Intracellular Localization of Two Betaine Lipids by Cell Fractionation and Immunomicroscopy

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The cellular localization of the betaine lipids diacylglyceryl-*N,N,N*-trimethylhomoserine (DGTS) and diacylglycerylhydroxymethyl-*N,N,N*-trimethyl-β-alanine (DGTA) was investigated by a) chemical analysis of subcellular fractions and b) immunochemical methods using specific antisera and either fluorescence microscopy or electron microscopy for detection of the label. A homogenate of *Lycopodium annotinum* (Pteridophyta) was fractionated by differential and density gradient centrifugation. The particulate fractions obtained were analyzed for chlorophyll, cyt c oxidase, NADH-cyt c reductase and DGTS. Non-plastidial fractions were enriched in DGTS and only minor amounts of this lipid could be attributed to chloroplasts. Anti-DGTS and anti-DGTA sera were produced by immunization of rabbits. The monospecificity of the antisera was examined with cells of *Chlamydomonas reinhardtii* (Chlorophyceae) containing DGTS, *Pavlova lutheri* (Haptophyceae) containing DGTA and *Ochromonas danica* (Chrysophyceae) containing both DGTS and DGTA. *Euglena gracilis* which is free of betaine lipids, was used as a control. For the test, a FITC-coupled goat anti-rabbit antibody was used and detected by fluorescence microscopy. Thin sections of *Ochromonas* and *Pavlova* were incubated first with the anti-lipid sera and subsequently with a gold-coupled anti-rabbit serum and then examined in the electron microscope. With *Ochromonas*, anti-DGTS as well as anti-DGTA sera gave an accumulation of gold label in the cytoplasmic space but not in the chloroplasts. Similar results were obtained with *Pavlova* using anti-DGTA serum. These results describe for the first time the cytochemical localization of DGTS and DGTA strongly suggesting both these lipids to be associated mainly with non-plastidial structures.

Introduction

The membrane lipid pattern of non-flowering plants including ferns, mosses, algae, lichens and fungi, considerably differs from that of higher plants by the presence of additional constituents and/or the absence of certain common lipids of higher plants. A typical feature of many of these organisms is the production of betaine lipids which beside glycosyl- and phosphoglycerides represent a third group of plant membrane lipids and which are generally absent from higher plants (Eichenberger, 1993). At the present time, three different compounds have been detected with the structure of diacylglyceryl-*N,N,N*-trimethylhomoserine (DGTS) (Brown and Elovson, 1974) diacylglycerylhydroxymethyl-*N,N,N*-trimethyl-β-alanine (DGTA) (Vogel *et al.,* 1990) and diacylglycerylcarboxy-*N*-hyroxymethylcholine (DGCC) (Kato *et al.,* 1996). Their common structural element is a polar moiety containing a trimethylammonium group in combination with a carboxyl group. This betaine-like zwitterionic polar part is linked to the glycerol moiety either by an *O*-ether bond as in DGTS and DGTA or by an acetal linkage as in DGCC. The structure and natural distribution of betaine lipids has recently been reviewed (Dembitsky, 1996; Eichenberger, 1993; Künzler and Eichenberger, 1997; Sato, 1992).

Abbreviations: Cyt *c*, cytochrome *c*; DGCC, diacylglycerylcarboxy-*N*-hydroxymethylcholine; DGDG, digalactosyldiacylglycerol; DGTA, diacylglycerylhydroxymethyl-*N,N,N*-trimethyl-β-alanine; DGTS, diacylglyceryl-*N,N,N*-trimethylhomoserine; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; MGDG, monogalactosyldiacylglycerol; PBS, phosphate buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol.

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The zwitterionic properties of betaine lipids resemble those of the common phospholipid phosphatidylcholine (PC) suggesting the two constituents to have the same or similar functions in the membrane. Evidence for such a complementary role was obtained from algae which produce betaine lipids but not PC in detectable amounts. Examples are the DGTS-producing green algae *Chlamydomonas reinhardtii* (Giroud *et al.*, 1988) and several species of the genus *Ulva*, then brown algae of the orders Fucales and Dictyotales which synthesize DGTA (Eichenberger *et al.*, 1993) and finally, members of the Haptophyceae which produce DGCC (Kato *et al.*, 1996). Betaine lipids are most likely involved in both desaturation and exchange of fatty acids in *Chlamydomonas* (Giroud and Eichenberger, 1989; Grenier *et al.*, 1991; Schlapfer and Eichenberger, 1983), *Ochromonas* (Vogel and Eichenberger, 1992), *Acetabularia* (Stirnimann, 1993) and *Pavlova* (Eichenberger and Gribi, 1997) suggesting betaine lipids to fulfill in these organisms a similar metabolic role as does PC in the eukaryotic pathway of lipid biosynthesis in plants (Roughan and Slack, 1982).

Despite several attempts based on subcellular fractionation of different organisms, the cellular site of betaine lipids remained a matter of controversy. Isolated chloroplasts from *Acetabularia mediterranea*, *Eremosphaera viridis*, *Chlamydomonas reinhardtii* (Chlorophyta), *Polytrichum commune* (Bryophyta), *Lycopodium annotinum* and *Equisetum maximum* (Pteridophyta) contained insignificant amounts of DGTS and suggested betaine lipids to be extraplastidial constituents (Eichenberger, 1993). Similar results with *Dunaliella salina* (Chlorophyceae) chloroplasts were obtained by Sheffer *et al.* (1986). In other experiments with *Chlamydomonas reinhardtii* (Janero and Barnett, 1982) and *Dunaliella salina* (Norman and Thompson, 1985), in contrast, appreciable amounts of DGTS could be attributed to the chloroplast. It is to mention that all these experiments had dealt with the localization of DGTS only.

Thus, we made a new attempt to elucidate the subcellular site of both DGTS and DGTA in appropriate organisms. On the one hand, subcellular fractionation was carried out using *Lycopodium annotinum* (Lycopodiaceae, Pteridophyta). On the other hand, immunochemical methods were applied based on the use of antisera specifically interacting with individual lipids as described by Radunz (1971, 1972, 1976) and Radunz and Berzborn (1970). For the detection of fluorescent lipid-coupled antibodies on whole cells, fluorescence microscopy was used. In addition gold-labelled antibodies were detected *in situ* on ultrathin sections of cells by electron microscopy.

Materials and Methods

Isolation of membranes

Field-grown plants (50 g) of *Lycopodium annotinum* collected one day before the isolation were ruptured by passing through a roller mill with 250 ml of buffer according to Lord (1987) and Pfaffmann (1988) containing 30 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid)-NaOH pH 7.6; 0.4 M sucrose, 5 mM ascorbic acid; 0.1% BSA; 5 mM cysteine; 1 mM EDTA; 10 mM KCl; 1 mM $MgCl_2$ and 2% polyvinylpyrrolidone (PVP insoluble, Polyclar AT SERVA). The suspension was then filtered through four layers of cheese cloth. After sedimentation of PVP (200 x *g*, 10 min) the homogenate was centrifuged at 2000 x *g* for 20 min. The pellet mainly containing chloroplasts was purified on a discontinuous sucrose gradient (10 ml of each 25%, 37% and 45% w/w) during 30 min at 2300 x *g*. The chloroplasts banded between the 25% and 37% layers and were recovered by diluting with water and centrifuging for 15 min at 10000 x *g* (fraction C).

The supernatant of the first run at 2000 x *g* was centrifuged at 10000 x *g* during 20 min and then at 20000 x *g* during 15 min to obtain two mitochondrial fractions (fractions 1 and 2). Two subsequent ultracentrifugations at 100000 x *g* for 1 h each yielded two microsomal fractions (fractions 3 + 4). The membranes were carefully suspended in the homogenization medium from which BSA and PVP were omitted. All the steps were carried out at 4 °C.

Characterization of membrane fractions

Protein was determined according to Bradford (1976), and chlorophyll according to Lichtenthaler *et al.* (1982). Cytochrome *c* oxidase was measured according to Tolbert *et al.* (1968) and cyanide-insensitive, antimycine-insensitive NADH-cyto-

chrome *c* reductase according to Briskin *et al.* (1987). All assays were carried out twice immediately after the membrane preparation at 20 °C.

Quantification of lipids

The membrane suspensions were extracted with at least 10 times the volume of MeOH containing 0.05% butylhydroxytoluene (BHT) as an antioxidant. To remove non-lipid constituents, extracts were evaporated to dryness and to the residue diethyl ether and saturated NaCl solution were added. This leads to a 2-phase system with the lipids dissolved in the upper ether phase which was dried under N_2 flow. Lipids (about 1 mg) were spotted on silicagel plates $60F_{254}$ (Merck, 1.05715) and separated with $CHCl_3$-MeOH-H_2O (65:25:4, v/v) (solvent I) in the 1st dimension and with $CHCl_3$-MeOH-isopropylamine-conc. NH_3 (65:35:0,5:5, v/v) (solvent II) in the 2nd dimension. For the identification of lipids, the plate was sprayed first with Dragendorff's reagent (Munier and Macheboeuf, 1951). DGTS and DGTA gave an orange color which was intensified by spraying the plate thereafter with molybdenum-blue reagent (Dittmer and Lester, 1964) which in the same time made appear the phospholipids as blue spots. For the quantification of lipids, the spots were vizualized first under UV 254 nm and 366 nm after spraying with 2',7'-dichlorofluorescein (0.05% in ethanol). The lipids were scraped off and measured photometrically by using the methods of Chen *et al.* (1956) for phospholipids and of Heinz (1967) for glycolipids. The betaine lipid DGTS was quantified by measuring its constituent fatty acids by GLC using arachidic (20:0) acid methyl ester as an internal standard (Vogel and Eichenberger, 1992).

Isolation of lipids used as antigens

For the isolation of DGTS and DGTA, lipid extracts from *Chlamydomonas reinhardtii* and from *Fucus serratus,* respectively, were used. Total lipid (40 mg) was dissolved in 2 ml dichloromethane and spotted on a TLC plate (silicagel G type 60, Merck 7731). Solvent I was used for the isolation of DGTS and solvent II for DGTA. Spots were detected by spraying the edge of the plate. Lipids were eluted with methanol and, after evaporation of the solvent, dissolved in toluene and stored under N_2 at -20 °C. A final examination of the purity on a TLC plate (Merck 5715) using solvent I gave a single spot.

Preparation of the antiserum

DGTS (2 mg) dissolved in 5 ml toluene and 1 mg methylated bovine serum albumin dissolved in 5 ml of physiological saline were emulgated with a few drops of ethanol by sonication. Toluene was removed from the mixture by repeated evaporation and addition of ethanol. Then, the volume of the mixture of DGTS and serum albumin was concentrated to at maximum 1 ml. This suspension was emulgated with 1 ml of Freund's adjuvant and injected subcutaneously at two different sites into the back of a rabbit. After 25 days, the antigen suspension without Freund's adjuvant was injected 6 times in a two-days rhythm into the ear vene of the rabbit. After the last injection, blood collection was started and continued in intervals of one week during 12 weeks. Control sera withdrawn from the animal before treatment and antisera were stored at −70 °C.

Testing of antiserum specificity

The specificity of the antisera was tested by using both the dot blot procedure (Voss *et al.,* 1992; Schmid *et al.,* 1993) and the precipitation reaction with an antigen-protein suspension. The protein suspension contained 2 mg lipid and 1 mg ovalbumin per ml.

Plant material

Chlamydomonas reinhardtii, cell wall-weaky mutant cw-15 (strain cc-1615 from Chlamydomonas Genetics Center, Duke University, Durham, NC, USA), was cultivated in a medium according to Sager (1954). *Euglena gracilis* (Algal Collection, University of Göttingen) was grown in Difco Bacto-Euglena Broth at 20 °C. *Ochromonas danica* (strain 933/2b, The Culture Centre of Algae and Protozoa, Cambridge, U. K.) was cultivated in a medium according to Aaronson and Baker (1959) at 24 °C. *Pavlova lutheri* (Droop) Green strain 931/1 (Culture Collection of Algae and Protozoa, Dunstaffnage Marine Laboratory, Scotland, U. K.) was grown in sea water (28‰ salinity) supplemented with PES according to Starrr and Zei-

kus (1993) at 20 °C. All organisms were grown in Erlenmeyer flasks (150 ml) in 35 ml medium under continuous white fluorescent light (60 μE m^{-2} s^{-1}) and shaking. Field-grown *Fucus serratus* was harvested at the French Brittany coast and then frozen in liquid N_2 and ground before extraction.

Immunofluorescence

The cells were centrifuged at 2000 x *g* (4200 rpm) in a Eppendorf Centrifuge 5415 and the pellet was washed twice with phosphate buffered saline (PBS, Dulbecco A, Nr. RO22485–002), pH 7.3). Then the cells were fixed by suspension in a solution of paraformaldehyde 3% for 15 min at 20 °C, washed with PBS and incubated with fish-skin gelatine 2% in order to block unspecific binding sites. The incubation with the rabbit antilipid serum (= first antibody, diluted 1:50 with PBS containing 0.5% fish-skin gelatine) was done for 1 hour at room temperature.

Cells were washed in PBS and then incubated with the second antibody which was a goat anti-rabbit IgG F(ab')2, conjugated with FITC (Pierce, Rockford, IL, USA) and diluted 1:200 with PBS. This step was carried out in the dark for 1 hour at room temperature. After washing the cells several times in PBS, the pellet was spotted on a coverslide and the fluorescence was observed in a fluorescence microscope (Zeiss Axiovert 35) at 450–490 nm with a LP 520 filter at a 1000-fold magnification. For photographs, a Kodak Ektachrom Elite 400 ASA film was used.

Immunoelectron microscopy

Fixation, dehydration, and embedding

All steps were carried out at 4 °C. The pellet of cell material was washed in 100 mM Na_2HPO_4 pH 7.0 (= EM-buffer) and fixed with EM-buffer containing 0.1% glutaraldehyde and 3% paraformaldehyde for 1 hour at 0 °C. Then the suspension was transferred into a aldehyde-blocking solution containing 50 mM glycine in EM-buffer for 1 hour at 0 °C. For the stepwise dehydration, cells were suspended for 10 min at -20 °C consecutively in 30%, 50%, 70% and 90% ethanol. Then the cells were transferred in acrylic resin (LR-White, medium grade) in a polypropylene tube and put on ice. The resin was replaced tree times every 12 hours. The specimen were purged with N_2, evacuated and polymerized at 60 °C during 12 hours. Ultrathin sections were obtained by cutting with a diamond knife (Diatome MC 368) on an ultramicrotome (Ultracut, Reichert & Jung). The sections were picked up with Ni-grids (200 mesh, 3 mm) coated with a Formvar carbon film.

Immunogold labelling

The side of grids holding the sections were layed on drops of blocking solution containing 0.5% fish-skin gelatine and 50 mM glycine in PBS for 1 hour at room temperature in a humid chamber. Then they were transferred on drops containing anti-DGTS- or anti-DGTA serum diluted 1:20 with blocking solution. After 1 hour of incubation, the grids were washed on drops of PBS and then incubated for 1 hour with the second antibody (goat anti-rabbit IgG, conjugated with 20 nm colloidal gold) diluted 1:50 with PBS containing 0.1% fish-skin gelatine and 0.05% Tween 20. After washing with PBS and drying, the preparations were stained using uranyl acetate 2% for 5 min in the dark. The grids were then washed in bidistilled water and transferred for at least 5 min on a solution of lead citrate 2% in a NaOH saturated chamber.

For electron microscopy, a TEM (Hitachi H 600) at 100 kV was used. Photographs were taken with Agfa Scientia EM-film 23D56 P3 AH, 9 x 12 cm.

Results

Subcellular fractionation

For the homogenization and subsequent isolation of subcellular fractions, *Lycopodium annotinum* was chosen, because field-grown plants are easily available and contain considerable amounts of DGTS. The different fractions obtained from the homogenized plant material were analyzed for chlorophyll, protein and the activity of both cyt *c* oxidase and NADH-cyt *c* reductase which are used as marker enzymes for mitochondria (Tolbert *et al.*, 1968) and endoplasmic reticulum (Briskin *et al.*, 1987), respectively.

The results, as presented in Table I indicate that the chlorophyll concentration was highest in Frac-

Table I. Content of markers in subcellular fractions of *Lycopodium annotinum*. Values are means of 3 experiments.

Marker	Fraction C	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Supernatant
Chlorophyll (μg/mg protein)	267	86	106	59	16	2
Cyt *c* oxidase (nmol/min x 100 μg protein)	–	62	45	98	3	–
Cyt *c* reductase (nmol/min x 100 μg protein)	83	23	17	61	117	29

tion C collected from the dark-green band of the sucrose density gradient.

Although this fraction was rich in unbroken chloroplasts, as observed in the light microscope, it contained considerable amounts of NADH-cyt *c* reductase indicating the presence of cytoplasmic impurities. Minor amounts of chlorophyll were also found in Fractions 1–3 and ascribed to chloroplast fragments. The activity of cyt *c* oxidase representing mitochondrial membranes were also distributed among Fractions 1–3 with the highest concentration in Fraction 3. Fraction 4 was enriched in NADH-cyt *c* reductase indicating that it mainly contained ER membranes.

The particle fractions obtained were analyzed for glyco- and phospholipids and for DGTS.

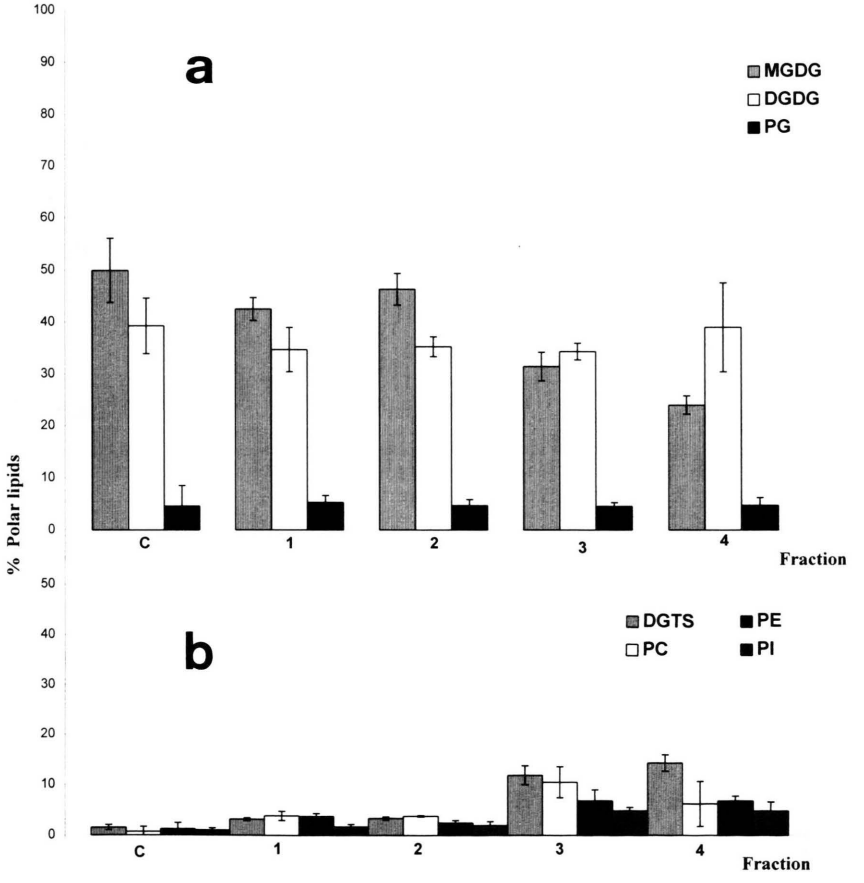


Fig. 1. MGDG, DGDG, PG, DGTS, PC, PE and PI in different fractions of *Lycopodium annotinum*. C = chloroplast fraction, 1 = 10000 x *g* pellet, 2 = 20000 x *g* pellet, 3 = first 100000 x *g* pellet, 4 = second 100000 x *g* pellet. Values are means of three experiments.

DGTS accumulated in Fractions 3 and 4 accounting for 11% and 14% of the polar lipids, respectively, as shown in Fig. 1b. The accumulation of both PE and PC in the same fractions is in keeping with the current view that PE (Mazliak, 1977) and most part of PC (Mudd, 1980) are localized in microsomal membranes. This strongly suggests DGTS to be a constituent of microsomal membranes. The small amount of PE and, hence, also of DGTS, in the chloroplast fraction most likely originates from microsomal impurities indicating that chloroplasts contain, if at all, very minor amounts of this lipid. The plastidial constituents MGDG and DGDG, like chlorophyll were present in all the particulate fractions (Fig. 1a). In Fractions C, 1 and 2, the molar ratio of MGDG/DGDG was around 1.25 according to the quantitative predominance of MGDG in chloroplasts as reported by Douce and Joyard (1980). The excess of DGDG in Fractions 3 and 4 might be explained by an accumulation of chloroplast envelope membranes which contain more DGDG than MGDG (Douce and Joyard, 1980). PG as expected, was found to be distributed among the different subcellular fractions.

Immunochemical localization of DGTS and DGTA

The immunochemical approach for localizing the betaine lipids within the cell was chosen mainly for two reasons. On the one hand, several studies on the cellular site of DGTS had been carried out (Eichenberger, 1993; Janero and Barnett, 1982; Norman and Thompson, 1985; Sheffer *et al.*, 1986), while no attempts had been undertaken for the localization of DGTA. The DGTA-containing organisms, however, belong to the chromophyte algae which by both their morphology and constituents are much different from green algae or vascular plants and therefore not appropriate for cell rupture and fractionation. On the other hand, specific antisera had been successfully used for the detection and localization of lipid constituents in the past (Radunz 1971, 1972, 1976; Radunz and Berzborn, 1970). Therefore, specific antisera were produced by immunization of rabbits with either DGTS or DGTA.

For the experiments, four different organisms were used, namely *Chlamydomonas reinhardtii* (Chlorophyceae) containing DGTS only, *Ochromonas danica* (Chrysophyceae) which contains both DGTS and DGTA, *Pavlova lutheri* (Haptophyceae) containing DGTA only and, as a control, *Euglena gracilis* (Euglenophyceae) which is free of betaine lipids. The specificity of the two antisera were examined by incubation of cells with either the anti-DGTS or the anti-DGTA serum. In order to facilitate the access of the antibodies to the membrane lipids, the cells were pretreated with paraformaldehyde solution. After the first incubation, the cells were incubated with a fluorescent goat anti-rabbit serum. Coupling of the second antibody could be detected in the fluorescence microscope by a typical bright yellow-green fluorescence. With controls, in contrast, the deep-red fluorescence of chlorophyll only could be observed. *Chlamydomonas* cells gave a positive reaction with anti-DGTS serum, as shown in Fig. 2.

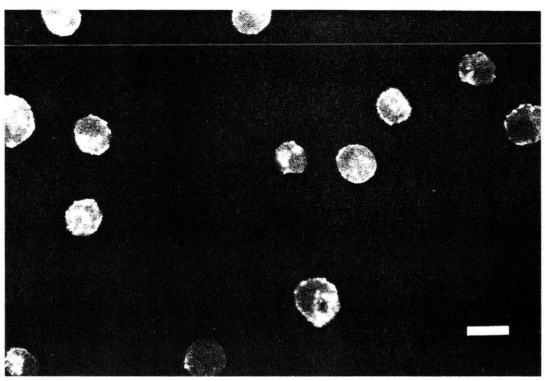


Fig. 2. Fluorescence micrograph of *Chlamydomonas reinhardtii* incubated with anti-DGTS serum and FITC-conjugated second antibody. Bar is equivalent to 10 μm.

No typical fluorescence was observed using anti-DGTA or control serum. Cells of *Ochromonas* reacted with both anti-DGTS and anti-DGTA serum, as expected for an organism containing both these lipids. *Pavlova*, finally, gave the typical fluorescence with anti-DGTA serum only (results not shown). With *Euglena* lacking any kind of betaine lipids, the deep-red fluorescence of chlorophyll only was observed, as shown in Fig. 3.

These results indicated the monospecifity of both the anti-DGTS and anti-DGTA sera which reacted exclusively with cells containing the corresponding lipid. Due to the low resolution of the light microscope, a further localization of the lipids within the cell was not possible with this method.

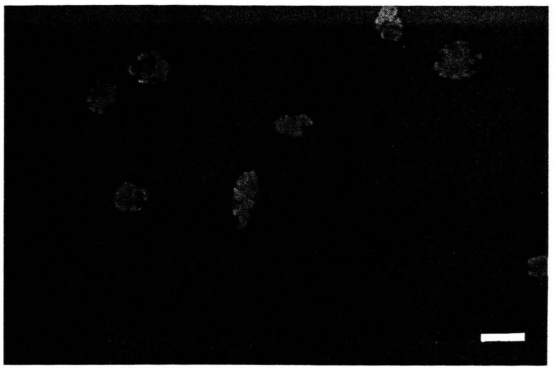
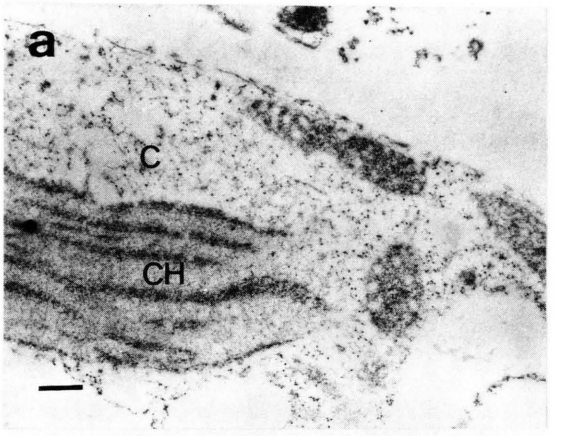


Fig. 3. Fluorescence micrograph of *Euglena gracilis* incubated with anti-DGTS-serum and FITC-conjugated second antibody. Bar is equivalent to 10 μm.



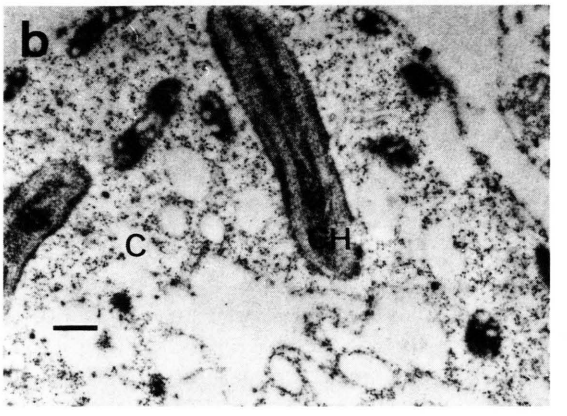


Fig. 4. Electron micrograph of *Ochromonas danica* incubated with
a) anti-DGTS-serum and gold-conjugated second antibody;
b) anti-DGTA-serum and gold-conjugated second antibody.
CH = chloroplast, C = cytoplasm. Bar is equivalent to 0.4 μm.

Consequently, the antigen-antibody coupling was applied to ultrathin sections of cells which had been embedded in acrylic resin before. The sections were treated first with the antiserum and then incubated with a second antibody conjugated with gold particles. After staining with uranyl acetate and lead citrate the samples were examined in the electron microscope.

In cells of *Ochromonas* treated with anti-DGTS serum, gold particles were concentrated in the extraplastidial regions, while only few of them appeared in the plastidial structures, as shown in Fig. 4a.

Similarly, in cells treated with anti-DGTA serum, gold particles also accumulated in non-plastidial structures, as shown in Fig. 4b.

Insignificant label and therefore no coupling of antibodies was found in *Euglena*, as shown in Fig. 5.

Correspondingly, thin sections of cells from *Pavlova* reacted with anti-DGTA only and the label accumulated in the non-plastidial compartment, too (not shown).

Discussion

The analysis of different subcellular fractions of *Lycopodium annotinum* strongly suggest the major part of DGTS to be localized in non-plastidial membranes. Chloroplasts, in contrast, contain only minor portions. Similar results are presented here for the first time also by using immunochemical methods. The monospecificity of the antisera against DGTS and DGTA was confirmed with whole cells by fluorescence microscopy. The antisera did not produce cross reactions, nor did they react with cells which do not contain betaine lipids.

It is interesting to note that with naked cells of *Ochromonas* containing both DGTS and DGTA, a positive reaction was obtained only after pretreatment of cells with paraformaldehyde as a fixative agent. Since, with intact cells, the plasma membrane only is exposed to the antibodies, this effect can be explained either by a non-accessibility of the lipid molecules for sterical reasons or by the absence of betaine lipids from the outer leaflet of the plasma membrane. Attempts to isolate

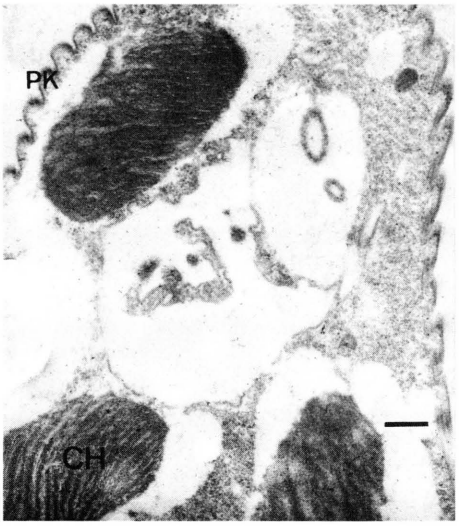


Fig. 5. Electron micrograph of *Euglena gracilis* incubated with anti-DGTS-serum and gold-conjugated second antibody. CH = chloroplast, PK = pellicle. Bar is equivalent to 0.44 μm.

plasma membranes from *Ochromonas* were not successful, because during cell disruption, structures were rapidly destroyed by the effect of very active hydrolases of this organism.

Immunoelectron microscopy with *Ochromonas* clearly showed an accumulation of gold label in the cytoplasmic compartment, while the label in chloroplastic structures was negligible. Since the same result was obtained with both anti-DGTS and anti-DGTA sera, we conclude that both these lipids are concentrated in non-plastidial membranes. As to DGTS, these findings are in full accordance with the data obtained by chemical analysis of subcellular fractions from *Acetabularia*, *Chlamydomonas*, *Eremosphaera*, *Polytrichum* and *Equisetum* (Eichenberger, 1993) and *Lycopodium*.

As to DGTA, the immunoelectron micrographs from *Ochromonas* for the first time demonstrate this lipid to be concentrated in the cytoplasmic compartment rather than in plastidial structures. The same subcellular distribution of DGTA was found in *Pavlova* which contains DGTA but no DGTS. It is interesting to note that with *Euglena* used as control, the immunolabelling was insignificant in all experiments. The view that DGTS and DGTA have to be attributed to the same subcellular compartment, is also in accordance with the fact that DGTS acts as a biochemical precursor of DGTA, as demonstrated in *Ochromonas* (Vogel and Eichenberger, 1990) and *Sphacelaria* (Eichenberger and Hofmann, 1992).

It is interesting to note that in *Pavlova*, the third betaine lipid DGCC, could be attributed to non-plastidial membranes, too (Eichenberger and Gribi, 1997). Further investigations will be necessary to clearly identify the membranes to which betaine lipids are associated.

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