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BIOSYNTHESIS OF EICOSAPENTAENOIC ACID (EPA) IN THE FRESHWATER EUSTIGMATOPHYTE *MONODUS SUBTERRANEUS* (EUSTIGMATOPHYCEAE)[1]

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In an attempt to elucidate the biosynthesis of the polyunsaturated fatty acid eicosapentaenoic acid (20:5ω3, EPA), we treated cultures of the eustigmatophyte *Monodus subterraneus* Peterson with either salicylhydroxamic acid or the herbicide SAN 9785. Labeled linoleic acid was incorporated into the cultures in the presence and absence of the latter inhibitor, and the redistribution of label was followed. Our results suggest that the major biosynthetic pathway leading to EPA involves fatty acids of the ω6 family. In the early stages of the biosynthesis, 18:1 is predominantly incorporated to the *sn*-2 position of phosphatidylcholine, where it is stepwise desaturated by the Δ12 and Δ6 desaturases to 18:3ω6. The latter is released from the lipid, elongated to 20:3ω6 and reincorporated to both positions of phosphatidylethanolamine (PE) where it is further desaturated by the Δ5 and ω3 desaturases to EPA. We suggest that PE is the donor of the 20:5/20:5 diacylglycerol that is imported to the chloroplast to form the eukaryotic-like molecular species of monogalactosyldiacylglycerol. Likewise, 20:3ω6 can be also incorporated into diacylglyceryltrimethylhomoserine, mostly to the *sn*-2 position and similarly desaturated to 20:4ω6 and 20:5ω3. These fatty acids can be exported and incorporated into the *sn*-1 position of the prokaryotic-like molecular species of the chloroplastic lipids. We thus suggest that both the eukaryotic-like and the prokaryotic-like molecular species are biosynthesized by different extraplastidial lipids.

Key index words: **algal lipids; EPA biosynthesis; eukaryotic pathway; fatty acid desaturation; LC-PUFA; *Monodus subterraneus*; salicylhydroxamic acid; SAN 9785**

Abbreviations: **C_n fatty acid, fatty acid with *n* carbon atoms; Δ*n*D, a fatty acid desaturase that introduces a double bond at the *n*th carbon atom from the carboxylic end; DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; DGTS, diacylglyceryltrimethylhomoserine; EPA, eicosapentaenoic acid (20:5ω3); MGDG, monogalactosyldiacylglycerol; MLC, medium to long chain (14–18 carbon atoms); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid; SHAM, salicylhydroxamic acid; TAG, triacylglycerols; ω3D, a fatty acid desaturase that introduces a double bond at a distance of 3 carbon atoms from the methyl end of the chain. In the shorthand numbering system used for identifying fatty acids, the figure preceding the colon indicates the number of carbon atoms in the fatty acid, whereas that after the colon represents the number of double bonds present. Pairs of numbers representing the fatty acids, when separated by a slash, designate the acyl groups residing in the *sn*-1 and *sn*-2 positions, respectively, of the molecular species. Unless otherwise mentioned, 20:4 and 20:5 designate the ω6 and ω3 isomers, respectively.**

The elucidation of the biosynthesis of polyunsaturated fatty acids (PUFAs) in higher plants has shown that PUFAs are produced in two pathways (Browse and Somerville 1994). In the prokaryotic pathway, C_{18} and C_{16} fatty acids are desaturated up to 18:3ω3 and 16:3ω3 while residing in the *sn*-1 and *sn*-2 positions, respectively, of chloroplastic lipids. In the eukaryotic pathway, 18:1 is incorporated to phospholipids, where it is stepwise desaturated to 18:3ω3. Diacylglycerols (DAG), mostly 18:2/18:2 and 16:0/18:2, are released from the phospholipid, exported into the chloroplast, incorporated into galactolipids, and further desaturated. The eukaryotic molecular species are characterized by the presence of C_{18} PUFAs in the *sn*-2 position of the galactolipid and either a C_{18} or a C_{16} fatty acid at the *sn*-1 position. However, although the pathways leading to these molecular species are distinct, there are no known differences in their respective roles.

The biosynthesis of PUFAs in algae is much more complicated. Unlike that of higher plants, algal PUFAs may contain up to six double bonds with chain lengths as long as 22 carbon atoms. Several pathways were suggested to describe PUFA biosynthesis at the fatty acid

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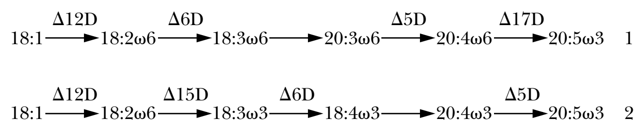
level in different algae. In the red microalga *Porphyridium cruentum,* 18:2 is desaturated to 18:3ω6, elongated, and stepwise desaturated to eicosapentaenoic acid (20:5ω3, EPA) (Nichols and Appleby 1969, Khozin et al. 1997) (sequence 1). A minor ω3 pathway, in which 18:2 was first desaturated to 18:3ω3, was also implied for this alga (Shiran et al. 1996) (sequence 2). In *Euglena,* 18:2 was proposed to be elongated to 20:2, before further desaturation took place (Nichols and Appleby 1969). In the diatom *Phaeodactylum tricornutum,* it was claimed that four different pathways were responsible for the production of EPA (Arao et al. 1994):



Even less is known about the lipids that participate in the various pathways. Phosphatidylcholine (PC) was suggested to be the carrier for the Δ12 and Δ6 desaturations of C_{18} fatty acids in the eustigmatophyte *Nannochloropsis* and phosphatidylethanolamine (PE) to be the substrate for the Δ5 and ω3 desaturations that result in the production of EPA (Schneider and Roessler 1994). In *Ochromonas danica* (Vogel and Eichenberger 1992) and the chlorophyte *Chlamydomonas reinhardtii* (Giroud and Eichenberger 1989) the Δ12, Δ15, and Δ6 desaturations operate on C_{18} fatty acids linked to the *sn*-2 position of diacylglyceryltrimethylhomoserine (DGTS) or PE. In the rhodophyte *P. cruentum,* PC is the major substrate for the Δ12, Δ6, and Δ5 desaturations, whereas the final Δ17 desaturation of 20:4 to 20:5 is predominantly chloroplastic, involving eukaryotic-like (monogalactosyldiacylglycerol [MGDG]) and prokaryotic-like (MGDG and digalactosyldiacylglycerol [DGDG]) molecular species (Khozin et al. 1997). We (Khozin-Goldberg et al. 2000) recently showed that triacylglycerols (TAG) can also contribute arachidonic acid (20:4ω6) and EPA for the construction of eukaryotic MGDG in the latter alga.

Because of the absence of appropriate molecular biology tools, biosynthesis of C_{20} PUFAs in microalgae relied mostly on radiolabeling and inhibitor studies. Recently, we found that salicylhydroxamic acid (SHAM) inhibits the Δ6 and possibly the Δ12 desaturation of PC in *P. cruentum* (Khozin-Goldberg et al. 1999). The substituted pyridazinone SAN 9785 (BASF 13-338, 4-chloro-5(dimethylamino)-2-phenyl-3(2H) pyridazinone) was shown (Murphy et al. 1985, Norman and St. John, 1986, 1987) to inhibit chloroplastic, but not cytoplasmic, desaturation of 18:2 to 18:3ω3 in both the prokaryotic and eukaryotic pathways of higher plants and algae (Murphy et al. 1985). SAN 9785 was also shown to inhibit the assembly of TAG in *Pavlova lutheri* (Siljegovich-Hanggi and Eichenberger 1998) and to decrease the amount of neutral lipids in *Nannochloropsis* (Henderson et al. 1990). In a preliminary study, we (Khozin and Cohen 1996) showed that in another eustigmatophyte, *Monodus subterraneus,* SAN 9785 surprisingly affected an increase in the proportion of EPA, especially in the galactolipids. Furthermore, in the major galactolipid, MGDG, the share of molecular species containing EPA in both the *sn*-1 and *sn*-2 positions (eukaryotic-like) increased at the expense of those containing a fatty acid with a shorter chain length at the *sn*-2 position (prokaryotic-like) (Khozin and Cohen 1996). These unexpected findings suggested the existence of a novel and interesting pathway. In this work, we attempt to elucidate the biosynthesis of EPA in *M. subterraneus* at the fatty acid, lipid, and molecular species level using SAN 9785 and SHAM. Our findings suggest that PC is mostly involved in the desaturation of C_{18} fatty acids, whereas PE and DGTS are involved in the desaturation of C_{20} PUFAs, leading to EPA. Furthermore, PE and DGTS are the apparent sources of EPA for the eukaryotic-like and prokaryotic-like molecular species of MGDG, respectively.

MATERIALS AND METHODS

Growth conditions. Cultures of *M. subterraneus* were cultivated on BG11 medium (Steiner et al. 1971) in 150-mL Erlenmeyer flasks or in 1-L tubes under an air-CO_2 (99:1) atmosphere. The flasks were placed in an incubator shaker at 25° C and illuminated from above at a light intensity of 115 μmol photons·m^{-2}·s^{-1}. Tubes were placed in the temperature regulated water bath and illuminated from aside at a light intensity of 180 μmol photons·m^{-2}·s^{-1}. Cultures were grown exponentially and were daily diluted to 3 μg Chl·mL^{-1} for at least 4 days before the onset of the experiment. Fatty acids analysis was carried out as previously described (Cohen et al. 1988). Stock solutions of 10 mM SAN 9785 or SHAM in DMSO were added to achieve final concentrations of 100 μM and 300 μM, respectively. Each experiment was repeated at least three times.

Radiolabeling experiments. [1-^{14}C]linoleic was purchased from NEN Research Products (Mississauga, Ontario, Canada). Cultures were labeled with 25 μCi of [1-^{14}C]linoleic acid. Labeled fatty acids were added as their ammonium salts, which were obtained by neutralization of the free fatty acids with an equimolar amount of 2 M ammonium hydroxide. Exponentially growing cells were concentrated to half the original volume and pulse labeled for 1 h. Cells were centrifuged and washed repeatedly with label-free medium, resuspended to the original volume, and cultivated as described above. Each experiment was repeated three times. Data are of a representative experiment.

Lipid extraction. The cell wall of cells of *M. subterraneus* contains an acetolysis-resistant material that is almost impermeable to most common solvents used for lipid extraction. Samples of lyophilized biomass (50 mg) were thus stirred with 0.5 mL DMSO at 70° C and were extracted for 1 h with methanol at 4° C. The mixture was centrifuged, the supernatant removed, and the residue was reextracted with a mixture of hexane and ether (1:1, v/v). Peroxide-free diethyl ether containing 0.01% butylated hydroxytoluene, hexane, and water were added to the supernatant to form a ratio of 1:1:1:1 (v/v/v/v). The mixture was shaken and then centrifuged for 5 min at 35 × 100 rpm, and the upper phase was collected. The water phase was acidified with 10% acetic acid to pH 4 and extracted again with a mixture of diethyl ether:hexane (1:1, v/v). The lipid containing phases were combined and evaporated to dryness.

Fatty acid and lipid analysis. Methyl esters were prepared by transmethylation of the lipid(s) with methanol and sulfuric acid as previously detailed (Cohen et al. 1988). The data shown represent mean values with a range of less than 5% for major peaks (over 15% of fatty acids) and 10% for smaller peaks, of at least two independent samples, each analyzed in duplicate. Methyl esters of fatty acids were separated by reverse-phase HPLC (RP-18 column, 5 μm, 250 mm, Lichrospher 100, Merck, Darm-

stadt, Germany) using a solvent system of methanol:acetonitrile:water (76:12:12, v/v/v), detected at 205 nm and 242 nm, respectively, and identified using authentic standards. Radioactivity of individual peaks was determined by a Flo-OneBeta series A-100 detector (Radiomatic Instruments and Chemical Co., Inc., Tampa, FL, USA). Distribution of radioactivity among individual lipids was assessed by TLC on 10 × 10-cm plates (Silica Gel 60, 0.25 mm thickness, Macherey-Nagel, Düren, Germany). Polar lipids were separated by two-dimensional TLC using a solvent system of chloroform:methanol:water (65:25:4, v/v/v) for the first direction and of chloroform:methanol:1-ethylpropylamine: conc. ammonia (65:35:0.5:5, v/v/v/v) for the second direction. Neutral lipids were resolved with petroleum ether:diethyl ether:acetic acid (80:20:1, v/v/v). Radioactivity was detected by autoradiography with x-ray films (X-OMAT AR, Kodak, Eastman Kodak, Rochester, NY, USA) exposed to the TLC plates for 24 h. Lipid spots were scraped directly into scintillation vials containing 1 mL of methanol and 2 mL of scintillation cocktail, and radioactivity was measured in a liquid scintillation counter (Rackbeta LKB, model 1217, LKB, Wallac Oy, Finland).

Molecular species analysis. Individual lipids eluted from TLC plates were purified by normal-phase HPLC (Lichrospher, Si 60, 5 μm, Merck), using a linear gradient from 100% mobile phase A (hexane:isopropanol, 40:60, v/v) to 100% mobile phase B (hexane:isopropanol:water, 40:53:7, v/v/v) over 40 min. Molecular species analysis of individual lipids was performed by reversed-phase HPLC (RP-18 column, 5 μm, 250 mm, Lichrospher 100, Merck) on a Hitachi L 7100 chromatograph (Merck, Waters, Milford, MA, USA) equipped with a UV detector (Lambda-Max 481, Millipore, Waters) and an evaporative light scattering detector (drift temperature tube 125° C, nebulizer gas flow 2.5 $L \cdot min^{-1}$, Varex, Burtonsville, MD, USA). Molecular species of MGDG and DGDG were resolved by reversed-phase HPLC using a methanol:water mixture (93:7, v/v) as mobile phase. Other polar lipids were separated using various mixtures of methanol:acetonitrile:water as follows: DGTS (80:12:8, v/v/v), PC (60:35:5, v/v/v), and PE (35:55:10, v/v/v). Ethanolamine (5 μM) was added to the solvent mixtures used for the separation of PC and PE (adapted from Brouwers et al. 1999). Molecular species were identified and quantified by collection of the peaks, transmethylation of the lipids, and GC analysis of their fatty acids in the presence of an internal standard. Molecular species composition of PE was estimated using a light-scattering detector. Each datum point represents a mean of three independent experiments varying by less than 5%.

Positional distribution analysis. Positional analysis of phospholipids (PC and PE) and DGTS was performed using phospholipase A_2 from *Crotalus adamanteus* venom (Sigma Chemical Co., St. Louis, MO, USA) (Jones and Harwood 1992) and lipase from *Rhizopus arrhizus* (Boehringer Mannheim, Mannheim, Germany) (Lynch and Thompson 1986), respectively.

RESULTS

SHAM. Treatment of cultures of *M. subterraneus* with SHAM (300 μM) for 2 days resulted in a growth inhibition of 50%. The proportion of TAG increased sharply from 36.3% to 50.9% (of total fatty acids) at the expense of most polar lipids (Table 1), except for PC and PE, which increased slightly. The major effect on the fatty acids was observed in a decrease in the proportion of 18:3ω6 and most C_{20} PUFAs and in an increase in 18:2 and its precursors. This effect was observed in every lipid (Table 1). In PC, 18:3ω6 decreased from 5.8% to 1.2%, whereas 18:2 increased from 8.6% to 32.0%. Similarly, the proportion of all the presumed down stream products, 20:3ω6, 20:4ω6, and 20:5ω3, decreased. The proportion of 18:3ω3, the

Table 1. Effect of SHAM (300 μM) and SAN 9785 (100 μM) on lipid distribution and fatty acid composition in *Monodus subterraneus.*

			Fatty acid composition (% of total fatty acids)																	
Lipid	Treatment	Lipid content[a]	14:0	16:0	16:1 ω7	16:1[b] ω5	16:2	16:3	18:0	18:1[c]	18:2 ω6	18:3 ω6	18:3 ω3	20:2 ω6	20:3 Δ5	20:3 ω6	20:4 ω6	20:4 Δ5	20:4 ω3	20:5 ω3
MGDG	CON	19.0	4.8	12.2	17.3	0.3	tr	—	0.3	3.1	0.5	0.5	0.3	—	—	0.4	4.6	—	0.2	55.3
	SHAM	13.5	4.4	14.4	21.6	0.3	tr	0.2	0.8	6.3	5.3	0.3	1.0	0.2	0.2	0.2	2.4	tr	—	42.4
	SAN	31.2	4.0	6.3	11.3	0.5	0.2	0.2	0.3	2.1	0.7	0.2	0.3	—	—	0.2	5.3	—	tr	68.2
DGDG	CON	12.6	2.6	26.9	43.7	0.2	0.2	tr	0.2	5.8	0.6	tr	0.6	0.3	—	tr	0.6	—	—	18.1
	SHAM	9.9	2.0	24.9	42.1	0.4	0.2	0.2	0.2	7.8	4.0	tr	0.9	0.4	tr	tr	0.4	tr	—	16.4
	SAN	17.8	2.8	21.1	45.9	0.3	0.4	0.2	0.2	6.6	1.1	tr	0.8	0.5	—	tr	0.9	—	—	18.9
SQDG	CON	6.4	3.7	50.2	37.5	0.4	—	tr	0.8	5.5	0.3	tr	—	tr	—	—	0.4	—	—	0.9
	SHAM	4.1	3.2	50.2	38.6	0.4	—	tr	0.4	5.8	0.6	—	—	0.3	—	—	tr	—	—	0.3
	SAN	6.5	3.6	48.4	38.0	1.5	—	0.2	0.4	5.7	0.3	—	—	1.0	—	—	0.2	—	—	0.7
PG	CON	1.8	1.0	19.3	5.9	28.3	0.4	tr	1.7	5.4	0.7	tr	0.3	—	—	tr	1.6	—	—	35.1
	SHAM	1.1	0.8	24.1	6.3	31.5	—	0.3	1.5	9.0	2.7	—	—	tr	—	—	0.9	—	tr	22.7
	SAN	3.3	1.1	11.2	8.1	38.6	1.2	0.2	1.2	7.7	1.4	—	0.2	—	—	—	2.3	—	0.3	26.6
DGTS	CON	12.3	2.5	30.0	26.3	0.2	0.3	tr	1.4	4.8	0.8	0.5	0.6	—	—	tr	8.4	—	tr	24.1
	SHAM	8.2	2.5	28.0	34.3	0.4	0.4	0.2	1.7	9.9	4.9	0.3	1.2	0.3	tr	tr	2.6	tr	—	13.1
	SAN	13.4	5.1	21.7	31.5	1.1	0.6	—	1.2	4.1	1.3	0.4	0.3	—	—	tr	8.7	—	—	23.2
PC	CON	4.1	1.2	32.2	11.7	1.7	1.0	0.0	3.4	8.8	8.6	11.9	—	—	—	0.4	4.7	—	—	13.2
	SHAM	4.5	1.0	32.0	11.4	0.7	0.6	0.2	2.2	15.5	32.0	1.2	1.0	—	—	—	0.4	—	—	1.6
	SAN	8.6	2.5	25.5	25.3	2.3	2.5	0.9	3.8	14.1	11.5	1.2	0.7	tr	—	—	2.7	—	—	7.0
PE	CON	1.5	tr	9.9	5.5	—	—	—	1.9	8.6	0.7	0.7	1.3	—	—	3.1	20.6	—	—	47.6
	SHAM	2.2	tr	7.3	7.0	1.0	—	0.7	1.6	15.5	10.8	0.4	0.3	0.9	0.6	1.3	9.3	0.3	0.4	42.5
	SAN	2.3	tr	6.3	5.0	—	—	—	0.8	6.1	0.4	0.2	1.7	0.6	—	0.8	20.2	—	—	57.9
TAG	CON	36.3	3.3	26.9	36.3	0.8	0.2	tr	0.6	23.1	0.8	0.7	0.2	0.2	0.9	—	1.6	—	tr	4.3
	SHAM	50.9	2.0	23.5	31.5	0.4	tr	0.4	0.2	32.8	5.9	0.2	0.4	0.6	0.2	tr	0.2	—	—	1.7
	SAN	7.6	2.8	19.3	32.7	1.2	0.5	0.7	1.5	17.8	3.0	0.2	0.3	0.2	0.3	—	4.6	—	—	14.8

[a] % of total fatty acids.
[b] In PG 16:1tΔ3.
[c] Total of two isomers (ω9+ω7).
tr, trace level (<0.1%).

ω3 desaturation product of 18:2, also increased from an undetectable level to 1.0%. These findings clearly indicate an inhibition of the Δ6 desaturation. In PE, 18:1 and 18:2 accumulated, but 18:3ω6 was already low in the control. We also observed the appearance of 20:2, the elongation product of 18:2, which was not detected in the control and of the relatively rare polymethylene interrupted fatty acids, 20:3Δ5 (Δ5,11,14) and 20:4Δ5 (Δ5,11,14,17).

SAN 9785. Treating *M. subterraneus* with 100 μM SAN 9785 for 2 days resulted in a reduction in the fatty acid content from 12.3% to 7.3% (of dry weight, data not shown). The herbicide affected a sharp decrease in the share of TAG from 36.3% to 7.6% (of fatty acids), whereas the proportions of the galactolipids MGDG and DGDG increased from 19.0% and 12.6% to 31.2% and 17.8%, respectively (Table 1). PC was doubled from 4.1% to 8.6%, and PE increased from 1.5% to 2.3%; however, DGTS did not change appreciably (from 12.3% to 13.4%).

The proportion of 20:5 increased from 25.9% (of fatty acids) in the control culture to 33.8% in the treated culture (data not shown). Contemporarily, decreases were noted in the proportions of 16:0, 16:1, and 18:1, fatty acids that are characteristics of TAG. In PC, we observed a sharp decrease in the proportion of 18:3ω6, from 11.9% to 0.3%, whereas that of the precursors, 18:1 and 18:2, increased (Table 1). In PE, the proportion of 20:5 increased from 47.6% to 57.9%. In MGDG, the proportion of 20:5 increased from 55.3% to 68.2% at the expense of 16:0 and 16:1, whereas in DGDG the proportion of 20:5 did not change significantly.

Molecular species composition of PC. The *sn*-1 position of PC was predominantly occupied by C_{16}-C_{18}, saturated and monounsaturated fatty acids with almost no PUFAs (Table 2). The *sn*-2 position was mostly occupied by 18:1-3 (58.9%) and 20:4-5 (35.5%). It thus follows that the fatty acids in the *sn*-2 position are responsible for most of this lipid involvement in the biosynthesis of EPA. SHAM affected an increase in the proportion of 18:1- and 18:2-containing molecular species, supporting the suggested inhibition of Δ6 desaturation. The total of 16:0/18:2, 18:1/18:2, and 18:2/18:2 increased from 14.6% to 75.1%, whereas 16:1/18:1 increased from 7.0% to 16.6%. The proportion of molecular species containing acyl groups with three or more double bonds was decimated from 54.1% to 5.1%. In the presence of SAN 9785, there was an increase in the proportion of 18:1- and 18:2-containing molecular species. The proportion of molecular species of the type 16:0-1/18:1-2 increased from 32.0% to 42.6%. The proportion of 20:5/20:5 increased from 1.7% to 6.6%. Concurrently, the proportion of four molecular species containing 18:3 decreased sharply from 24.3% to 2.1%.

Molecular species composition of PE. PE of the control culture consisted mostly of three major molecular species containing 20:4 and 20:5. Because of the small amount of this lipid, it was difficult to isolate and identify the various C_{16}- and C_{18}-containing molecular species, which, according to Table 1, must be present to a low extent. The excess of 18:1 and 18:2 that resulted from the SHAM treatment was manifested in the appearance of 18:1/20:5 and 18:2/20:5 (Table 3). However, the proportion of 20:5/20:5 was not affected. In the presence of SAN 9785, there was an apparent increase in Δ5 desaturation, as evidenced by the increase in the proportion of 20:5/20:5 from 48.4% to 59.4%.

Molecular species composition of DGTS. The major molecular species in the control contained a C_{14}-C_{16} fatty

TABLE 2. Effect of SHAM (300 μM) and SAN 9785 (100 μM) on the molecular species composition of PC in *M. subterraneus.*

Molecular species (*sn*-1/*sn*-2)	Molecular species composition (% of total fatty acids)		
	Control	SHAM	SAN 9785
16:0/18:1 + 18:1/18:1	9.3	1.0	9.8
16:0/18:2	12.0	53.6	19.6
16:0/18:3ω6	17.6	—	2.1
16:0/20:4	8.0	1.0	1.0
16:0/20:5	19.2	2.7	8.1
16:0/16:1[a]	2.0	2.0	19.6
16:1/16:1[a]	2.0	—	17.8
16:1/18:0[a]	1.6	—	—
16:1/18:1[a]	7.0	15.6	13.2
16:1/18:2	3.7	—	tr
16:1/18:3ω6	4.5	1.0[b]	—
16:1/20:4	2.4	—	1.0
16:1/20:5	2.2	1.3	1.0
18:0/18:3ω6	1.0	—	—
18:1/18:2[a]	2.6	9.2	—
18:1/20:4	1.7	—	tr
18:2/18:2	—	12.3	—
20:4/20:4	tr	tr	tr
20:5/18:3ω6	1.2	—	—
20:5/20:4[a]	0.3	—	tr
20:5/20:5	1.7	0.4	6.6

PC was separated by RP-HPLC. Individual peaks were transmethylated and analyzed by GC. Data were confirmed by positional analysis of total PC.

[a] Positional distribution not determined.

[b] 18:3ω3.

tr, trace level (<0.2%).

TABLE 3. Effect of SHAM (300 μM) and SAN 9785 (100 μM) on the major molecular species composition of PE in *Monodus subterraneus.*

Molecular species[a]	Molecular species composition (% of total peak area)		
	Control	SHAM	SAN 9785
16:0/20:5	1.8	4.1	0.4
18:1/20:5	—	14.3	—
18:2/20:5	—	10.0	—
20:4/20:4	9.0	2.7	4.5
20:4/20:5	40.8	24.2	35.7
20:5/20:5	48.4	44.8	59.4

PE was separated by RP-HPLC. Peak identities were determined as in Table 2. Quantitative composition was estimated by integrating peak areas obtained using an evaporative light-scattering detector (ELSD).

[a] Positional distribution not determined.

TABLE 4. Effect of SAN 9785 (100 μM) on the molecular species composition of DGTS in *Monodus subterraneus.*

Molecular species (*sn*-1/*sn*-2)	Molecular species composition (% of total fatty acids)	
	Control	SAN 9785
14:0/20:4	6.1	1.6
14:0/20:5[a]	0.8	1.3
16:0/16:1 + 16:1/16:0	27.3	25.8
16:0/18:3ω6	0.6	0
16:0/18:3ω3	0.2	0
16:0/20:5	13.7	3.8
16:1/16:1	15.9	20.1
16:1/18:1	tr	2.2
16:1/18:3ω3	0.4	0.3
16:1/18:3ω6	1.3	0.6
16:1/20:4ω6	0.8	0.3
16:1/20:5	11.8	18.9
18:0/20:5	2.5	0.8
18:1/20:4	4.4	3.5
18:1/20:5	1.2	2.7
20:4/20:5 + 20:5/20:4	5.2	6.4
20:5/18:3ω6	0.2	0.4
20:5/18:3ω3	0.4	1.3
20:5/20:5	8.5	8.9
x/20:4	tr	0.3

[a] Positional distribution not determined.
tr, trace level (<0.2%); x, unidentified fatty acid.

TABLE 5. Effect of SAN 9785 (100 μM) on the molecular species composition of MGDG and DGDG in *Monodus subterraneus.*

Molecular species (*sn*-1/*sn*-2)	Molecular species composition[a]			
	MGDG		DGDG	
	Control	SAN 9785	Control	SAN 9785
16:0/16:1[b]	4.7	0.7	44.3	25.8
16:1/16:1	0.7	0.8	10.3	18.3
20:4/16:0	tr	—	tr	tr
20:4/16:1	tr	—	—	—
20:4/18:2[b]	—	tr	—	—
20:5/14:0	7.4	2.6	tr	tr
20:5/16:0	3.2	1.1	6.9	9.2
20:5/16:1	14.0	4.2	38.5	47.8
20:5/18:1[b]	—	tr	—	—
20:5/18:2[b]	—	tr	—	—
20:5/18:3ω3[b]	tr	tr	—	—
20:5/20:4[b]	1.2	2.6	—	—
20:5/20:5	68.0	87.8	—	—

[a] Molecular species were analyzed using a light-scattering detector without calibration. Data are percentages of total signal area.
[b] Positional distribution not determined.
tr, trace level (<0.2%).

acid at the *sn*-1 position and either a C_{16} or C_{20} fatty acid at the *sn*-2 position (Table 4). We could not find any 18:2-containing molecular species and very little of those containing 18:3(ω6+ω3). In the presence of SAN 9785, the proportion of most molecular species containing a C_{20} acyl group at the *sn*-2 position decreased, whereas that of shorter chained molecular species increased. For example, 16:0/20:5 and 14:0/20:4 decreased sharply from 13.7% and 6.1% to 3.8% and 1.6%, respectively. However, 16:1/20:5 increased from 11.8% to 18.9%. In the control, there were also lower proportions of 20:4/20:5 (5.2%) and 20:5/20:5 (8.5%) that were not significantly affected by the herbicide.

Molecular species composition of MGDG and DGDG. MGDG consists of two eukaryotic-like molecular species containing C_{20} in both positions (20/20), 20:5/20:5 and 20:5/20:4, and several prokaryotic-like molecular species containing medium to long chain (MLC, C_{14}-C_{18}), mostly saturated or monounsaturated, acyl groups at the *sn*-2 position (20/MLC and MLC/MLC). DGDG is exclusively made of the second type (16/16 and 20/16) (Table 5). In the presence of SAN 9785, there was a sharp increase in the proportion of the 20/20 molecular species of MGDG from 69.2% to 90.4% at the expense of the 20/MLC and MLC/MLC molecular species. In DGDG, 20:5/16:1 increased at the expense of 16:0/16:1.

Radiolabeling experiments. Pulse-chase experiments with [2-^{14}C]acetate were used to follow EPA biosynthesis. As expected, the label was mainly incorporated into the products of the *de novo* fatty acid synthesis, 14:0, 16:0, 16:1, and 18:1 (data not shown). However, C_{20} fatty acids, 20:3ω6, arachidonic acid (20:4ω6), and EPA were already labeled after the 2-h pulse. A similar finding was obtained after the incorporation of radiolabeled acetate into *Nannochloropsis* sp. (Schneider and Roessler 1994). Apparently, the involvement of acetate in fatty acid elongation is responsible for this phenomenon. Attempts to label with 18:1 failed because the label was mostly incorporated into neutral lipids. Indeed, data obtained from the incorporation of externally labeled fatty acids should be cautiously regarded, because the fatty acids may not accurately reflect the *de novo* pathway. We thus compared the kinetics obtained after the label with linoleic acid with that of acetate labeling to exclude any possible artifacts (data not shown).

After pulse labeling with [1-^{14}C]linoleic acid, the label was rapidly turned over to the fatty acids indicated in sequence 1 (data not shown). Immediately after the pulse, TAG was the most labeled lipid (Fig. 1). PC was the highest labeled polar lipid but lost its label gradually with the time course. DGTS, PE, and PG were labeled next, whereas MGDG and finally DGDG were the last ones to be labeled. Similar results were obtained for the acetate labeling experiments (data not shown). This sequence of events suggests that early steps of the biosynthesis of EPA occur using PC as a substrate, whereas DGTS, PE, and finally MGDG and DGDG, are used only later.

In the presence of SAN 9785, the incorporation of label into TAG was significantly reduced, in comparison with the control, by ca. 50% (Fig. 1), whereas its apparent precursor, DAG, was accumulated. In contrast to the control, PE was more labeled than DGTS throughout the time course, whereas the label of PC was much less affected. MGDG was labeled faster and eventually higher, whereas DGDG was somewhat less labeled. We infer these findings as an indication to the existence of a pathway involving PE and MGDG.

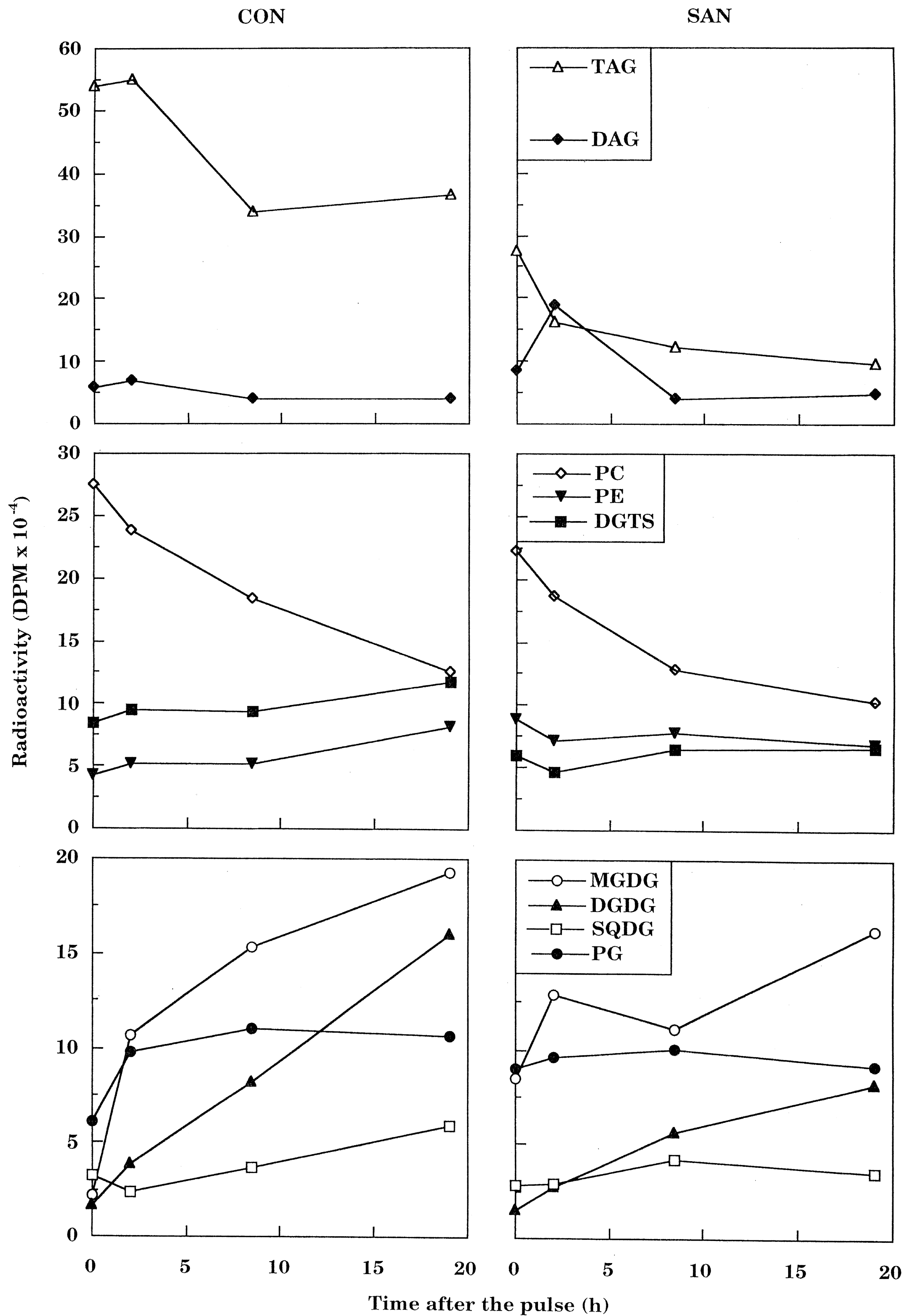


FIG. 1. Redistribution of radioactivity in lipids of *Monodus subterraneus* after labeling with [1-^{14}C] linoleic acid and exposure to 100 μM SAN 9785.

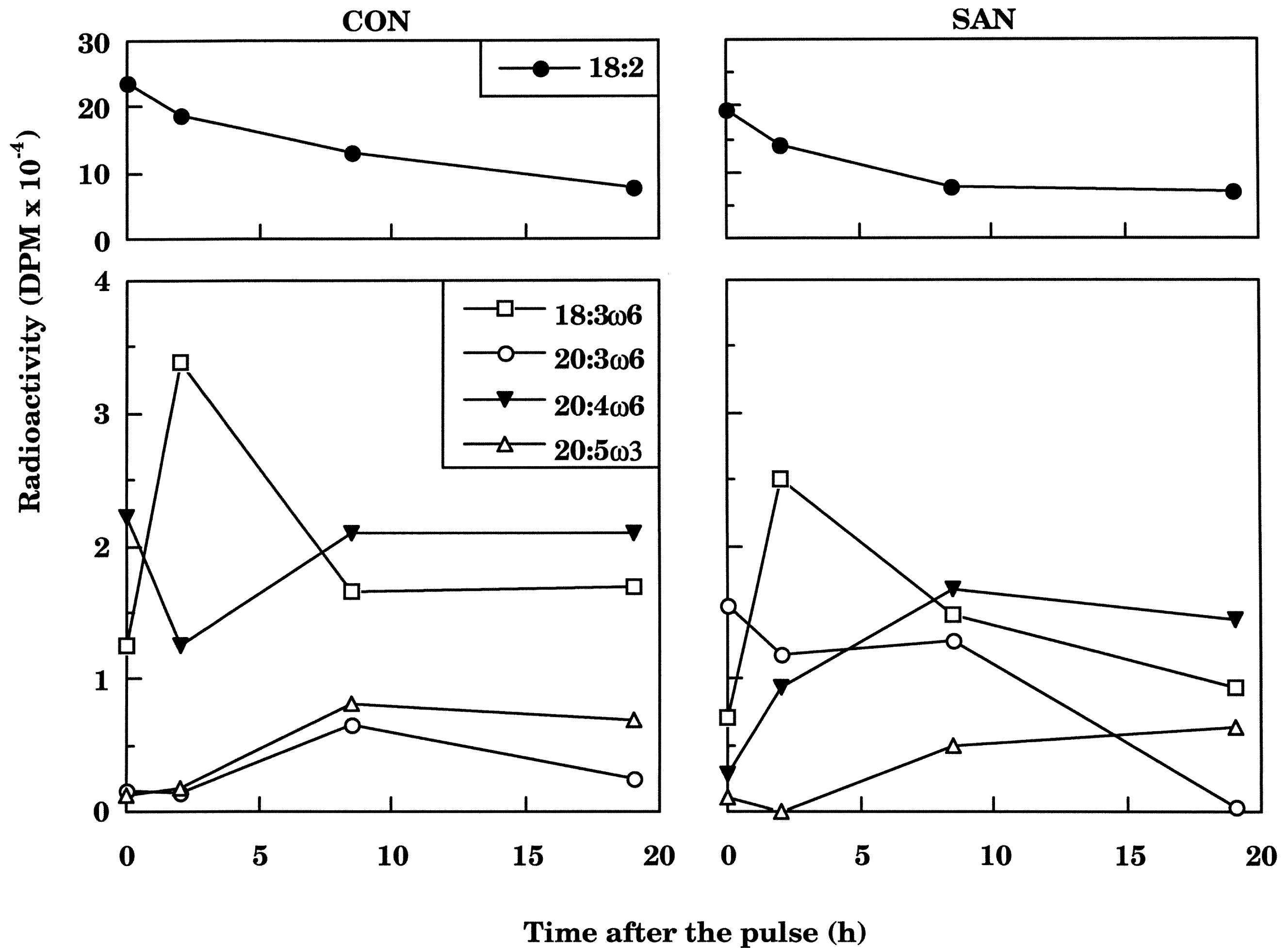


FIG. 2. Redistribution of radioactivity in the fatty acids of PC in *Monodus subterraneus* after labeling with [1-^{14}C]linoleic acid and exposure to 100 μM SAN 9785. Fatty acids were determined by radio-HPLC as their methyl esters.

In the first hours after the pulse, label of 18:2 in PC, which was initially similar to that of the control, decreased more quickly (Fig. 2). The label of its Δ6 desaturation product, 18:3ω6, was also lower. These findings could suggest a faster turnover in the presence of the inhibitor. Indeed, label of the elongation product, 20:3ω6, was much higher and reached its peak immediately after the pulse, in comparison with 8 h in the control. Turnover of label was also much faster in PE (Fig. 3). Label of 18:2 decreased faster, that of 18:3 peaked already after 2 h, and that of 20:4ω6 and 20:5ω3 after 9 h, in comparison with 9 and 18 h, respectively, in the control. The increase in the label of 20:3ω6 toward the end of the chase is likely an artifact resulting from fatty acid oxidation, because labeling with acetate did not result in elevated levels of 20:3ω6 at any time (data not shown).

In DGTS however, less 18:2 was incorporated, 18:3ω6 peaked only after 18 h, in comparison with 9 h in the control (Fig. 4). Label of 20:4ω6 and 20:5ω3 was significantly lower throughout the time course. We interpret these findings as an indication of a faster flow of label through PE, at the expense of DGTS. In MGDG, initial label of 20:4ω6 and 20:5ω3 was similar to that of the control; however, after 18 h, 20:4ω6 was less and 20:5ω3 was more labeled than in the control (Fig. 5).

DISCUSSION

The time course changes in the label of fatty acids after the incorporation of radiolabeled acetate and 18:2 indicated that the major fatty acids that participate in the biosynthesis of EPA in *M. subterraneus* are of the ω6 family, supporting sequence 1. However, the presence in PE of low proportions of the ω3 fatty acids, 18:3ω3 and 20:4ω3, suggests that the existence of a minor pathway utilizing ω3 fatty acids (sequence 2) cannot be excluded. Possibly, the desaturase that converts 20:4ω6 to 20:5ω3 is not a chain length specific Δ17D but an ω3D that can desaturate also 18:2 to 18:3ω3. This desaturation is the first and only dedicated step of the ω3 pathway. Further steps in this

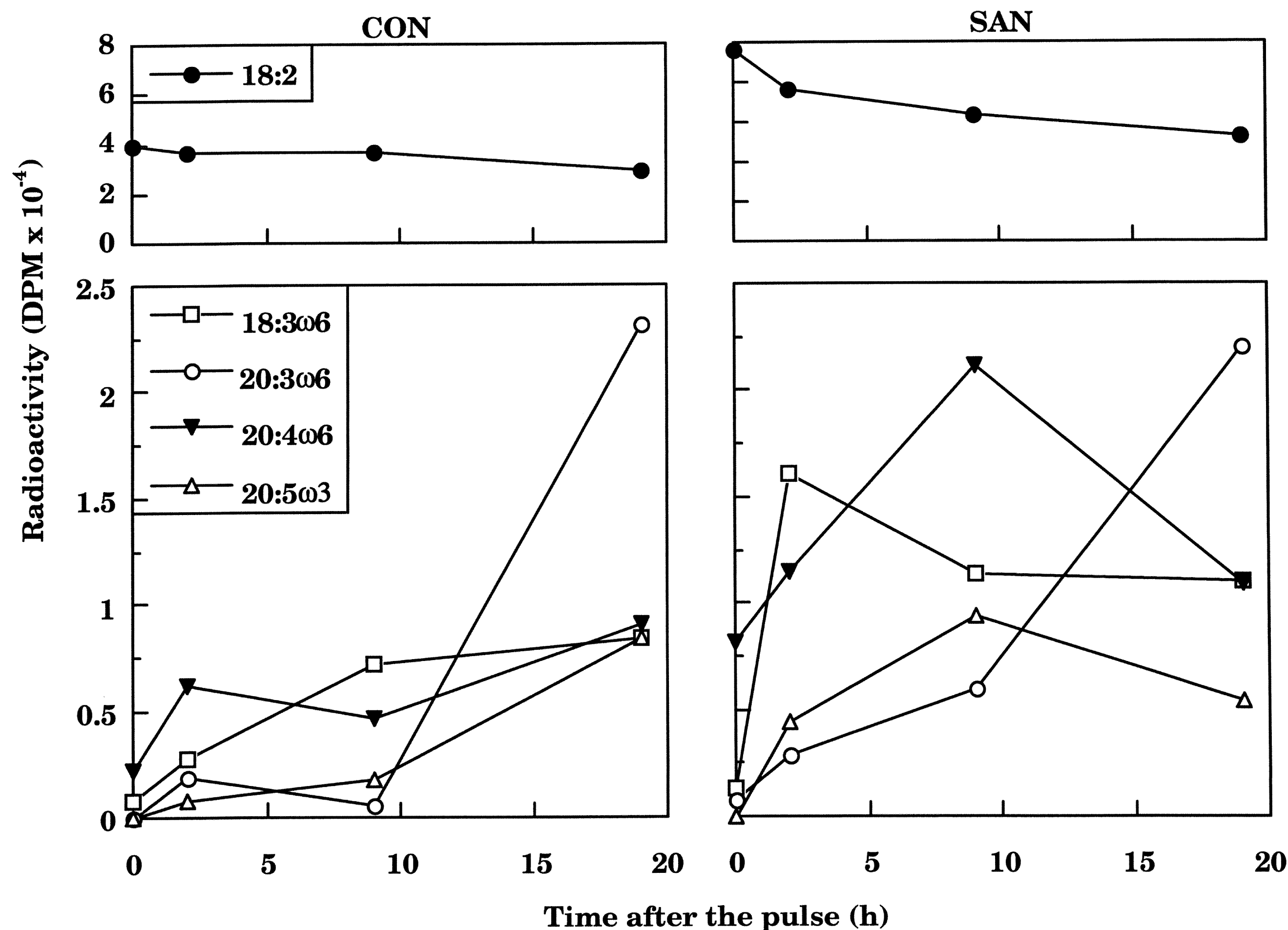


FIG. 3. Redistribution of radioactivity in the fatty acids of PE in *Monodus subterraneus* after labeling with [1-^{14}C]linoleic acid and exposure to 100 μM SAN 9785. Fatty acids were determined by radio-HPLC as their methyl esters.

pathway presumably use the same Δ6 and Δ5 desaturases of the Δ6 pathway. Indications to the existence of such pathway were previously demonstrated in *P. cruentum* (Shiran et al. 1996).

The labeling kinetics of the lipids, after the incorporation of [1-^{14}C]linoleic acid, show that PC was the first polar lipid to be labeled, indicating this lipid as a likely substrate in the early stages of EPA biosynthesis. Furthermore, the kinetics also suggests that the following steps involve PE and DGTS and finally also MGDG and DGDG. Similar results were obtained after acetate labeling (data not shown).

The data in Table 1 clearly show that SHAM inhibits the Δ6 and perhaps also the Δ12 desaturations of *M. subterraneus.* PC, which is richest in C_{18} fatty acids, is also the most affected lipid, indicating it as the likeliest substrate for the stepwise Δ12 and Δ6 desaturation of 18:1 to 18:3ω6. Similarly, we recently showed (Khozin-Goldberg et al. 1999) that SHAM inhibits the Δ6 and possibly the Δ12 desaturation of PC in *P. cruentum.* Furthermore, the molecular species composition of PC indicates that these desaturases show high preference to the *sn*-2 position. Although there was some 18:1 in the *sn*-1 position, there was very little 18:2 and no 18:3 in this position.

We previously showed (Cohen et al. 1997) that the inhibition of the Δ6 desaturation results in the accumulation of 18:2 and its elongation product, 20:2ω6. The latter can compete with 20:3ω6 for the Δ5 desaturase and be desaturated to the polymethylene-interrupted fatty acid, 20:3Δ5. Similarly, by competition with 20:4ω6 for the ω3 desaturation, 20:3Δ5 can be further desaturated to 20:4Δ5. The finding that, in the presence of SHAM, PE is the only cytoplasmic lipid to contain the two Δ5 fatty acids indicates that this lipid is apparently the major substrate for both the Δ5 and ω3 desaturations. Over 70% of the fatty acids of PE are C_{20} PUFAs. PE is thus the major acceptor of 20:3ω6, obtained from the elongation of 18:3. In the presence of SHAM, the availability of 18:3ω6, and therefore of 20:3ω6, is significantly reduced. Instead, 18:1 and 18:2 are apparently released from PC to the free fatty acid pool and from it to PE. Interestingly, in PE as well as in MGDG, C_{18} replace C_{20}, but not C_{16}

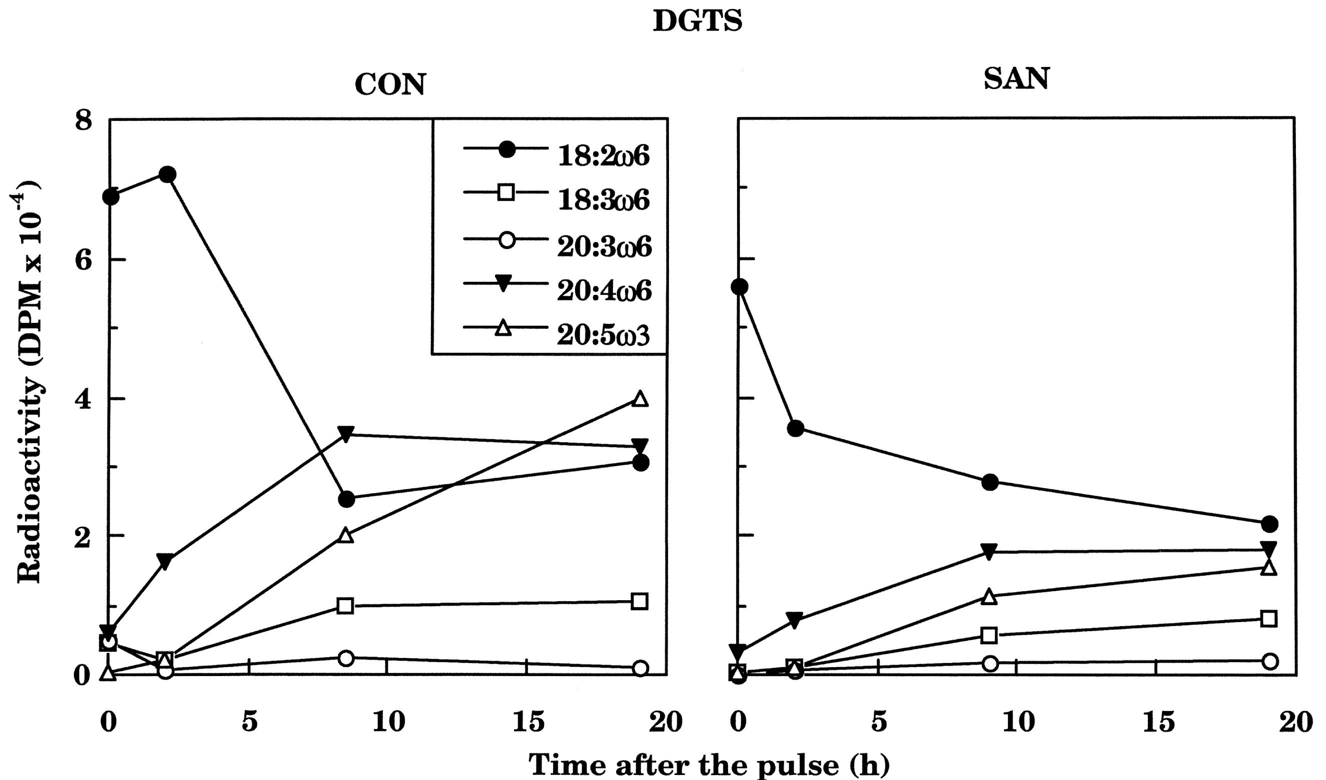


FIG. 4. Redistribution of radioactivity in the fatty acids of DGTS in *Monodus subterraneus* after labeling with [1-^{14}C]linoleic acid and exposure to 100 μM SAN 9785. Fatty acids were determined by radio-HPLC as their methyl esters.

fatty acids. As a result, the share of the 20/18 molecular species increased at the expense of the 20/20 molecular species. These findings are in keeping with a sequence by which 20/20 DAGs are imported from PE to produce the 20/20 molecular species of MGDG. If free fatty acids were imported, one would have expected C_{16} fatty acids to be replaced as well. DGTS contains both C_{18} and C_{20}; however, the very low proportions of 18:2 and 18:3ω6, under control conditions, suggest that it has only a minor role in the desaturation of C_{18} fatty acids. The much higher content of 20:4ω6 and 20:5ω3 (total of 29%) indicates this lipid as a possible substrate for the Δ5 and ω3 desaturations. Indeed, after the label with linoleic acid, desaturation of 20:3ω6 to 20:4ω6 and 20:5ω3 could clearly be observed.

A similar usage of PC and PE for the early and late desaturations, respectively, was suggested to occur in another eustigmatophyte, *Nannochloropsis* (Schneider and Roessler 1994). However, in contrast to *M. subterraneus,* the EPA content of DGTS of the former alga can be as high as 58% (Schneider et al. 1995) or even 90% (Haigh et al. 1996). These significantly higher figures suggest that the role of DGTS in the biosynthesis of EPA may be different in the two algae. Still different lipid combinations are used by other algae as substrates for the various C_{18} and C_{20} desaturations. In *Ochromonas danica* (Vogel and Eichenberger 1992) and the chlorophyte *Chlamydomonas reinhardtii* (Giroud and Eichenberger 1989) the Δ12, Δ15, and Δ6 desaturations operate on C_{18} fatty acids linked to the *sn*-2 position of DGTS or PE. In the rhodophyte *P. cruentum,* PC is the major substrate for the Δ12, Δ6, and Δ5 desaturation, whereas the final Δ17 desaturation of 20:4 to 20:5 is predominantly chloroplastic, involving 20/20 eukaryotic-like (MGDG) and 20/16 prokaryotic-like (MGDG and DGDG) molecular species (Khozin et al. 1997).

Phospholipids of higher plants are made of two types of molecular species combinations that can be termed 16/18 and 18/18, according to the chain length of the acyl groups residing in the *sn*-1 and *sn*-2 position, respectively. Generally, PC, the major substrate of the extra chloroplastic desaturations, is made of both types. These combinations are the consequence of the specificities of the acyltransferases that introduces acyl groups in the biosynthetic pathway of phosphatidic acid, the source of DAG for the production of phospholipids. Subsequently, C_{18} desaturation can take place at both positions, depending on the molecular species. In *M. subterraneus,* the early desaturations apparently use only the 16/18 combination of PC. However, later desaturations using 20/20 PE as its substrate occur at both positions, apparently requiring acyl transferases with a high specificity to 20:3 (Table 3). Similarly, in *P. cruentum,* C_{18} desaturations take

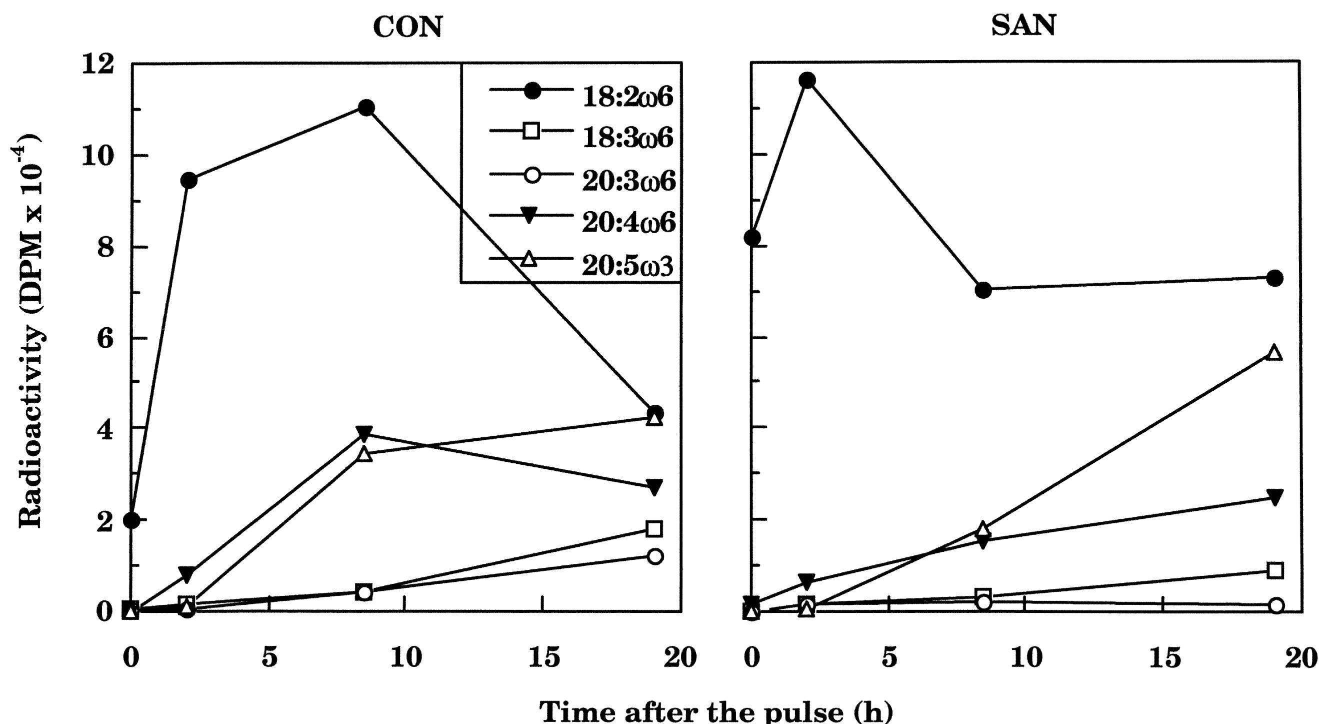


FIG. 5. Redistribution of radioactivity in the fatty acids of MGDG in *Monodus subterraneus* after labeling with [1-^{14}C]linoleic acid and exposure to 100 μM SAN 9785. Fatty acids were determined by radio-HPLC as their methyl esters.

place only at the *sn-2* position, whereas C_{20} desaturations can occur at both positions. Likewise, the positional distribution of fatty acids of PC in *P. tricornutum* (Arao et al. 1987) and *Porphyra yezoenzis* (Araki et al. 1987) suggests that C_{18} desaturations occur only at the *sn*-2 position of PC in these organisms.

Chloroplastic lipids of higher plants are also divided into two groups. Prokaryotic lipids are characterized by the presence of C_{16} acyl group in the *sn*-2 position and have the 18/16 structure, whereas eukaryotic molecular species consist mostly of the 18/18 and partly of the 16/18 combination. The prokaryotic-eukaryotic notation was adopted for algae, based on the structural resemblance between the 20/20 and the 18/18 combinations and between the 18/16 and the 20/16 molecular species. In analogy, molecular species of chloroplastic lipids of algae that contain a C_{16} acyl group at the *sn*-2 position and a C_{20} acyl at the *sn*-1 position were referred to as prokaryotic, whereas those having C_{20} acyls in both positions were termed eukaryotic (Roessler 1990). However, the biosynthetic pathway leading to the production of prokaryotic molecular species in higher plants is entirely chloroplastic, whereas it appears that in algae, the production of C_{20} fatty acids of both the eukaryotic-like and the prokaryotic-like molecular species is extrachloroplastic. Therefore, although the prokaryotic-like molecular species are structurally similar to those of higher plants, they are biosynthetically very different. To avoid this confusion, we suggest that molecular species of chloroplastic lipids in algae should be referred to as 20/20 or 20/MLC rather than eukaryotic and prokaryotic, respectively.

The products of the *de novo* pathway of fatty acid biosynthesis, 16:0, 16:1, and 18:1, are divided between TAG and polar lipids. In the presence of SAN 9785, the assembly of TAG is inhibited. A similar inhibition was shown to occur in *Pavlova lutheri* (Siljegovich-Hanggi and Eichenberger 1998) and in the eustigmatophyte *Nannochloropsis* (Henderson et al. 1990). In the former, an increase in label of EPA in the presence of the herbicide was also reported; however, the report did not specify whether EPA increased because of the decrease in PUFA-poor TAG or as a result of an enhanced production of EPA. The increase in the share of PE and of 20:5 in PE resulted in an almost 100% increase in the amount of EPA deposited in PE (from 0.7% to 1.3% of total fatty acids). The concurrent increase in the proportion of 20/20 MGDG may allude to a possible role of former as the source and the latter as the sink for 20:5/20:5 DAGs (Fig. 6). In DGTS, however, C_{20} PUFAs are more abundant at the *sn*-2 position than at the *sn*-1 position, making it a less likely source of 20/20 DAG. The herbicide affected a decrease in the proportion of the MLC/20 (except for 16:1/20:5) molecular species of DGTS. The corresponding decrease in the proportion of 20/MLC molecular species of MGDG could indicate DGTS as a possible donor of 20:4ω6 and 20:5ω3 that can be inserted into the *sn*-1 position of the 20/MLC combina-

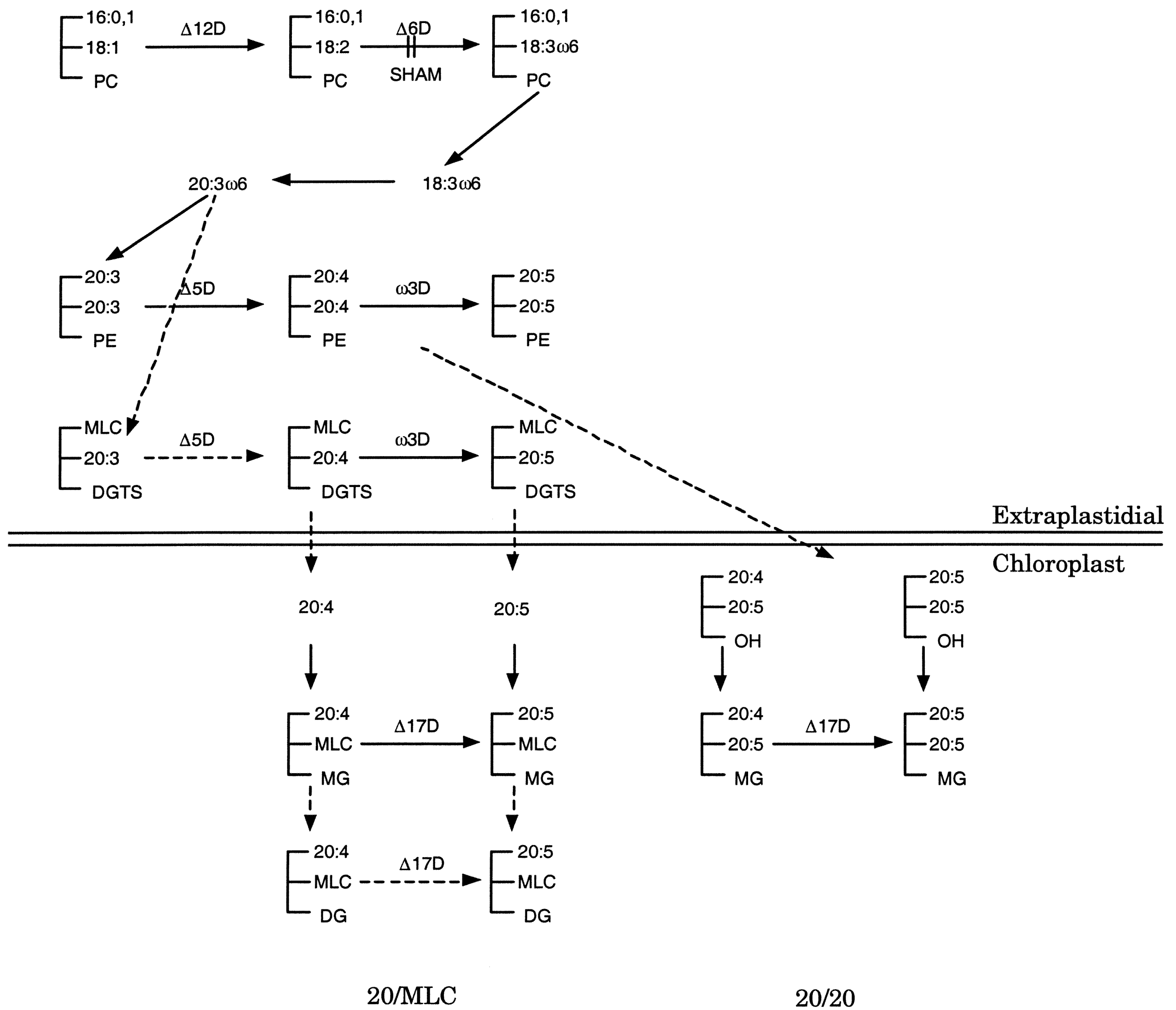


FIG. 6. Likely pathways in the biosynthesis of EPA in *Monodus subterraneus.* MLC, medium to long chain acyl groups (14–18 carbon atoms). Broken arrows denote hypothetical steps.

tions of chloroplastic lipids. The concurrent decreases in the proportion and label of DGTS lends further support to this novel pathway (Fig. 6). We thus suggest that in *M. subterraneus*, PC is the substrate of choice for C_{18} desaturations, whereas PE and DGTS are involved with that of C_{20}, producing the acyl moieties for the 20/20 (PE) and 20/16 and 20/14 (DGTS) molecular species of chloroplastic lipids (Fig. 6).

In higher plants, the differences between the eukaryotic and prokaryotic molecular species are generally only in the chain length of the *sn*-2 constituent. Consequently, there are no known differences between their roles in chloroplastic membranes. However, in algae, the 20/20 and 20/16 molecular species differ in up to five double bonds and four carbon atoms. Adlerstein et al. (1997) showed that in *P. cruentum*, the ratio of the 20/20 to 20/16 molecular species of MGDG increases significantly with decreasing temperature. The requirement for two types of molecular species in different environmental conditions may have resulted in the development of discrete pathways. The prokaryotic and eukaryotic molecular species of higher plants could possibly be remnants of the 20/20 and 20/16 molecular species of algae.

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