Isolation and characterization of an ether-linked homoserine lipid from the thylakoid membrane of *Chlamydomonas reinhardtii* 137$^+$

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Abstract A polar diacylglycerolipid isolated from the phototrophic green alga *Chlamydomonas reinhardtii* 137$^+$ (wild-type) displays chromatographic and chemical identity with the ether-linked homoserine lipid 1(3),2-diacylglyceryl-(3)-*O*-4′(*N*,*N*,*N*-trimethyl)homoserine (DGTS) of the golden alga *Ochromonas danica*. Subcellularly, DGTS is a constituent of a fraction of thylakoid membranes purified from *Chlamydomonas* whole-cell homogenates. The proportion of DGTS in the photosynthetic lamellae is ~40% of the total found in the alga. Cellular and thylakoid-membrane DGTS both have an unsaturated:saturated ratio of about 1.8 and contain predominantly hexadecanoic and octadecanoic fatty acids. Quantitatively, the fatty acid complement of thylakoid DGTS is distinct from the cellular DGTS fatty acid profile. The results provide the first demonstrations that DGTS is a bona fide membrane lipid and, specifically, that ether-linked homoserine lipid is a component of the thylakoid membrane of a phototrophic green-plant cell.—**Janero, D. R., and R. Barrnett.** Isolation and characterization of an ether-linked homoserine lipid from the thylakoid membrane of *Chlamydomonas reinhardtii* 137$^+$. *J. Lipid Res.* 1982. **23:** 307–316.

Supplementary key words green alga • photosynthetic membrane • diacylglycerolipid • fatty acids

Studies on the lipid biochemistry of green tissue from higher plants have led to a general conclusion that the photosynthetic, or thylakoid, membrane contains mostly glycolipid and relatively little phospholipid. Three glycoglycerolipids, monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SL), account for well over 80% of the acyl lipids of higher green-plant thylakoids. Phosphatidylglycerol (PG) is the only significant phospholipid constituent of the most homogeneous thylakoid preparations (1).

At the whole-cell level, these four lipids are found in the limited number of green algae whose lipid biochemistry is reasonably well documented (2). From this, it has been largely inferred that the polar acylglycerolipids in the thylakoids of lower green plant cells are identical to those in higher green plant photosynthetic lamellae. Direct support for such a conclusion is lacking, since very few studies on the lipids of isolated green-algal thylakoids have been carried out, and indirect analyses, such as comparisons of lipid biochemistry among algae under varying growth conditions that influence the ability to produce photosynthetic membrane, are almost always employed.

During our investigations on the lipid biochemistry of the green phytoflagellate *Chlamydomonas reinhardtii*, an unusual polar diacylglycerolipid was found in the phototrophic, wild-type alga (strain 137$^+$). Subcellular studies designed to augment the limited information on the lipids of lower green-plant thylakoids uncovered an association between a significant portion of this cellular lipid and the alga's thylakoid membrane. We report here the identification of the lipid as 1(3),2-diacylglyceryl-(3)-*O*-4′-(*N*,*N*,*N*-trimethyl)homoserine (DGTS) (cf. 3), the quantitative biochemistry of DGTS in photosynthetically-cultured *Chlamydomonas reinhardtii* 137$^+$, and the first isolation and analysis of DGTS from thylakoid membrane. A preliminary account of some of these findings has appeared in abstract.[2]

EXPERIMENTAL PROCEDURES

Cell culture and fractionation

Cultures of *Ochromonas danica* Pringsheim (University of Texas Algal Culture Collection; UTEX-1298)

Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SL, sulfoquinovosyldiacylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; DGTS, 1(3),2-diacylglyceryl-(3)-*O*-4′-(*N*,*N*,*N*-trimethyl)homoserine; NL, neutral lipid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylene diaminetetraacetic acid; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

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[2] Janero, D. R., and R. Barrnett. 1979. *J. Cell Biol.* **83:** 360a (abstract).

were grown in the light under defined conditions (4). *Chlamydomonas reinhardtii*, strain 137^+ (wild-type), was cultured phototrophically and asynchronously in axenic, log-phase culture as described (5). Cells were harvested by low-speed centrifugation. Thylakoid membranes were purified from *Chlamydomonas* homogenates according to Chua and Bennoun (6).

Isopycnic sucrose gradient analysis

Chlamydomonas cultures were exposed to [^{3}H]arginine (New England Nuclear, Boston, MA; sp act 28.2 Ci/mmol; 2.5 M final concentration) for one generation (9.5 hr (7)). The cells were then chilled, pelleted, washed three times with fresh, sterile medium containing a 200-fold excess of nonlabeled arginine, and fractionated to obtain a thylakoid membrane preparation. Samples of the thylakoid preparation were layered onto 5-ml linear gradients from 1.0 to 1.5 M in sucrose containing 5 mM HEPES–10 mM EDTA, pH 7.5. Centrifugation of the gradients in a Beckman SW 50.1 rotor, 1.49×10^5 g_{avg}, at 2°C for 21 hr was sufficient for the thylakoids to reach their equilibrium density (cf. 8), after which time a sharp green band forms. Fractionation of one such gradient yielded minimally 25 fractions of about 0.2 ml each. The density of each fraction was calculated from the refractive index determined with a Bausch and Lomb refractometer, and portions of each were assayed chemically for chlorophyll (9) and protein (10). Incorporation of [^{3}H]arginine into acid-precipitable protein (recovery > 98% with respect to that loaded onto the gradient) was assayed radiochemically by a filter-paper technique (11). Biofluor (New England Nuclear) was used as scintillant, and the efficiency of scintillation counting (>55%) was determined by both internal and external standardizations (12).

Lipid isolation and deacylation

Purification of DGTS from *Ochromonas* was carried out chromatographically (3). *Chlamydomonas* lipids were extracted and purified from cells and fractions by a modified Bligh-Dyer method (5). Lipids in the chloroform extract (recovery > 98%) were separated by thin-layer chromatography (TLC) on Type-60 gel with fluorescent indicator (Merck, Darmstadt, Germany) (13). The resolved lipids were visualized nondestructively under ultraviolet light or destructively after charring with acid bichromate (5). When necessary, lipid was completely eluted from absorbent by extracting the gel (5). Deacylation of DGTS from both algae was carried out by mild base saponification (14). Water-soluble deacylation products, chromatographed on paper in a variety of solvents (15), were visualized under ultraviolet light after soaking the chromatogram with Rhodamine 6G (16) or by exposing the paper to iodine vapors (17). Chromatographic stains for phosphorus (18), sugars (19), and quarternary amino groups (20), were prepared and used as detailed; that for sulfur was purchased commercially ("Sulfvis"; Supelco, Bellefonte, PA).

Radiocompositional lipid analysis

Chlamydomonas cultures were exposed to [^{3}H]acetate, sodium salt (New England Nuclear, sp act 2.0 Ci/mmol; 800 μM final concentration) for one generation. Labeled cells were harvested and washed three times with fresh, sterile medium containing a 200-fold excess of nonlabeled sodium acetate. Total cellular lipid was extracted and purified from a portion of the washed algae. The remaining cells were fractionated to yield a thylakoid membrane preparation whose lipids were likewise purified. All other cellular material from the thylakoid isolation was similarly extracted, and its purified lipids were pooled to constitute the lipid routinely discarded during preparation of the thylakoid fraction. Cellular lipids and the lipids in the thylakoid and "pooled discard" subfractions were resolved on TLC and were detected as above. Lipid spots recovered from the plates were assayed for radioactivity by liquid scintillation spectrometry as previously described. The recovery of radioactivity through this procedure was >97%.

Fatty acid ester preparation and analysis

Purified DGTS was transesterified in 0.5 N sodium methoxide (21), and total fatty acid methyl esters were fractionated into subclasses based on unsaturation by argentation TLC (22). The fatty acid methyl esters, recovered quantitatively (>98% based on an internal standard of di[1-^{14}C]palmitoyl-PC (New England Nuclear)), were separated by gas–liquid chromatography on a 10% stabilized diethylene glycol succinate glass column (Supelco) in an HP 5830A gas chromatograph (Hewlett-Packard, Chicago, IL) operated isothermally at 200°C with carrier nitrogen flow at 25 ± 1 cc/minute. The response of the detector was calibrated with standard fatty acid methyl ester mixtures (Supelco; Analabs, North Haven, CT; Alltech, Arlington Heights, IL); all analyses were carried out well within the linear response range. Identification of fatty acids was the result of combined information from several sources, principally retention times of known commercial ester standards and mathematical analyses of retention time-chain length relationships under the conditions employed (cf. 23). Quantitation of peak areas on resulting chromatograms was through computer integration, and conversion of relative

ester areas to mole-percent composition was based on response factors obtained with the quantitative standards (23).

Nuclear magnetic resonance (NMR) spectra

Spectra of intact lipids were run in deuteriochloroform. Water-soluble deacylation products of mild alkaline hydrolysis (14) were purified by ion-exchange column chromatography on Bio-Rad 50W-X4 resin (Bio-Rad Laboratories, Richmond, CA) as described (3). Spectra of deacylated lipids were run in deuterium oxide. All spectra were obtained on Jeol FT-90Q and Bruker 270 Fourier-transform spectrometers with identical results, and peak area integration was computerized in all cases.

Lipid and enzyme assays

Quantitation of lipid recovered from TLC plates was done with a hydroxamate method (24), and lipid nitrogen was determined by microanalysis (25). Lipid phosphorus (26), sugar (27), and sulfur (28) were assayed spectrophotometrically.

5′-Nucleotidase (EC 3.1.3.5) activity was estimated as the inorganic phosphate liberated from 5′-AMP in the presence of sodium-potassium tartrate (10 mM, final concentration) to inhibit acid phosphatases (29). NADH cytochrome c reductases (EC 1.6.2.2) were assayed spectrophotometrically by following the reduction of cytochrome c in the absence or presence (1.0 μM, final concentration) of antimycin A (30). Nucleoside diphosphatase (EC 3.6.1.6) liberation of orthophosphate from inosine-5′-diphosphate was measured as described (31). Cytochrome c oxidase (EC 1.9.3.1) activity was assayed spectrophotometrically by following the oxidation of cytochrome c in the reaction mixture of Peters, Muller, and de Duve (32). For each enzyme, reported results were obtained under the specified assay conditions, within which linearity of activity with respect to time, protein concentration, and pH were verified for the tissue and fractions therefrom. Substrates were purchased from Sigma (St. Louis, MO).

Electron microscopy

Thylakoid membranes were fixed as pellets and embedded and stained for electron microscopy as described (8). Membrane areas were estimated by morphometry (33).

Statistical analyses

Statistical significance of the difference between two means was assessed with a Student-type *t*-test (34); the significance level was set at the ninety-fifth confidence interval, $P < 0.05$ indicating a statistically-significant difference.

RESULTS

Properties of the thylakoid fraction

A French-pressure-cell homogenate of *Chlamydomonas* carried through the series of differential and sucrose-gradient centrifugations described (6) produces in high yield a thylakoid membrane fraction representing over 75% of the 95% of chlorophyll recovered in the procedure. Since the protein-to-chlorophyll ratio in whole cells is 14.4 ± 1.1 (S.D.; n = 5) and in the thylakoid fraction is 5.3 ± 0.2 (S.D.; n = 5), the photosynthetic membrane is purified some 2.7-fold over the starting homogenate, on the reasonable assumption that cellular chlorophyll is exclusively localized in that membrane. The isolated thylakoids and the cell as a whole have identical chlorophyll *a* to chlorophyll *b* ratios, 2.3 ± 0.1 (S.D.; n = 5). Comparison between chromograms (A) and (B) of **Fig. 1** reveals that phosphatidylethanolamine (PE) and phosphatidylcholine (PC), prominent in extra-thylakoid membranes and often used as negative thylakoid lipid markers (35), are not found in the thylakoid fraction under conditions of chromatogram loading whereby a lipid component comprising less than 0.25% of the mixture applied can readily be resolved and detected.

Isopycnic sucrose density gradient analysis of the thylakoid membrane fraction demonstrates that its protein content, assayed chemically and radiochemically, parallels its chlorophyll content across the gradient. Constancy in the ratio of protein concentration to chlorophyll concentration through the membrane band and with the fraction loaded onto the gradient (~5.3 in all cases) indicates a high degree of fraction homogeneity. The membrane reaches equilibrium density at ~1.18 g/cm^2 (1.3–1.4 M sucrose), as has been reported for spinach photosynthetic lamellae (36) and for the thylakoids of *Chlamydomonas* isolated by a similar procedure (8). Therefore, non-green, extra-thylakoid membrane having a density significantly different from that of the photosynthetic membrane was not detectable in our thylakoid fraction.

Electron microscopic examination of the pelleted thylakoid preparation revealed a uniform population of swollen, vesiculated thylakoids which appear homogeneous in regard to membrane type and are easily identified as such (**Fig. 2**). Morphologically recognizable chloroplast contaminants were rare, consisting of an occasional starch plate. Chloroplast envelope fragments,

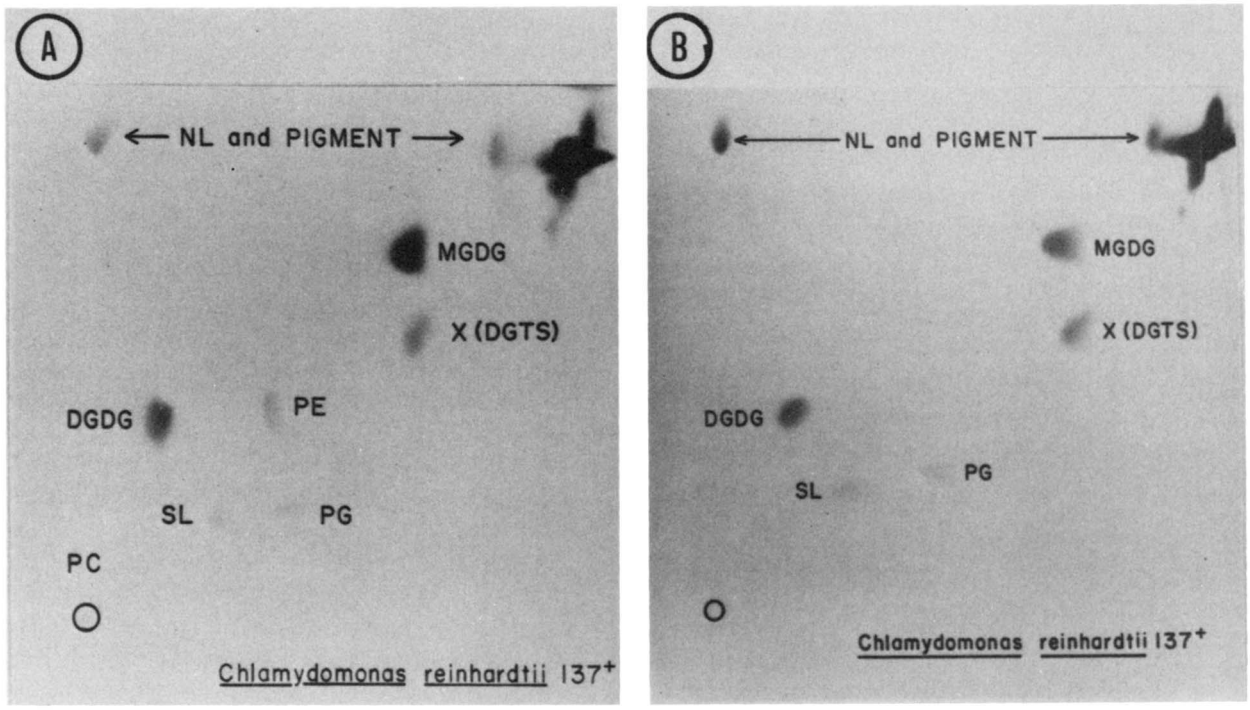


Fig. 1. Chromatograms of cellular (A) and thylakoid-membrane (B) lipids of *C. reinhardtii* 137+ resolved as described (13). O marks the origin. Detection by acid-bichromate charring.

which from *Chlamydomonas* are easily spotted as flattened, collapsed vesicles with dense crossbars spanning the opposed membranes (cf. 37), were absent in virtually every field examined. The total membrane area of all envelope remnants encountered in a fraction was far less than 0.5% of the total area of the thylakoid membrane vesicles in any one field. Extra-chloroplast contaminants, and the only significant contributor of non-thylakoid membrane, appeared limited to occasional swollen mitochondria. Recognizable rough microsomes (viz., rough endoplasmic reticulum), dictyosomes, nuclear fragments, cell walls, and flagellae were not encountered.

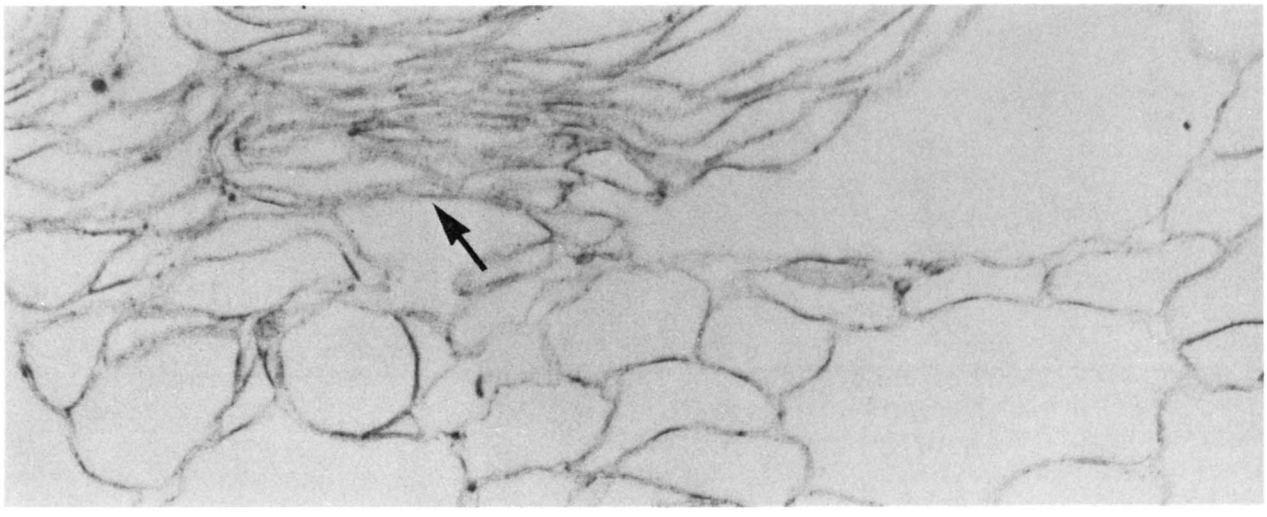


Fig. 2. Electron micrograph of the thylakoid fraction from *Chlamydomonas*. The field represents a pelleted thylakoid fraction isolated from the alga and prepared for microscopy as described in the text. Profiles appear homogeneous with respect to membrane type. An arrow highlights a slightly swollen and dismantled granum stack surrounded by more extensively swollen, deformed thylakoids still piled up as in the chloroplast in situ. X 21,000.

TABLE 1. Distribution of marker enzyme activities during *Chlamydomonas* fractionation

Fraction	Enzyme Activities Relative to Homogenate		
	Inosine diphosphatase	5'-Nucleotidase	Cytochrome c oxidase
Homogenate	100	100	100
Pooled differential centrifugation supernatants	52.6 ± 2.9	94.1 ± 6.1	78.2 ± 1.3
Gradient pellet + 1.8 M and 0.5 M sucrose layers	47.3 ± 2.6	8.5 ± 0.6	16.7 ± 1.0
Thylakoid fraction	n.d.[a]	n.d.[a]	3.9 ± 0.1
Activity recovered	99.9 ± 4.5	102.6 ± 6.0	98.8 ± 2.1

[a] n.d., none detected.

Enzymes were assayed under conditions specified (Experimental Procedures) in the three subcellular fractions generated during the purification of thylakoid membrane by the Chua and Bennoun procedure (6). Fraction activities are expressed as percent of total homogenate activity and have been summed to give a percent recovery figure. All numbers are means ± S.D. (n = 3). Results of other markers assayed are detailed in the text.

To quantitatively assess the contribution of any extrathylakoid membrane to the isolated thylakoids, a number of "marker enzymes" were assayed throughout the fractionation (**Table 1**). The enzymes assayed may be reliably used as negative markers for the thylakoid fraction, for none are found in the photosynthetic membrane (38). Quantitative recovery of inosine diphosphatase activity was achieved, and none was detected in the thylakoid fraction. Since at least some of the diphosphatase activity is associated with plant Golgi membrane (39), the biochemical results support the morphological absence of dictyosome membrane contamination in the photosynthetic lamellar fraction. Similarly, the quantitative recovery of 5'-nucleotidase activity and its absence in the thylakoid fraction demonstrate the lack of plasma membrane contamination (cf. 40).

In plant cells, antimycin A-insensitive NADH cytochrome c reductase is mainly associated with the endoplasmic reticulum-nuclear envelope system and also with the outer mitochondrial membrane, whereas an antimycin A-sensitive activity is in the inner mitochondrial membrane (38). Of the more than 98% of the total activity recovered in the fractionation, 11.5% was antimycin A-sensitive. No antimycin A-insensitive activity could be detected in the thylakoid fraction, demonstrating an absence of endoplasmic reticulum-nuclear envelope membrane therein. However, 3.4 ± 0.2% (S.D.; n = 3) of the total antimycin A-sensitive activity was found in the fraction, suggesting a contamination of isolated photosynthetic membrane by some 3–4% of the total mitochondrial population. Since mitochondria are the only organelles that would be expected to have a density similar to thylakoids in isopycnic centrifugations (38), mitochondrial membrane is the most likely contaminant of a thylakoid preparation, as has been demonstrated for a similar thylakoid fraction from *Chlamydomonas* (8). Therefore, a second mitochondrial marker, cytochrome c oxidase, was also assayed (Table 1). Of the >98% activity recovered, 3.9 ± 0.1% (S.D.; n = 3) is associated with the thylakoid fraction, confirming a general conclusion that 3–4% of the total cellular mitochondrial population is the only significant non-green membrane contamination of the isolated thylakoids.

Identification of DGTS in *C. reinhardtii* 137$^+$

Thin-layer chromatography of total *Chlamydomonas* cellular lipid (Fig. 1A) and total thylakoid-membrane lipid (Fig. 1B) in the system of Allen and Good reproducibly resolved a lipid spot at location "X" with R_f(x, y) of (0.61, 0.52). The lipid and its water-soluble deacylation product were negative for the presence of phosphorus, sugar, and sulfur as determined by both specific dyeing reagents and chemical assays. However, the lipid (intact or deacylated) was positive to detection reagent specific for quarternary amino groups. The intact form of the lipid purified from either *Chlamydomonas* cells or isolated thylakoids co-migrated with cellular DGTS from *Ochromonas* in a number of solvent systems, as did the respective water-soluble deacylation products. Quantitative correlation between the amount of nitrogen in the deacylation product and the amount of fatty acid released therefrom (either as methyl esters by GLC or as hydroxamic acids by hydroxamate assay) gave an intramolecular ratio among fatty acids:ester groups:nitrogen of 2:2:1, respectively.

The intact lipids from *Chlamydomonas* (whole cells and isolated thylakoids) and authentic DGTS from *Ochromonas* cells were analyzed in parallel by proton NMR with identical results at 90 and 270 MHz, save for some spin-spin splittings more noticeable at the higher ra-

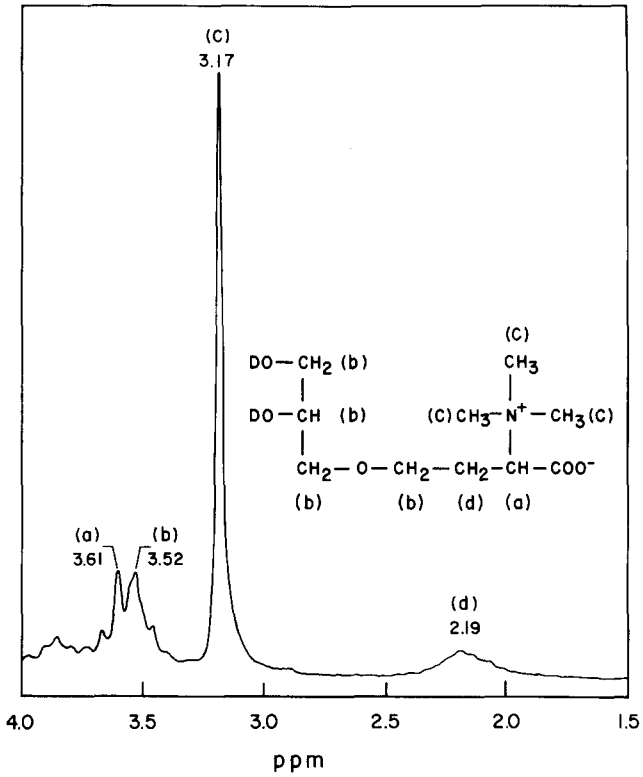
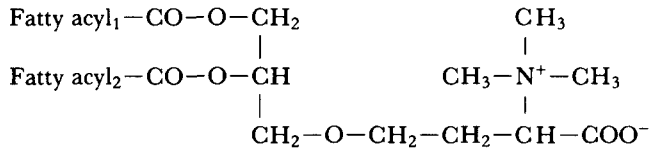


Fig. 3. NMR spectrum of deacylated, presumptive DGTS from the thylakoids of *Chlamydomonas*. Lipids of the alga's thylakoid fraction were chromatographed on TLC (13). The lipid spot at R_f(x, y) (0.61, 0.52) demonstrating chromatographic and chemical properties similar to *Ochromonas* DGTS was recovered and deacylated, and the water-soluble deacylation product was purified by ion-exchange chromatography and analyzed by Fourier-transform proton NMR as detailed under Experimental Procedures. Structural assignment of the spectral peaks has been made as indicated to the protons of deacylated *Ochromonas* DGTS in deuterium oxide. Chemical shifts are expressed as ppm from an external standard of tetramethylsilane. Identical spectra were obtained for presumptive *Chlamydomonas* cellular DGTS and known *Ochromonas* DGTS, when deacylated.

diofrequency (cf. 41). Spectra of the three intact lipids all displayed a major singlet at ~3.17 ppm which was attributable to the nine protons of the (*N,N,N*)-trimethyl moiety of authentic DGTS and which was in about the same position as that reported for PC (42). The intact lipid spectra also displayed a series of dominant peaks in the 0.80–2.5 ppm range, indicative of fatty-acyl group protons (41, 42). A collection of peaks at 3.4–3.7 ppm contained contributions from the protons of a glycerol moiety, as comparison with the spectrum of glycerolphosphorylcholine (42) indicated. The spectra of authentic *Ochromonas* and presumptive *Chlamydomonas* cellular and thylakoid DGTS, when deacylated, consisted of four prominent resonances at 3.61, 3.52, 3.17, and 2.19 ppm (**Fig. 3**). The singlet at 3.17 ppm, also prominent in the spectra of the three intact lipids, was now dominant and, as with the intact lipids, was attributable to the nine (*N,N,N*)-trimethyl protons (42). Peaks

at 3.61 (C—CH—COO⁻), 3.52 (O—CH), and 2.19 (—CH_2—) ppm can be assigned to the indicated protons in these moieties of a glycerol-homoserine residue (3, 41, 42). Computer integration gave a ratio of areas under these four peaks (a):(b):(c):(d), at 3.61 ppm, 3.52 ppm, 3.17 ppm, and 2.19 ppm (Fig. 3). of 1.17:7.21:9.07:2.09, respectively. This ratio is in reasonable agreement with the actual mass ratio of proton-types in deacylated *Ochromonas* DGTS, 1:7:9:2, for protons assigned to peaks (a):(b):(c):(d), respectively (Fig. 3). This chemical evidence, all fully in accord with our chromatographic and biochemical data and the data presented for the *Ochromonas* lipid (3), was considered sufficient to demonstrate structurally that the wild-type *Chlamydomonas* cellular and thylakoid-membrane lipids are DGTS and to assign to them the structure below:



Quantitation of DGTS in *C. reinhardtii* 137+

Chlamydomonas DGTS, resolved by TLC, was recovered and quantitated by hydroxamic acid ester analysis (**Table 2**). At the whole-cell level, DGTS comprised about 13% of the total polar acyl lipid mass. Although the cellular glycolipid complement was some five-fold greater, the amount of the ether lipid was equal to that of total phospholipid. Subcellularly, glycolipid again predominated in the thylakoids, yet DGTS comprised a significant portion (~10%) of thylakoid polar lipid, actually exceeding the amount of phospholipid (i.e., PG (43)) present in the membrane. On the assumption that chlorophyll is localized exclusively in the thylakoids, the ratio between the mass of DGTS in the thylakoids as standardized to chlorophyll and the mass in the cell so standardized should allow some assessment of the intracellular distribution of *Chlamydomonas* DGTS. This ratio, 0.38 ± 0.02 (S.D.; n = 5), indicates that about 40% of the cellular DGTS complement is found in the thylakoid membrane.

Since the present state of development of this alga's subcellular fractionation allows its analytical use only in the case of photosynthetic membrane (8), definitive localization of extra-thylakoid DGTS can not yet be

TABLE 2. Quantitation of DGTS in *C. reinhardtii* 137+

Lipid	Whole Cells		Thylakoid Membrane		"Pooled Remainder"
	Chlorophyll	Polar Acyl Lipid	Chlorophyll	Polar Acyl Lipid	Polar Acyl Lipid
	μg/mg	%	*μg/mg*	%	%
Total glycolipid	1940.8 ± 110.8	68.2 ± 4.0	1028.6 ± 37.7	80.4 ± 3.4	62.9 ± 5.0
Total phospholipid	391.3 ± 29.8	13.6 ± 1.0	118.8 ± 3.5	8.9 ± 0.3	23.3 ± 1.2
DGTS	344.5 ± 25.1	13.8 ± 0.9	131.4 ± 6.3	9.8 ± 0.5	13.1 ± 1.0
		Sum: 95.6 ± 6.2		Sum: 99.1 ± 3.6	Sum: 99.3 ± 4.2

Lipids from whole algal cells, either labeled for one generation with [^{3}H]acetate or not, and thylakoids isolated therefrom were extracted, chromatographed on TLC, and quantitated by a hydroxamate method and radiochemically as described under Experimental Procedures. Results are expressed as lipid mass normalized to chlorophyll mass and as % of total polar acyl lipid; this total is summed over the three lipid classes. Additionally, a "pooled remainder" fraction, consisting of all the cellular material discarded during thylakoid purification, was prepared from the radiolabeled cells, and its lipid profile was assessed radiochemically after TLC of the extracted lipids. Radiochemical % composition was taken as the distribution of label from [^{3}H]acetate among the chromatographically-resolved lipids; total recovery of label from the chromatograms was always >97%. The percent-composition figures for the whole cells and the thylakoid membrane represent combined biochemical and radiochemical data. Total glycolipid and total phospholipid represent sums of the various individual lipids of each type resolved by TLC (see Fig. 1). All numbers are means ± S.D. ($n \geq 3$).

made. Nonetheless, we have carried out a radiochemical analysis of the lipid profile of the cellular components discarded during thylakoid purification (the "pooled remainder") along with a corresponding lipid radioanalysis of the whole cell and the thylakoid fraction (Table 2). Lipids in the pooled remainder fraction are mainly from the cellular complement of non-green organelles, although some green membrane (represented by ~10% of the cellular chlorophyll) is also present therein. The "pooled remainder" fraction is markedly enriched in phospholipid over the thylakoids, consistent with the presence of PE and PC in the cell, but not in its thylakoids (Fig. 1). Conversely, galactolipid constitutes a higher (by ~23%) proportion of lipid in the thylakoids than in the discarded material. The focus of this study, DGTS, is found in greater proportion (by 1.3 ± 0.1-fold (S.D.; n = 3)) in the pooled remainder than in the isolated thylakoids. Therefore, the fractional contribution of DGTS outside the thylakoid membrane is somewhat greater than in the membrane itself.

Fatty acids of cellular and thylakoid DGTS

The extent to which the DGTS found in the thylakoids is representative of that in the cell as a whole was investigated through quantitative GLC analysis of methyl ester derivatives of the fatty acids esterified to DGTS from both levels (**Table 3**). A range of fatty acids, from C-14[3] to C-22, was found in cellular and thylakoid

TABLE 3. Major fatty acids of DGTS from *C. reinhardtii* 137+

Fatty Acid	Mole-percent in Cellular DGTS	Mole-percent in Thylakoid DGTS
14:0[c]	1.63 ± 0.15	2.23 ± 0.09
14:1[b]	1.02 ± 0.18	1.47 ± 0.05
14:3	1.54 ± 0.06	<1.0
16:0[d]	26.49 ± 0.89	25.91 ± 0.02
16:1[c]	2.90 ± 0.13	3.82 ± 0.14
16:2[d]	2.15 ± 0.27	2.01 ± 0.13
16:3[c]	1.38 ± 0.16	3.49 ± 0.25
18:0[c]	3.72 ± 0.18	5.65 ± 0.34
18:1[c]	6.91 ± 0.48	7.72 ± 0.06
18:2[d]	7.55 ± 0.22	7.27 ± 0.54
18:3[c]	2.34 ± 0.16	8.74 ± 0.77
18:4[a]	16.25 ± 1.59	13.19 ± 0.36
20:0[c]	1.44 ± 0.09	2.69 ± 0.10
20:1[c]	12.27 ± 0.87	5.48 ± 0.14
20:2[c]	6.52 ± 0.51	4.55 ± 0.08
20:3	<1.0	1.35 ± 0.08
20:4[d]	1.12 ± 0.03	1.08 ± 0.03
22:2[c]	2.07 ± 0.07	1.22 ± 0.07
	Sum: 97.28 ± 3.02	Sum: 97.87 ± 2.83
Unsaturated/saturated:[d]	1.92 ± 0.14	1.68 ± 0.15

[a] $P < 0.05$, Student's *t*-test.
[b] $P < 0.01$.
[c] $P < 0.001$.
[d] Statistically insignificant difference ($P \geq 0.05$).

Methyl ester derivatives of fatty acids prepared and analyzed as described (Experimental Procedures) and comprising >1.0 mole-percent of the total cellular and thylakoid-membrane DGTS fatty acid complements are quantitated. The sum of the tabulated mean mole-percentages is indicated for each group, as is the ratio of unsaturated-to-saturated fatty acids therein. All values are the mean ± S.D. for eight determinations. *t*-Test *P*-values indicate the significance of the difference between the cellular and thylakoid means for any one fatty acid.

[3] Two shorthand notations for fatty acids are used. The number of carbon atoms in a fatty acid family is designated numerically (e.g., C-16 denotes the hexadecanoic series). Individual fatty acids are denoted by two numbers separated by a colon. The first number signifies the carbon chain length; the second, the number of *cis* unsaturated bonds.

DGTS. Cellular DGTS contained the greater proportion of C-20 acids, but less fatty acids of the C-18 family. Although there were no significant differences in the amounts of saturated and tetraene fatty acids, DGTS of the thylakoids was relatively richer in trienes. However, thylakoid DGTS contained less monoenes and dienes than cellular DGTS as a whole, making for an unsaturated:saturated ratio of about 1.8 at both levels. No *trans* unsaturated acids were detected by argentation TLC of the total complement of DGTS fatty acid esters.

Fatty acids of the C-16 and C-18 families comprised about 70–75% of the total fatty acids at both levels, with 16:0 and 18:4 predominant. As indicated by the *t*-test *P*-values, significant quantitative differences exist in the distributions of most of the C-16 and C-18 acids (16:0, 16:2, and 18:2 as exceptions). Cellular DGTS had the greater amount of 18:4, whereas thylakoid DGTS was notably richer in 14:0, 16:1, 16:3, 18:0, 18:1, and 18:3.

DISCUSSION

Detection of the ether-linked homoserine lipid DGTS in an arginine-requiring, streptomycin-resistant mutant of *Chlamydomonas* (strain sr_3 (44)) suggested that the lipid may not be limited to *Ochromonas* (3), but may also augment the phospho- and glycolipids usually found in green plant tissue and in the higher algae. The qualitative finding of DGTS in crude photosystem I and II particles prepared from this *Chlamydomonas* mutant (45) raised a further possibility that DGTS might be a constituent of the thylakoid membrane. Strong alternatives could not be ruled out, however, that the lipid might be an artifact or a peculiarity of the mixotrophically-grown mutant, since one study on the lipids of wild-type *Chlamydomonas* (strain 137^+ (46)) and others on a spontaneous yellow mutant therefrom (strain y-1 (37, 47)) had not detected it (cf. 1).

Our studies establish that DGTS, constituting some 13% of the polar lipid complement, is a prominent cellular lipid in photosynthetic, wild-type *Chlamydomonas*. The contribution of DGTS to the total polar lipid mass of *Chlamydomonas* is some four-fold less than to that of *Ochromonas*, due, at least in part, to the striking paucity of phospholipid in the golden alga (3). Detailed molecular comparisons among the fatty acids of DGTS from different organisms must be deferred until sufficient data are available regarding *Ochromonas* and the *Chlamydomonas* mutant.

In view of the demonstrated integrity and virtual homogeneity that characterize the analyzed thylakoid preparation and of the results of our chemical and biophysical analyses, we conclude that DGTS is a significant polar acyl lipid of the chloroplast thylakoid membrane in the green alga *Chlamydomonas*. Such an association between DGTS and the photosynthetic membrane of any plant cell has not been reported, but has been speculated upon (1). Although *Ochromonas*, the only other cell in which DGTS has been definitely identified, contains abundant thylakoid membrane when light-grown (48), subcellular fractionation has not yet been applied to this alga in an attempt to purify photosynthetic (or any other intracellular) membrane. Study of the flagellar membrane lipid of *Ochromonas* (49), however, had not identified DGTS in that subcellular structure. Our results thus establish DGTS as a bona fide membrane lipid.

The original identification of DGTS in *Ochromonas* cells which, because of growth under minimal (i.e., ambient) light, had developed only a rudimentary thylakoid system prompted speculation (3) that DGTS in this Chrysophyte may be localized in extra-thylakoid membrane as a substitute for phospholipid. The amounts of phospholipid in the photosynthetic membrane and, especially, in the "pooled remainder" fraction from wild-type *Chlamydomonas* do not support a conclusion that DGTS substitutes for phospholipid in this green plant cell. An intriguing possibility is that the postulated (50) parallel divergent evolution which the Chrysophytes and Chlorophytes are thought to have undergone may be manifest in lesser amounts of DGTS, but greater amounts of phospholipid, in *Chlamydomonas* relative to *Ochromonas*. The most noteworthy role of *Chlamydomonas* DGTS would appear to be as a zwitterionic contributor of charge to the photosynthetic membrane, in which uncharged glycolipids predominate (51).

Quantitative differences in esterified fatty acids between DGTS from whole *Chlamydomonas* and from its thylakoid membrane indicate that thylakoid DGTS forms a distinctive subpopulation in situ at the molecular level. Since the synthetic pathway of this lipid and the intracellular site(s) of its synthesis are not known, one can only speculate as to how the alga discriminates between the species of DGTS destined for the thylakoid membrane and those presumably destined for other supramolecular assemblies. Our study, therefore, raises questions about the metabolism of DGTS and the role of this lipid in thylakoid biogenesis that will be addressed in subsequent reports.

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