, La Jolla, California

SUMMARY The plant sulfolipid has been isolated from *Chlorella pyrenoidosa* cells and from alfalfa leaves by chromatography on Florisil and DEAE cellulose columns. The galactolipids, galactosyl diglyceride and digalactosyl diglyceride were isolated by further chromatography on silicic acid columns. The galactolipids from alfalfa leaves were highly unsaturated and contained 87–94% linolenic acid, while the sulfolipid contained approximately equal amounts of palmitic and linolenic acids.

THE PLANT SULFOLIPID 6-sulfo-α-D-quinovopyranosyl-(1→1′)-diglyceride (SQDG) occurs in plants in concentrations comparable to those of the phosphatides (1, 2). In this paper a procedure is given for the preparative isolation of SQDG, galactosyl diglyceride (GDG), and digalactosyl diglyceride (GGDG) from alfalfa leaves and *Chlorella pyrenoidosa*. The fatty acid compositions of these lipids are also reported.

MATERIALS AND METHODS

Extraction

Alfalfa leaves were collected in Carmel Valley, Del Mar, Calif., in July and August. *Chlorella pyrenoidosa* was grown in inorganic medium for 6 days under incandescent illumination in a 15-liter bottle. The fresh wet leaves and harvested cells were extracted by homogenization in ten volumes of chloroform–methanol (C–M) 2:1 under nitrogen. All solvents were redistilled prior to use. The extract was filtered; the residue was reextracted with 5 volumes of C–M 2:1 and the second extract filtered. The combined filtrates were evaporated to dryness in vacuo at room temperature in a rotary evaporator. The lipid extract was kept in a nitrogen atmosphere as much as possible throughout the isolation and analysis procedures. The extract was dried in vacuo for an 18 hr period and taken up in C–M 2:1 and insoluble material, presumably mainly proteolipid, was filtered off. The lipid extract was then evaporated to dryness and portions of the dried extract were taken for column chromatography.

Column Chromatography

Lipid isolation was carried out using a combination of Florisil, diethylaminoethyl (DEAE) cellulose, and silicic acid column chromatographic procedures similar to those described by Rouser et al. (3, 4) for brain lipid separations. The details of these procedures are as follows.

Two hundred grams of Florisil (60–100 mesh, Floridin Co., Tallahassee, Fla.) was washed with 10 liters of distilled water on a filter funnel to remove water-soluble impurities. The absorbent was spread on aluminum foil, dried at 120° for 18 hr, and cooled in a stoppered flask. The product was stored under methanol and poured as a slurry to a height of 15 cm over a glass wool plug in a glass column, 50 × 4.5 cm (i.d.) equipped with a Teflon stopcock. The methanol was replaced by chloroform, and 1.0 g of lipid extract was applied to the column in 10 ml of C–M 9:1 containing 5% 2,2-dimethoxypropane (2,2-DMP) (Dow Chemical Co., Midland, Mich.). The column was eluted with 1.0 liter of C–M 9:1 plus 5% 2,2-DMP and 1.5 liters of C–M 2:1 plus 5% 2,2-DMP. The C–M 9:1 eluate contained carotenoids and most of the chlorophyll. The C–M 2:1 eluate contained GDG, GGDG, and SQDG plus some

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† Visiting Scientist, Scripps Institution of Oceanography, 1963. Present address: Department of Pathology and Medicine, University of Southern California. School of Medicine, Los Angeles, California.

chlorophyll. Phospholipids were not eluted from the Florisil column. The C–M 2:1 eluate was poured directly onto a column of DEAE cellulose, prepared as follows.

One hundred grams of DEAE (Standard Selectacel, Schleicher and Schuell, Keene, N.H.) was washed on a filter funnel with 1 liter of 1 N HCl, distilled water until the washings were neutral, 1 liter of 10% aqueous KOH, and distilled water until the washings were neutral. This cycle was repeated three times. DEAE was not allowed to be in contact with the acid for prolonged periods, since hydrolysis occurs. The ion-exchanger was then washed with acetone, chloroform, and methanol (1 liter each) and poured as a slurry in methanol to a height of 25 cm over a glass wool plug in a column similar to that described above using nitrogen pressure (2.5 psi) to ensure firm packing. The column was then washed with 1 liter of glacial acetic acid and allowed to stand overnight. The acetic acid was washed out with 1.5 liters of methanol and the methanol removed with 1 liter of chloroform.

The C–M 2:1 eluate from the Florisil column was passed through the DEAE column followed by an additional 300 ml of C–M 2:1. The eluate contained the neutral glycolipids (GDG and GGDG) plus some chlorophyll. The column was washed with 1 liter of glacial acetic acid to elute any remaining chlorophyll and any free fatty acids present. The acetic acid was removed with 1 liter of methanol and the sulfolipid (SQDG) was eluted with 2 liters of C–M–concd NH_4OH (4:1:0.2). The flow rates for both the Florisil and DEAE columns were approximately 10 ml/min. The DEAE column was prepared for re-use by washing out the C–M–NH_4OH with 500 ml of methanol, passing 1 liter of glacial acetic acid through the column and letting it stand for at least 12 hr. Florisil columns must be prepared fresh each time.

After evaporation to dryness, the C–M 2:1 fraction could be separated into a pigment fraction, a fraction containing GDG, and one containing GGDG by chromatography on a silicic acid column. Two hundred grams of silicic acid (Mallinkrodt, 120 mesh) was washed with 1 liter of 6 N HCl, followed by distilled water until the wash had the pH of the water, and then dried for 16 hr at 120°. The absorbent was cooled in a stoppered flask and methanol was added. The column was prepared by pouring a slurry of silicic acid in methanol to a height of 15 cm over a glass wool plug in a glass column 50 × 2.5 cm (i.d.) equipped with a Teflon stopcock. Nitrogen pressure of 2.5 psi was used in packing the column and in maintaining a flow rate of 2 ml/min during elution. Methanol was washed out of the column with chloroform and 60 mg of the C–M 2:1 fraction from the DEAE column was applied to the silicic acid column in C–M 9:1. Ten-milliliter fractions were collected, and elution was monitored with the solids test described previously (4), using 0.5 ml aliquots. The first 100 ml contained pigment and was free of glycolipid while the next 200 ml contained GDG, free of pigment, and GGDG. Two hundred milliliters of C–M 4:1 were then passed through the column to elute GGDG, which was free of contamination.

The C–M–NH_4OH fraction from the DEAE column contained SQDG plus a large quantity of ammonium acetate generated by the ion-exchange process. This fraction was evaporated to dryness and ammonium acetate was removed by a dialysis procedure described recently for cerebroside sulfate isolation (5). S^{35}-Labeled SQDG, prepared by incubating $Na_2S^{35}O_4$ with a culture of *Chlorella*, was isolated and dialyzed using this procedure. No radioactivity could be detected in the dialysate of the SQDG-S^{35}, demonstrating that no loss of SQDG occurred during dialysis.

Analytical Methods

The purity of SQDG, GDG, and GGDG was assessed by chromatographing each lipid on silicic acid impregnated paper (3) in C–M 9:1 and staining with triaminodiphenyltolylcarbinol (*p*-rosaniline) (4, 5). In this system the R_F values were: GDG 0.9; GGDG 0.5; and SQDG 0.4.

Hexose was determined by the anthrone method of Radin, Lavin, and Brown (6). Sulfur and phosphorus analysis using the X-ray fluorescence method and infrared spectroscopy were kindly performed by Mr. George Alexander (U.C.L.A. School of Medicine).

Fatty Acid Analysis

A measured portion of each pure lipid was heated in anhydrous 5% methanolic HCl at 60° for 1 hr. The methyl esters were extracted into petroleum ether (bp 30–60°) and weighed after evaporation of the solvent. They were analyzed by gas–liquid chromatography (GLC) on polyesters of diethylene glycol succinate and also hydrogenated over palladium black catalyst to determine unsaturation. The retention time of each peak was compared to those of known fatty esters and the chromatograms were compared before and after hydrogenation to identify the fatty ester peaks. Quantification of each fatty ester was made by triangulation. A comparison of the peak areas obtained before and after hydrogenation showed good agreement, i.e., the sum of the 18:0, 18:1, 18:2, and 18:3 peak areas[1] before hydrogenation was 96% of the peak area of 18:0 after hydrogenation, indicating that the GLC detector response for the C_{18} unsaturated acids was very close to that for stearic acid.

[1] The chain length is given by the numeral before the colon and the number of double bonds by the numeral after it.

TABLE 1 ANALYTICAL DATA FOR ALFALFA LEAF LIPIDS*

	GDG	GGDG	SQDG
Fatty acid methyl esters, %†	73 (76)	64 (63)	69 (71)
Molar ratio, fatty ester: galactose	2.0:0.95	1.0:0.90	2.0:0.98
Sulfur, %	—	—	4.2 (3.8)
Phosphorus	—	—	0.0 (0.0)

* Values in parenthesis are data calculated for galactosyl dilinolenin, dilinolenyl digalactosyl diglyceride, and palmitoyl-linolenyl sulfoquinovosyldiglyceride (ammonium salt).

† By weighing methyl esters after methanolysis.

RESULTS

Sulfoquinovosyldiglyceride from alfalfa leaves or *Chlorella* is a colorless translucent waxy solid. Its solubility distribution between water and different solvents was tested using S^{35}-labeled SQDG (*Chlorella pyrenoidosa*). Alfalfa leaf SQDG as the ammonium salt (mainly linolenyl-palmitoyl) was mixed with a small amount of SQDG-S^{35} from *Chlorella*-S^{35}. This mixture was dissolved in chloroform at a concentration of 2 mg/ml and two volumes of water were added. After complete equilibration (18 hr), 64% of the radioactivity was found in the chloroform phase (1.3 mg/ml) and 36% in the aqueous phase (0.35 mg/ml). With benzene as the organic solvent, 98% of the radioactivity was found in the aqueous phase (0.9 mg/ml) while with hexane all of the radioactivity was found in the aqueous phase (1.0 mg/ml).

Examination of SQDG, GDG, and GGDG by chromatography on silicic acid impregnated paper revealed the absence of other lipids in SQDG and GGDG. However, a small extraneous spot, estimated to be approximately 2% of the total, was seen in chromatograms of GDG from alfalfa leaves. This spot moved between GDG and GGDG in the system described, and may be one of the minor glycolipids reported to be present in leaves (7, 8). Analytical data on each lipid (Table 1) gave results corresponding closely to theoretical. The somewhat high sulfur value is within the experimental error of the method, the error being higher than usual in this case since only 1.6 mg was used for analysis. Infared analysis of SQDG closely resembled that reported previously for sulfoquinovosylglycerol (9) except for a strong ester carbonyl band in SQDG arising from fatty ester absorption.

The quantities of these lipids found in alfalfa leaves and *Chlorella* are given in Table 2. In each case the concentration of GGDG was approximately twice that of GDG. SQDG occurred in lower concentrations. Larger amounts of each glycolipid were present in *Chlorella* when expressed on a dry weight basis, but when expressed as per cent of total lipid, alfalfa leaves and *Chlorella* extracts gave almost equal values for each glycolipid. This was due to the larger content of nonlipid residue in the leaves.

The fatty acid composition of these three glycolipids is presented in Table 3. In alfalfa leaves, both in July and August harvested plants, SQDG showed almost equal quantities of 16:0 and 18:3 acids. GDG from alfalfa leaves was more highly unsaturated, containing 95% of the 18:3 acid. GGDG from alfalfa was slightly less unsaturated, containing 82% of 18:3, the remainder being mainly 16:0. The same lipids from *Chlorella* showed a different fatty acid pattern. SQDG contained mainly 16:0, 18:1, and 18:3 acids; while GDG and GGDG were similar to each other, containing mainly 18:1, 18:3, 16:1, and 16:0 acids.

TABLE 2 CONCENTRATION OF PLANT LIPIDS IN ALFALFA LEAVES AND CHLORELLA PYRENOIDOSA*

	GDG	GGDG	SQDG
Alfalfa leaves (July)	—	—	4.4 (3.0)
Alfalfa leaves (August)	4.5, 4.7 (4.2, 4.4)	9.3 (8.6)	2.3 (2.1)
Chlorella pyrenoidosa	14.0 (4.2)	24.0 (7.2)	6.4 (1.9)

* In mg/g dry weight. Values in parenthesis are data calculated as per cent of total extractable lipid.

DISCUSSION

The procedures used to isolate SQDG, GDG, and GGDG gave sufficient quantities of these lipids in pure form for fatty acid analysis and future studies of their physical, biological, and chemical properties. Since these methods had given quantitative results for brain lipids (3–5), the procedures were expected to be suitable for this purpose in plants. A further indication that reliable quantification is possible was evident when 99% of the S^{35} radioactivity in the *Chlorella*-S^{35} lipid extract was recovered as SQDG-S^{35} after column chromatography.

The fatty acid compositions of GDG and GGDG were very similar to those reported previously from leaves. Weenink (11) and Sastry and Kates (12) reported that GDG from red clover and runner-bean leaves contained 96% of 18:3. However, the GDG from wheat flour contained mainly 18:2, 18:1, and 16:0 (13); and from *Chlorella* (Table 3) mainly 18:1, 18:3, 16:1, and 16:0. GGDG from runner-bean leaves (12) and alfalfa leaves contained 93 and 82% of 18:3 respectively, the remaining acids being 16:0 and 18:0 in each case. GGDG from wheat flour (13) was more saturated, containing mainly 16:0, 18:1, and 18:2 acids; while from *Chlorella* it contained mainly 18:1, 18:3, 16:0, and 16:1.

TABLE 3 FATTY ACID COMPOSITION OF PLANT LIPIDS*

Fatty Acid	July Alfalfa Leaves	August Alfalfa Leaves			*Chlorella pyrenoidosa*		
	SQDG	SQDG	GDG	GGDG	SQDG	GDG	GGDG
14:0	0.4	tr.	tr.	tr.	tr.	tr.	tr.
15:0	0.4	tr.	tr.	—	tr.	tr.	tr.
16:0	42.8	37.4	2.7	14.0	67.5	2.7	11.6
16:1	tr.	1.9	tr.	tr.	1.6	9.7	9.5
17:0	0.1	tr.	tr.	0.7	—	1.5	1.2
18:0	3.2	7.1	0.2	3.3	0.4	0.3	0.4
18:1	1.5	2.6	0.3	0.4	18.3	40.5	36.8
18:2	4.4	1.9	1.7	0.8	2.0	4.5	5.8
18:3	47.2	49.0	95.0	82.0	9.8	26.8	27.0
19:0 (19.0)†	—	—	—	—	—	12.0	3.3
18:4 (21.3)†	—	—	—	—	0.8	2.6	3.3

* Each peak is expressed as per cent of the total peak area.
† Carbon number (10) on diethylene glycol succinate polyester.

GDG and GGDG resemble each other closely in fatty acid composition. These findings support the hypothesis that GDG is converted to GGDG by galactosylation (14, 15). SQDG, on the other hand, is much more saturated and could either arise from an independent diglyceride pool, or be acted upon by fatty acid reacylating enzymes, described for phosphoglycerides (16), which change the fatty acid composition after synthesis. The latter possibility must be borne in mind since Yagi and Benson (17) have demonstrated the presence of a sulfolipase in plants which hydrolyzes SQDG to lysosulfolipid (sulfoquinovosylmonoglyceride). The enzyme failed to hydrolyze GDG.

The fatty acid composition of SQDG from alfalfa leaves resembles that of the glycerophosphatides since it contains approximately equimolar proportions of a saturated and an unsaturated fatty acid. One might expect that this anionic lipid would be deacylated by phospholipase A (snake venom). However, it is uniquely resistant to hydrolysis by this enzyme despite its appropriate charge and fatty acid composition (17). It was therefore not possible to determine the positional distribution of fatty acids in SQDG by snake venom hydrolysis.

The high proportions of 18:3 in alfalfa leaf GDG and GGDG are of interest for several reasons. First, these lipids must exist almost entirely in fully unsaturated form, i.e. containing two unsaturated fatty ester residues, in the legumes so far studied [alfalfa, red clover (11), and runner-bean leaves (12)]. Hanahan and co-workers (18) showed that the majority of the lecithins from various sources (liver, egg) contained one unsaturated and one saturated fatty acid. Privett and Blank (19) have recently shown that small proportions of lecithins from egg yolk and spinal cord are fully unsaturated; while much larger proportions of this type of lecithin are present in soybean and wheat germ. Fully unsaturated lecithins (20) and phosphatidyl ethanolamines (21, 22) have also been isolated. The fact that legume leaf galactolipids are almost exclusively in fully unsaturated form is of interest since lecithin from the same source contains 35% saturated acids (16:0 and 18:0) (12).

Secondly, it is tempting to suggest that the high content of linolenic acid in the galactolipids, which occur almost exclusively in the chloroplast (23), may be related to a functional role of these lipids in photosynthesis. The relationship between linolenic acid content and photosynthesis has recently been pointed out by Erwin and Bloch (24), who found that the chloroplast lipids of photosynthetic bacteria account for over 85% of the linolenic acid content of the cells grown in the light, and that linolenic acid is a major fatty acid in light-grown cells and a minor fatty acid in dark-grown cells. These authors suggest that linolenic acid may be chemically involved in electron transport or is a necessary physical component of the oxygen-evolving reaction in photosynthesis. An alternative explanation is that oxygen may be required for the phytosynthesis of linolenic acid from oleic acid as it is in yeast (25), and that the increased synthesis of this fatty acid after photosynthesis begins may be merely a reflection of the requirement for oxygen. Despite the alternative explanations, the facts that linolenic acid is the predominant fatty acid of both GDG and GGDG in a variety of legume leaves (alfalfa, red clover, and runner-bean) (11, 12) and that these lipids are the predominant lipids in the chloroplast (23) suggest that future studies may reveal specific requirements for these lipids in chloroplast lipoproteins.

Finally, it is surprising that lipids with such a high degree of unsaturation are present at the site of oxygen evolution and exposed to the light, conditions which favor oxidation of unsaturated fatty acids. Failure to find appreciable quantities of oxidized fatty acids in leaf lipid extracts in this study indicates that the unsaturated

fatty acids are protected from oxidation by the presence of antioxidants and by their inaccessibility within the chloroplast lipoprotein.

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