Plant Cell Physiol. 33(1): 41–48 (1992)

Lipid Bodies in *Eremosphaera viridis* De Bary (Chlorophyceae)

Burkhard Vechtel[1], Waldemar Eichenberger[2] and Hans Georg Ruppel[1]

[1] *Department of Biology, University of Bielefeld, D-4800 Bielefeld, F.R. Germany*
[2] *Department of Biochemistry, University of Bern, CH-3012 Bern, Switzerland*

Under conditions of stress, e.g., nitrogen deficiency, *Eremosphaera viridis* De Bary (Chlorophyceae, Chlorococcales) synthesized secondary carotenoids and large amounts of triacylglycerols forming orange-red, cytosolic lipid bodies. Additionally, four polypeptides (28, 26, 25 and 23 kDa) as well as traces of chlorophyll *a* and *b*, of violaxanthin, neoxanthin and zeaxanthin, and of membrane lipids could be demonstrated in isolated lipid bodies. No membrane could be shown around the lipid bodies by the use of electron microscopy. The formation of lipid bodies in *Eremosphaera* is discussed as a bulging of the chloroplast envelope membranes.

Key words: Chloroplast — *Eremosphaera viridis* — Lipid body — Secondary carotenoids — Triacylglycerols.

When stressed by nutrient limiting conditions, algae synthesize large amounts of TAG and accumulate them mostly in discrete cytosolic droplets variously referred to as oil bodies, spherosomes, oleosomes or lipid bodies, the term to be used here (Czygan 1968, Shifrin and Chisholm 1981, Piorreck et al. 1984, Suen et al. 1987, Guckert and Cooksey 1990). Our knowledge of algal lipids has turned out to be surprisingly poor, and even less is known about the chemical composition of lipid bodies in algae. Some more information is available about oil bodies in higher plants, particulary in seeds.

Higher plants store TAG in so called oil bodies, spherosomes or oleosomes (Gurr et al. 1974, Bergfeld et al. 1978). They are associated with some characteristic proteins not extractable with detergents (Herman 1987, Murphy and Cummins 1989). It was suggested that oil bodies are surrounded by a half-unit-membrane (Yatsu and Jacks 1972, Moreau et al. 1989), or denied (Bergfeld et al. 1978). They are considered to form freely in the cytosol (Murphy et al. 1989), or to be derived from endoplasmatic reticulum or chloroplasts (Schwarzenbach 1971, Wanner et al. 1981). The oil bodies of seeds of higher plants serve as a source of energy during germination (Qu et al. 1986), whereas lipid bodies in algae are divided, not consumed during regreening after nitrogen limitations (Shifrin and Chisholm 1981).

In the chlorococcalean green alga *Eremosphaera viridis* De Bary (Chlorophyceae), under nitrogen deficiency numerous lipid bodies occurred which contained SC (Vechtel et al. 1991a) suggested to be synthesized in chloroplasts (Vechtel et al. 1991b). The purpose of this study was to examine the chemical composition of the lipid bodies in order to elucidate their ontogeny and to find indications on the means of transport of SC from chloroplasts into these cytosolic lipid bodies.

Materials and Methods

Algal culture—Eremosphaera viridis De Bary (Algal Coll. Goettingen F.R.G., SAG LB-221) was grown as described by Weidinger and Ruppel (1985). The N-deficient conditions of algal culture were employed according to Vechtel et al. (1991a).

Lipid bodies were isolated from algae grown under N-deficiency for 40 d according to Vechtel et al. (1991a) and, if necessary, incubated for 30 min at 25°C with 0.25% SDS or 0.61% Triton X-100 according to Murphy and Cummins (1989). Isolated lipid bodies were extracted twice with acetone and centrifuged at 4,300 × *g* for 5 min. The pellet was solubilized and used for SDS-PAGE to separate proteins according to Laemmli (1970). The proteins were stained by a combined commassie blue-silver stain procedure (De Moreno et al. 1985). The supernatant was

Abbreviations: DGDG, digalactosyldiacylglycerol; DGTS, diacylglycerol-*N,N,N*-trimethylhomoserine; HPLC, high pressure liquid chromatography; MGDG, monogalactosyldiacylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SC, secondary carotenoids; TAG, triacylglycerol; TLC, thin layer chromatography; d, day(s).

dried under N_2, and the residue redissolved in chloroform for lipid analysis.

Lipid analysis—Lipids were determined on silica gel TLC plates (Merck, F.R.G.). For separation, the following eluents and sprays, resp., were used: for TAG: petrol ether (60–80°KP)/diethylether/acetic acid (90 : 10 : 1 v/v), and bromothymol blue (0.4 mg ml^{-1} 0.1 N NaOH), resp.; for phospholipids: chloroform/methanol/H_2O (65 : 25 : 4 v/v), and molybdenum blue, resp.; for glycolipids: chloroform/methanol/acetic acid/H_2O (85 : 15 : 10 : 3.5 v/v), and phosphomolybdic acid, resp. All spray reagents were obtained from Sigma, F.R.G.

Fatty acid methyl esters from lipids isolated by TLC were obtained and identified according to Giroud et al. (1988). Chlorophyll content was determined according to Schmid (1971). Secondary carotenoids were separated by TLC and HPLC according to Vechtel et al. (1991a). IR-spektra from isolated lipids were made with a Mattson Polaris FT IR-spectronic.

Electron microscopy—For electron microscopy thin sections were prepared according to Weidinger and Ruppel (1985). Additionally, samples were fixed with 5% $KMnO_4$ for 30–60 min without staining with uranyl acetate or lead citrate. Thin sections were observed and recorded on a Hitachi H 500 electron microscope at 75 kV.

Results

Eremosphaera viridis De Bary contained about 200 chloroplasts within its giant cell of 100–200 μm diameter. Thin sections of *Eremosphaera* cells grown in complete medium showed chloroplasts similar to those of higher plants (Fig. 1). Most of the thylakoids appeared as pairs throughout the chloroplasts. Over a wide range grana stacks of about six thylakoids could be seen, whereas stroma-thylakoids were observed rarely. Starch grains occurred exclusively within the chloroplasts. Under nitrogen-free conditions thylakoids disintegrated and only a few could be found after 15 d of nitrogen deficiency, mostly in pairs as stroma-thylakoids (Fig. 2A). Starch grains often filled chloroplasts almost completely and lipid bodies appeared in the cytosol next to plastids. After 40 d of N-deficiency thylakoids were reduced to a minimum and lipid bodies reached or even surpassed the size of chloroplasts. After fixing with glutaraldehyde/OsO_4, no membrane or boundary could be detected around the lipid bodies from *Eremosphaera* (Fig. 2B). However, in cells of *Eremosphaera* fixed with $KMnO_4$, each lipid body was surrounded by a electron-dense coat, known as a deposition membrane of pyrolusite, and with marked differences from a normal bilayer membrane (Fig. 3A and B).

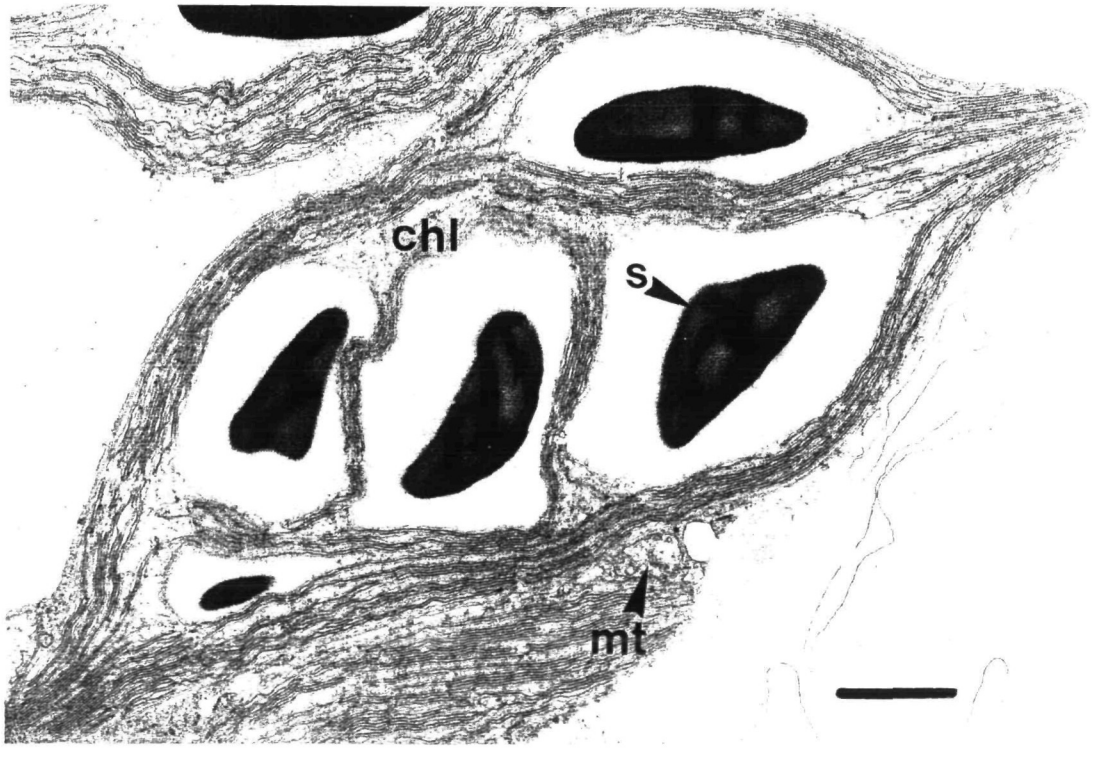

Fig. 1 Thin sections of cells of *Eremosphaera* grown in complete medium, fixed with glutaraldehyde/OsO_4 (bar = 1 μm). CHL = chloroplast, MT = mitochondrion, S = starch grain.

In isolated lipid bodies of *Eremosphaera*, TAG represented the main part (Table 1) with oleic acid (18:1) as the main fatty acid (Table 2). Most of the SC, synthesized under N-deficiency, β-carotene and lutein were found in isolated lipid bodies. Traces of chlorophyll (a/b ratio of 1.5), xanthophylls (neoxanthin, violaxanthin and zeaxanthin) and small amounts of MGDG, DGDG and PG could be detected. PE and the betaine lipid DGTS both of which are present in whole cells of this alga, could not be detected in lipid bodies. Four polypeptides of 28, 26, 25 and 23 kDa

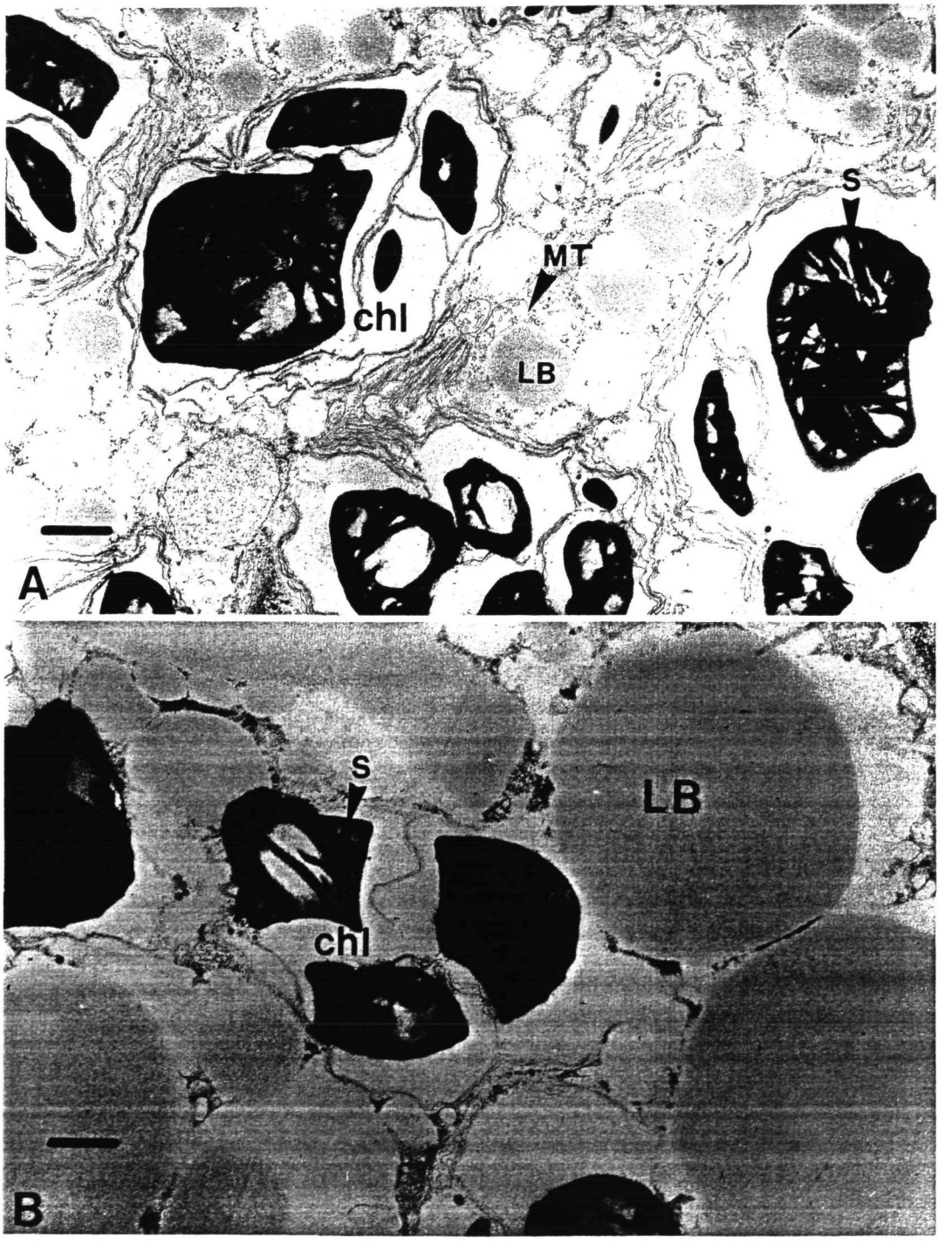

Fig. 2 As Fig. 1, grown in nitrogen-free medium for 9 d (A) and 40 d (B). (bar = 1 μm). LB = lipid body.

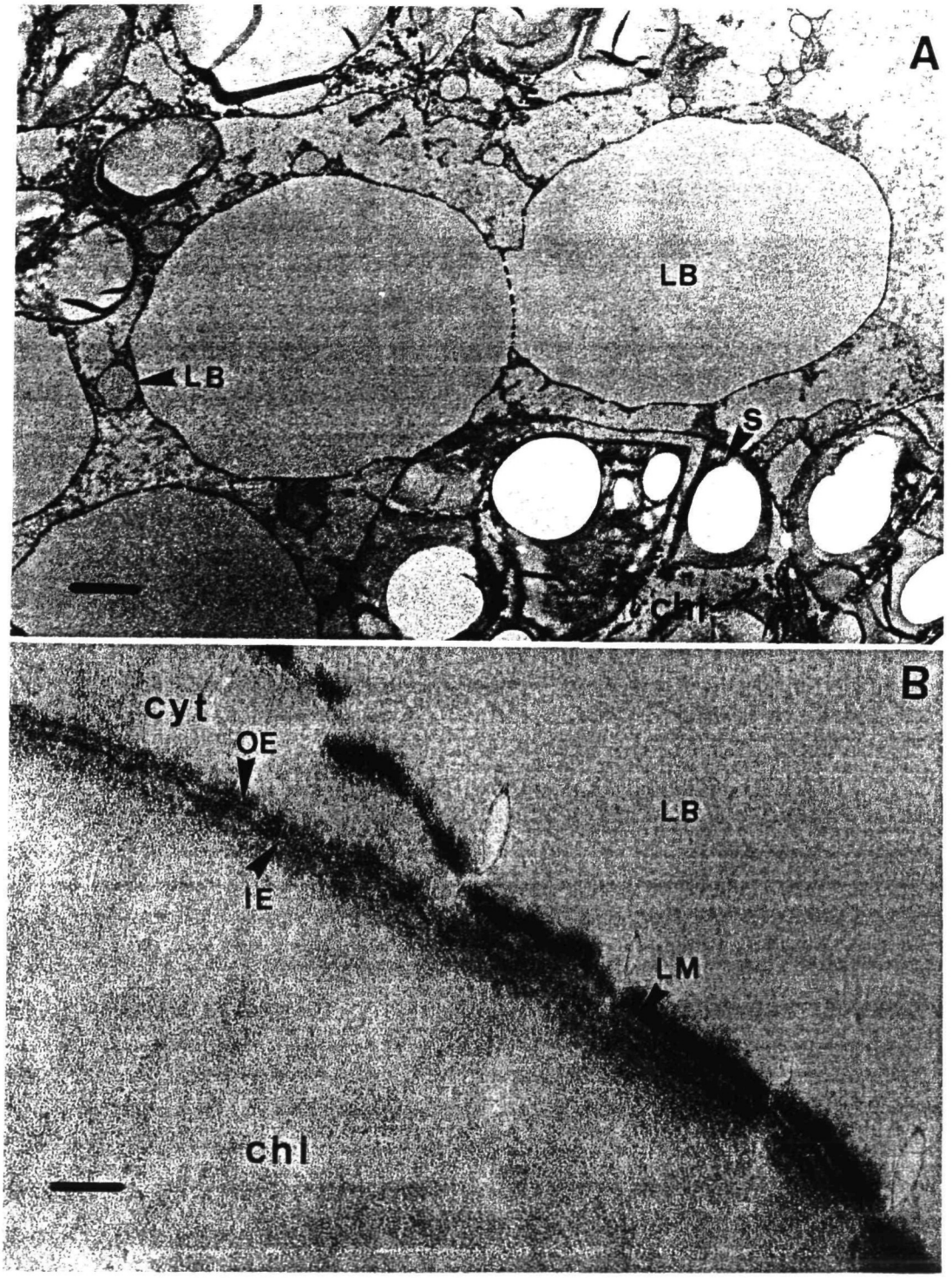

Fig. 3 Thin sections of cells of *Eremosphaera* grown in nitrogen-free medium for 40 d, fixed with $KMnO_4$. A: lipid bodies (1 μm = bar), B: detail of border zone between lipid body and chloroplast (0.04 μm = bar). CHL = chloroplast, CYT = cytosol, IE = inner envelope, LB = lipid body, M = lipid body membrane, OE = outer envelope, S = starch grain.

Table 1 Chemical composition of lipid bodies isolated from cells of *Eremosphaera* grown under N-deficiency for 40 d

Triacylglycerols	80%
Trialkylethers	10%
Secondary carotenoids	7%
Chlorophylls Phophatidylglycerol Monogalactosyldiacylglycerol Digalactosyldiacylglycerol	3%
Polypeptides: 28, 26, 25, 23 kDa	

Percentage of w/w, estimated.

molecular weight could be shown to be associated with the isolated lipid bodies (Fig. 4). Additionally, a fraction was isolated from lipid bodies by TLC containing ether bonds detected by IR-spectra (Fig. 5). We assume that these trialkylethers are derivatives from the phytols liberated by chlorophyll degradation during N-deficiency (Vechtel et al. 1991a).

Incubation with detergents, SDS or Triton X-100, did not result in fusion of the isolated lipid bodies, but led to the eluation of the proteins chlorophylls, xanthophylls and phospho- and glycolipids into the rinsing buffer. In contrast, TAG, trialkylethers and secondary carotenoids remained within the lipid bodies where they were demonstrated by TLC.

To investigate the process of lipid body formation, cells of *Eremosphaera* were grown under N-deficiency for several days and periodically observed by electron microscopy. From day 3 on, cytosolic lipid bodies appeared,

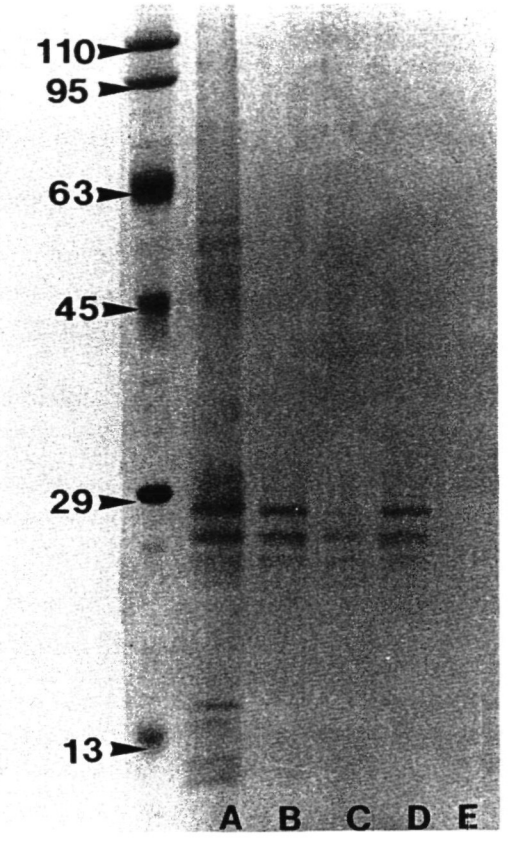

Fig. 4 SDS-PAGE of proteins attached to isolated lipid bodies. lane A: of isolated lipid bodies. lane B: after two and lane C after four times of washing of isolated lipid bodies. lane D: after extraction from rinsing buffer containing detergents. E: of isolated lipid bodies after incubation with detergents.

Table 2 Fatty acid composition in per cent (w/w) of triacylglycerols of lipid bodies isolated from cells of *Eremosphaera* grown under N-deficiency for 40 d

Fatty acid composition of triacylglycerols [% mol of total fatty acids]	
C-14:0	0.9%
C-16:0	8.0%
C-16:1	—
C-16:3	0.7%
C-18:0	1.5%
C-18:1	67.0%
C-18:2	16.6%
C-18:3	—
C-18:4	1.1%
C-20:4	0.9%
C-20:5	0.6%

always having a distinct minimum of size, and smaller ones could not be detected. On the other hand, after two days of growing in nitrogen-free medium, cells showed a "bulging" of chloroplast envelope membranes, especially of the outer one (Fig. 6A and B). Vesicles germinating from thylakoids seemed to migrate towards the "bulging site" (Fig. 6B), possibly one of the first stages of developing lipid bodies. Often the inner envelope was clearly to be seen, whereas the outer one began to disintegrate (Fig. 6B).

Discussion

In *Eremosphaera* the accumulation of TAG under N-deficiency leads to the formation of lipid bodies, common to other algae as well (Czygan 1968, Shifrin and Chisholm 1981, Ben-Amotz et al. 1982, Cooksey et al. 1987, Suen et al. 1987, Lers et al. 1990). Like in the other algae studied, the TAG of *Eremosphaera* mainly consists of C-18:1 fatty

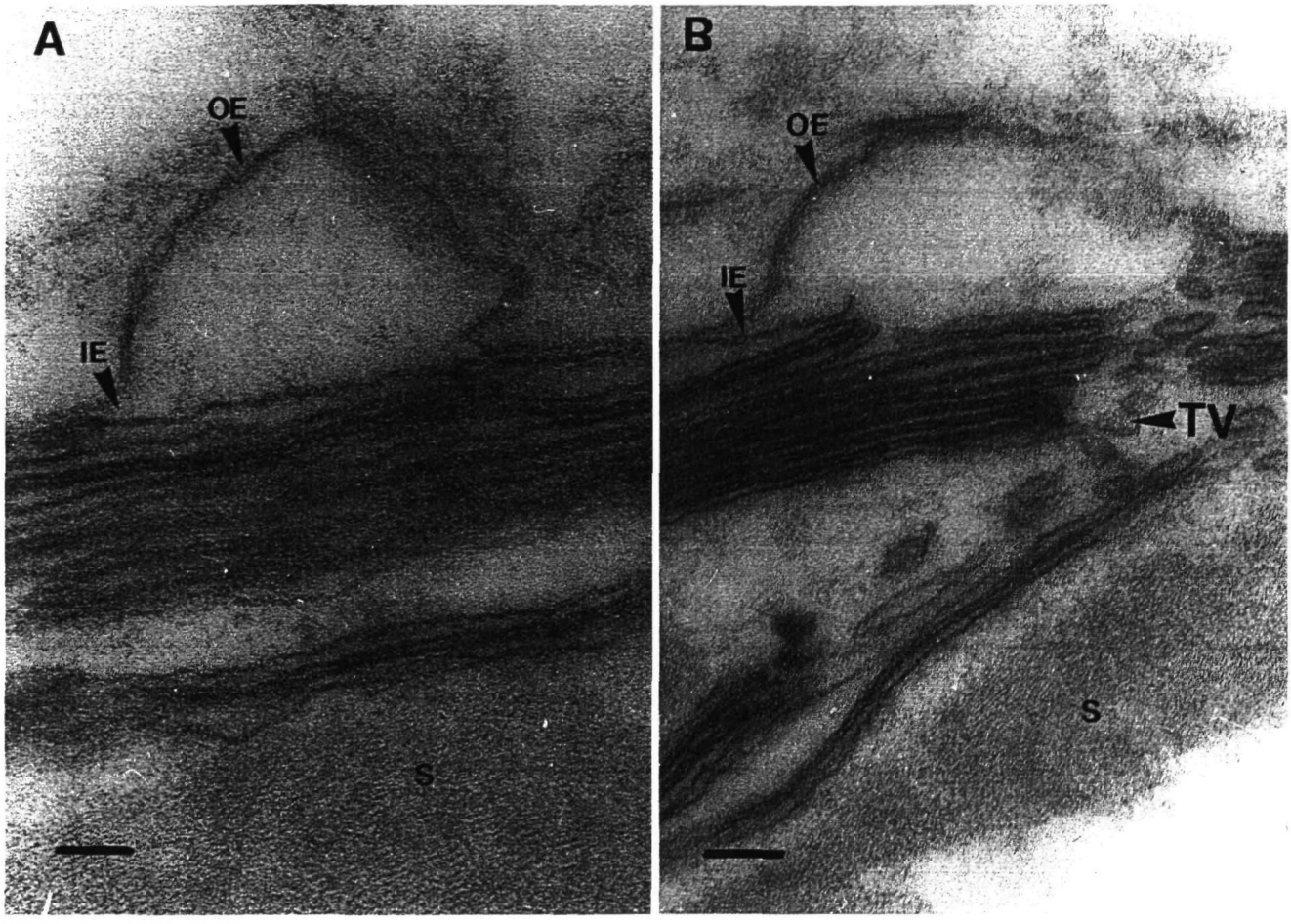

Fig. 5 As Fig. 3, grown in nitrogen-free medium for 2 d. A and B. possible stages of lipid body formation (bar = 0.15 μm). TV = thylakoid vesicles.

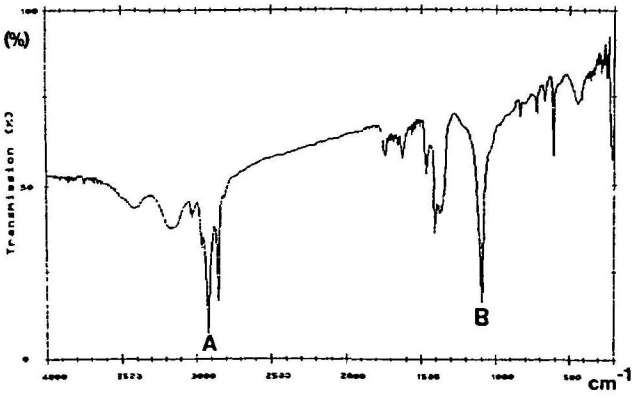

Fig. 6 IR-spectra of trialkylethers isolated by TLC from lipid bodies of cells of *Eremosphaera* grown under N-deficiency for 40 d. A: 2,960, 2,930 and 2,860 cm^{-1} (CH_2 and CH_3); B: 1,120 cm^{-1} (C-O-C ether).

acid which under conditions of stress appears in even larger proportions than under normal conditions (Piorreck et al. 1984). It was suggested that inhibition of cell division and interruption of the cell cycle at a stage of increased lipid synthesis leads to the formation of lipid bodies (Coleman et al. 1988).

Although lipid bodies of *Dunaliella bardawil* (Ben-Amotz et al. 1982) and of *Eremosphaera* seem not to be surrounded by a membrane, some indications for remnants of membrane compounds exist. Membrane lipids, together with proteins and chlorophylls are found in lipid bodies of *Dunaliella* (Ben-Amotz et al. 1982) and in those of *Eremosphaera* as well. This could be explained as a contamination caused by their attachment to the surface of the lipid bodies during isolation (Steinmüller and Tevini 1985). On the other hand, the proteins, together with the chlorophylls and the xanthophylls might be derived from light-harvesting complexes from the chloroplasts. Additionally, MGDG and DGDG which are exclusively located

in chloroplast membranes (Joyard and Douce 1987) are present in isolated lipid bodies and the trialkylethers, if they are derived from phytols of degraded chlorophylls, also have plastidic origin. Since, on the other hand, PE and DGTS both of which are constituents characteristic of the ER (Eichenberger and Gerber 1987), are absent from lipid bodies, these results indicate an involvement of plastids in the formation of lipid bodies in *Eremosphaera*.

It is accepted today that plastids play a distinctive role in lipid biosynthesis. Chloroplast envelopes are postulated as a major site for biosynthesis of membrane lipids like glyco- and phospholipids or of TAG (for review see: Douce and Joyard 1990). In addition, there is the possibility that plastids convert diacylglycerols into TAG as described from plastids of spinach leaves under stress conditions (Sakaki et al. 1990).

In 1981, Wanner et al. published their model of lipid body formation by plastids in oil plants suggesting budding off from the outer envelope. In agreement with this hypothesis, we now assume that in *Eremosphaera* the increased synthesis, together with a conversion of diacylglycerols to TAG, leads to the accumulation of neutral lipids between inner and outer envelope resulting in bulging especially of the outer membrane. Then the envelope membranes disintegrate.—Additionally, the TAG content of chloroplasts isolated from cells grown under N-deficiency for 3 d was higher than of chloroplasts from normal grown cells (data not shown).—Thereafter, lipid bodies of a particular size could be demonstrated in close proximity to chloroplasts by electron microscopy. Unfortunately, the transient stage of lipid body formation between bulging sites and individual droplets has not yet been observed.

Our hypothesis is in accordance with observations by Lichtenthaler and Peveling (1966), describing lipid globules leaving chloroplasts of *Hoya carnosa*, and Miyake et al. (1989), reporting plastoglobuli containing TAG released from plastids into the vacuole.

Our assumption explains the transport of SC out of chloroplasts into cytosolic lipid bodies as a simple diffusion of carotenodis into the hydrophobic bulging sites of the plastids, lateron forming individual lipid bodies.

We wish to thank Dr. Bodo Müller, Department of Biology, University of Bielefeld, for critical reading of manuscript.

References

Ben-Amotz, A., Katz, A. and Avron, M. (1982) Accumulation of β-carotene in halotolerant algae: purification and characterization of β-carotene-rich globules from *Dunaliella bardawil*. *J. Phycol.* 18: 529–537.

Bergfeld, R., Hong, Y. N., Kühnl, T. and Schopfer, P. (1978) Formation of oleosomes (storage lipid bodies) during embryogenesis and their breakdown during seedling development in cotyledons of *Sinapis alba*. *Planta* 143: 297–307.

Coleman, L. W., Rosen, B. H. and Schwartzbach, S. D. (1988) Environmental control of carbohydrate and lipid synthesis in *Euglena*. *Plant Cell Physiol.* 29: 423–432.

Cooksey, K. E., Guckert, J. B., Williams, S. A. and Callis, P. R. (1987) Fluorometric determination of neutral lipid content of microalgal cells using Nile Red. *J. Microbiol. Methods* 6: 333–345.

Czygan, F. C. (1978) Sekundärcarotinoide in Grünalgen. *Arch. Mikrobiol.* 61: 81–102.

De Moreno, M. R., Smith, J. F. and Smith, R. V. (1985) Silver staining of proteins in polyacrylamid gels: increased sensitivity through a combined coomassie blue-silver stain procedure. *Anal. Biochem.* 151: 466–470.

Douce, R. and Joyard, J. (1990) Biochemistry and function of the plastid envelope. *Annu. Rev. Cell Biol.* 6: 173–216.

Eichenberger, W. and Gerber, A. (1987) Lipids of *Acetabularia mediterranea*. Composition, cellular localization and biosynthesis. *In* Metabolism, Structure and Function of Plant Lipids. Edited by Stumpf, P. K., Mudd, J. B. and Nes, N. D. pp. 637–639. Plenum Press, New York.

Giroud, C., Gerber, A. and Eichenberger, W. (1988) Lipids of *Chlamydomonas reinhardtii*. Analysis of molecular species and intracellular site(s) of biosynthesis. *Plant cell Physiol.* 29: 587–595.

Guckert, J. B. and Cooksey, K. E. (1990) Triglyceride accumulation and fatty acid profile changes in *Chlorella* during high pH-induced cell cycle inhibition. *J. Phycol.* 26: 72–79.

Gurr, M. I., Blades, J., Appleby, R. S. and Smith, C. G. (1974) Studies on seed-oil triglycerides. *Eur. J. Biochem.* 43: 281–290.

Herman, E. M. (1987) Immunogold-localization and synthesis of an oil-body membrane protein in developing soybean seeds. *Planta* 172: 336–345.

Joyard, J. and Douce, R. (1987) Galactolipid synthesis. *In* The Biochemistry of Plants. Edited by Stumpf, P. K. 9: 215–274.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.

Lers, A., Biener, Y. and Zamir, A. (1990) Photoinduction of massive β-carotene accumulation by the alga *Dunaliella bardawil*. *Plant Physiol.* 93: 389–395.

Lichtenthaler, H. K. and Peveling, E. (1966) Osmiophile Lipideinschlüsse in den Chloroplasten und im Cytoplasma von *Hoya carnosa* R. Br. *Naturwissenschaften* 20: 534–535.

Miyake, H., Matsumura, H., Fujinuma, Y. and Totsuka, T. (1989) Effects of low concentrations of ozone on the fine structure of radish leaves. *New Phytol.* 111: 187–195.

Moreau, R. A., Liu, K. D. F. and Huang, A. H. C. (1980) Spherosomes of castor bean endosperm, membrane components, formation and degradation. *Plant Physiol.* 65: 1176–1180.

Murphy, D. J. and Cummins, I. (1989) Seed oil-bodies: isolation, composition and role of oil-body apolipoproteins. *Phyto-*

chemistry 28: 2063–2069.

Murphy, D. J., Cummins, I. and Kang, A. S. (1989) Synthesis of the major oil-body membrane protein in developing rapeseed (*Brassica napus*) embryos. *Biochem. J.* 258: 285–293.

Piorreck, M., Baasch, K. H. and Pohl, P. (1984) Biomass production, total protein, chlorophylls, lipids and fatty acids of freshwater green and blue-green algae under different nitrogen regimes. *Phytochemistry* 23: 207–216.

Qu, R., Wang, S., Lin, Y., Vance, V. B. and Huang, A. H. C. (1986) Characteristics and biosynthesis of membrane proteins of lipid bodies in the scutella of maize (*Zea mays* L.). *Biochem. J.* 235: 57–65.

Sakaki, T., Saito, K., Kawagushi, A., Kondo, A. and Yamada, M. (1990) Conversion of monogalactosylglycerols to triacylglycerols in ozone-fumigated spinach leaves. *Plant Physiol.* 94: 766–772.

Schmid, G. H. (1971) Origin and properties of mutant plants: yellow tobacco. *Methods Enzymol.* 23A: 171–194.

Schwarzenbach, A. M. (1971) Observations on spherosomal membranes. *Cytobiologie* 4: 145–147.

Shifrin, N. S. and Chisholm, S. W. (1981) Phytoplankton lipids: interspecific differences and effects of nitrate, silicate and light-dark cycles. *J. Phycol.* 17: 374–384.

Steinmüller, D. and Tevini, M. (1985) Composition and function of plastoglobuli. I. Isolation and purification from chloroplasts and chromoplasts. *Planta* 163: 201–207.

Suen, Y., Hubbard, J. S., Holzer, G. and Tornabene, T. G. (1987) Total lipid production of the green alga *Nannochloropsis* sp. QII under different nitrogen regimes. *J. Phycol.* 23: 289–296.

Vechtel, B., Kahmann, U. and Ruppel, H. G. (1991a) Secondary carotenoids of *Eremosphaera viridis* De Bary (Chlorophyceae) under nitrogen deficiency. *Bot. Acta* 104: (in press).

Vechtel, B., Pistorius, E. K. and Ruppel, H. G. (1991b) Occurrence of secondary carotenoids in PS I complexes from *Eremosphaera viridis* De Bary (Chlorophyceae). *Z. Naturforsch.* (in press).

Wanner, G., Formanek, H. and Theimer, R. R. (1981) The ontogeny of lipid bodies (spherosomes) in plant cells. *Planta* 151: 109–123.

Weidinger, M. and Ruppel, H. G. (1985) Ca^{2+}-requirement for a blue light-induced chloroplast translocation in *Eremosphaera viridis*. *Protoplasma* 124: 184–187.

Yatsu, L. Y. and Jacks, T. J. (1972) Spherosome membranes. *Plant Physiol.* 49: 937–943.

(Received June 25, 1991; Accepted October 26, 1991)