Plant Physiol. (1997) 114: 223–230

# Elucidation of the Biosynthesis of Eicosapentaenoic Acid in the Microalga *Porphyridium cruentum*[1]

## II. Studies with Radiolabeled Precursors

**Inna Khozin, Daniel Adlerstein, Chiara Bigongo, Yair M. Heimer, and Zvi Cohen***

The Laboratory for Microalgal Biotechnology (I.K., D.A. C.B., Z.C.), and the Albert Katz Center for Desert Agrobiology (Y.M.H.), Jacob Blaustein Institute for Desert Research, Ben-Gurion University of the Negev, Sde-Boker Campus 84990, Israel

**In the course of the study of the biosynthesis of the fatty acid eicosapentaenoic acid (EPA) in the microalga *Porphyridium cruentum*, cells were pulse-labeled with various radiolabeled fatty acid precursors. Our data show that the major end products of the biosynthesis are EPA-containing galactolipids of a eukaryotic and prokaryotic nature. The prokaryotic molecular species contain EPA and arachidonic acid at the *sn*-1 position and $C_{16}$ fatty acids, mainly 16:0, at the *sn*-2 positions, whereas in the eukaryotic species both positions are occupied by EPA or arachidonic acid. However, we suggest that both the eukaryotic and prokaryotic molecular species are formed in two pathways, ω6 and ω3, which involve cytoplasmic and chloroplastic lipids. In the ω6 pathway, cytoplasmic 18:2-phosphatidylcholine (PC) is converted to 20:4ω6-PC by a sequence that includes a Δ6 desaturase, an elongation step, and a Δ5 desaturase. In the minor ω3 pathway, 18:2-PC is presumably desaturated to 18:3ω3, which is sequentially converted by the enzymatic sequence of the ω6 pathway to 20:5ω3-PC. The products of both pathways are exported, as their diacylglycerol moieties, to the chloroplast to be galactosylated into their respective monogalactosyldiacylglycerol molecular species. The 20:4ω6 in both eukaryotic and prokaryotic monogalactosyldiacylglycerol can be further desaturated to EPA by a chloroplastic Δ17 (ω3) desaturase.**

The biosynthetic pathways leading to PUFA in leaves of higher plants were recently reviewed (Browse and Somerville, 1991). In the prokaryotic pathway, 18:1 linked to a galactolipid is stepwise desaturated to 18:3ω3. In the eukaryotic pathway, 18:1 produced in the chloroplast is exported to the cytoplasm, and after linking to PC it is similarly desaturated to 18:2 and 18:3ω3. The 18:2-containing DAG moieties of PC are reimported to the chloroplast, galactosylated, and further desaturated to form the 18:3ω3/18:3ω3 eukaryotic molecular species of galactolipids. Prokaryotic species typically contain $C_{18}$ and $C_{16}$ fatty acids in their *sn*-1 and *sn*-2 positions, respectively, whereas the eukaryotic species are characterized by the presence of $C_{18}$ fatty acids in the *sn*-2 position.

The respective pathways in algae that produce PUFA of no more than 18 carbon atoms are presumed to be similar to those of higher plants (Norman et al., 1985). However, the biosynthesis of $C_{20}$ PUFA in various algae appears to be different and more complex. Nichols and Appleby (1969) claimed that in *Euglena* the elongation of 18:2ω6 to 20:2ω6 precedes the desaturation to 20:3ω6 according to Sequence I, whereas in the red microalga *Porphyridium cruentum*, 18:2 is first desaturated to 18:3ω6 according to Sequence II. The few detailed studies published so far suggest that, in the examined strains, EPA biosynthesis is entirely cytoplasmic. Thus, in the heterotrophic alga *Crypthecodinium cohnii*, which does not produce galactolipids, EPA is synthesized via PC-linked fatty acids (Henderson and Mackinlay, 1991). Schneider and Roessler (1994) have shown that in the eustigmatophyte *Nannochloropsis*, the desaturation sites of $C_{18}$ and $C_{20}$ fatty acids are PC and phosphatidylethanolamine, respectively. Arao et al. (1994) and Arao and Yamada (1994) have shown that in the diatom *Phaeodactylum tricornutum*, 18:2/18:2 PC is converted to EPA via a complex network of cytoplasmic pathways that involves ω3 and Δ6 desaturations and an elongation step in no particular order. Differential responses to the herbicide SAN 9785 (Sandoz, Basel, Switzerland) have shown that the ω3 desaturation of 20:4ω6 to 20:5ω3 can be cytoplasmic in some algae and chloroplastic in others, e.g. *P. cruentum* (Khozin and Cohen, 1996). Feeding fatty acids to *P. cruentum* revealed two possible pathways (Shiran et al., 1996). In the ω6 pathway,

[1] This research was supported in part by the Israeli Ministry of Art and Sciences. I.K. was supported in part by a fellowship from the Israeli Ministry of Absorption. This is contribution no. 85 from the Laboratory for Microalgal Biotechnology, Jacob Blaustein Institute for Desert Research.

* Corresponding author; e-mail cohen@bgumail.bgu.ac.il; fax 972-7-657-0198.

Abbreviations: AA, arachidonic acid (20:4ω6); $C_n$ 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; EPA, eicosapentaenoic acid (20:5ω3); MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PUFA, polyunsaturated fatty acids; TAG, triacylglycerol; X:Y, a fatty acyl group containing X carbon atoms and Y *cis* double bonds. Pairs of numbers representing the fatty acids, when separated by a slash, designate the components in the *sn*-1 and *sn*-2 positions, respectively, of the molecular species (except for 20:4/20:5, which is a mixture of two isomers).

18:2 is desaturated to 18:3ω6, elongated to 20:3ω6, and subsequently desaturated to 20:4ω6 and to 20:5ω3 (Sequence III). In the ω3 pathway, 18:2 is first desaturated to 18:3ω3, which is sequentially converted to 18:4ω3, 20:4ω3, and 20:5ω3, presumably by the same enzymatic sequence as that of the ω6 pathway (Sequence IV).

$$\underset{9,12}{18{:}2\omega6} \xrightarrow{\Delta 8D} \underset{11,14}{20{:}2\omega6} \xrightarrow{} \underset{8,11,14}{20{:}3\omega6} \xrightarrow{\Delta 5D} \underset{5,8,11,14}{20{:}4\omega6}$$

**Sequence I**

$$\underset{9,12}{18{:}2\omega6} \xrightarrow{\Delta 6D} \underset{6,9,12}{18{:}3\omega6} \xrightarrow{} \underset{8,11,14}{20{:}3\omega6} \xrightarrow{\Delta 5D} \underset{5,8,11,14}{20{:}4\omega6}$$

**Sequence II**

$$\underset{9,12}{18{:}2\omega6} \xrightarrow{\Delta 6D} \underset{6,9,12}{18{:}3\omega6} \xrightarrow{} \underset{8,11,14}{20{:}3\omega6} \xrightarrow{\Delta 5D} \underset{5,8,11,14}{20{:}4\omega6} \xrightarrow{\Delta 17D} \underset{5,8,11,14,17}{20{:}5\omega3}$$

**Sequence III**

$$\underset{9,12}{18{:}2\omega6} \xrightarrow{\Delta 15D} \underset{9,12,15}{18{:}3\omega3} \xrightarrow{\Delta 6D} \underset{6,9,12,15}{18{:}4\omega3} \xrightarrow{} \underset{8,11,14,17}{20{:}4\omega3} \xrightarrow{\Delta 5D} \underset{5,8,11,14,17}{20{:}5\omega3}$$

**Sequence IV**

The emerging pharmaceutical importance of PUFA (Simopoulos, 1991) and the indications that microalgae can serve as a source for PUFA (Cohen et al., 1995) have rekindled the interest in the biosynthesis of these fatty acids. In this study we have attempted to obtain more insight (Shiran et al., 1996) into the various pathways of EPA biosynthesis at the fatty acid, lipid, and molecular species level in *P. cruentum* by pulse-labeling with various radioactive fatty acid precursors.

## MATERIALS AND METHODS

### Growth Conditions

*Porphyridium cruentum* strain 1380.1d was obtained from the Göttingen Algal Culture Collection (Göttingen, Germany) and was grown on Jones' medium (Jones et al., 1983) as previously described (Cohen et al., 1988) in Erlenmeyer flasks under an air:$CO_2$ (99:1, v/v) atmosphere at 25°C. The flasks were placed in an incubator shaker and illuminated from above at a light intensity of 115 μmol quanta $m^{-2}$ $s^{-1}$. Cultures were grown exponentially (with proper dilution) for at least 4 d prior to the onset of the experiment.

### Labeling Experiments

[1-$^{14}$C]AA (specific activity 58 mCi/mmol) was obtained from Amersham, and [1-$^{14}$C]linoleic and [1-$^{14}$C]α-linolenic acids (specific activity 53 mCi/mmol) were obtained from NEN Research Products. Ammonium salts of labeled fatty acids, 25 μCi of [1-$^{14}$C]linoleic acid, 25 μCi of [1-$^{14}$C]linolenic acids, or 10 μCi of [1-$^{14}$C]AA were obtained by neutralization of the free fatty acids with equimolar amounts of 2 M ammonium hydroxide. Cultures were pulse-labeled for 30 min, centrifuged, washed repeatedly with label-free medium, resuspended in one-half of the original volume, and cultivated in Erlenmeyer flasks as previously described (Cohen et al., 1988). Aliquots were taken at various times after the pulse.

### Lipid Extraction and Analysis

Lipids were extracted using the procedure of Bligh and Dyer (1959). Fatty acid methyl esters of total and individual lipids were obtained by transmethylation with 2% sulfuric acid in methanol. Fatty acid methyl esters were separated by reverse-phase HPLC on a reverse-phase-18, 5-μm column (250 mm, Lichrospher 100, Merck, Darmstadt, Germany) using a solvent system of methanol:acetonitrile: water (76:12:12, v/v), detected at 205 nm and identified using authentic standards. Radioactivity of individual peaks was determined by a detector (Flo-One β series A-100, Radiomatic Instruments and Chemical Co., Inc., Tampa, FL).

Distribution of radioactivity among individual lipids was assessed by TLC on 10- × 10-cm plates (0.25 mm thickness, Silica Gel 60, Macherey-Nagel, Duren, Germany). Two-dimensional separations of polar lipids were carried out using a solvent system of chloroform:methanol: water (65:25:4, v/v) for the first direction and of chloroform:methanol:1-ethylpropylamine:concentrated ammonia (65:35:0.5:5, v/v) for the second direction. To estimate distribution of label in neutral lipids, aliquots of total lipid extracts were separated by TLC using a solvent system of petroleum ether:diethyl ether:acetic acid (80:20:1, v/v). Lipids were visualized by brief exposure to $I_2$ vapors. Radioactivity was detected by autoradiography with x-ray films (X-Omat AR, Kodak) exposed to the TLC plates for 17 h. Lipid spots were scraped directly into scintillation vials containing 1 mL of methanol, a scintillation cocktail was added, and radioactivity was measured in a liquid-scintillation counter (Rackbeta, model 1217 [LKB, Wallac Oy, Finland]). MGDG extracted from the silica-gel plates was separated to the constituent molecular species by reverse-phase HPLC (column as mentioned above) with a solvent mixture of methanol:water (95:5, v/v) (Lynch et al., 1983) on a Waters (Millipore) chromatograph, equipped with UV and radioactivity detectors. Quantitative analysis of nonlabeled molecular species of MGDG was performed as previously described (Shiran et al., 1996).

PC molecular species were similarly analyzed using 30 mM choline chloride in methanol:water:acetonitrile (70:5: 25, v/v) as an eluent (Demandre et al., 1985). Positional analysis of the molecular species of MGDG and PC was

performed as previously described (Lynch and Thompson, 1986; Jones and Harwood, 1992) using lipase from *Rhizopus arrhizus* (Boehringer Mannheim) and phospholipase $A_2$ from *Crotalus adamanteus* venom (Sigma), respectively. For autoradiography, x-ray films were exposed for 48 to 72 h at −20°C to TLC plates containing resolved bands of free fatty acids and lysolipids. We have also compared the HPLC mobilities of the DAG derivatives, obtained by a phospholipase C (from *C. perfringens*, Sigma) treatment, with that of available DAGs.

## RESULTS

Initially we labeled cultures of *P. cruentum* with acetate; however, the extent of incorporation into polar lipids was too low to allow for reliable resolution (data not shown). Thus, we chose to label the cultures with long-chain fatty acids, although we were aware that the incorporation of externally introduced fatty acids may not completely image the de novo pathway and the positional specificities. The fatty acids we utilized were linoleic acid, which is the branching point between the suggested ω6 and ω3 pathways (Sequences III and IV), and AA and α-linolenic acid, which are intermediates in these pathways, respectively. The cultures were pulse-labeled with these fatty acids as described in "Materials and Methods," and changes in the label of lipid, fatty acid, and molecular species, as well as the positional specificity in MGDG and PC, were monitored using two-dimensional TLC and radio HPLC.

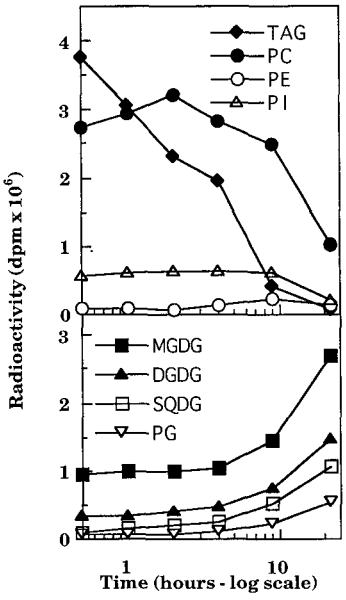


**Figure 1.** Redistribution of total radioactivity in cytoplasmic (top) and chloroplastic (bottom) lipids of *P. cruentum* after labeling with [1-$^{14}$C]linoleic acid. Exponentially cultivated algal cultures were labeled with [1-$^{14}$C]linoleic acid for 30 min and resuspended in label-free medium at time 0 as described in "Materials and Methods." PE, Phosphatidylethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerol.

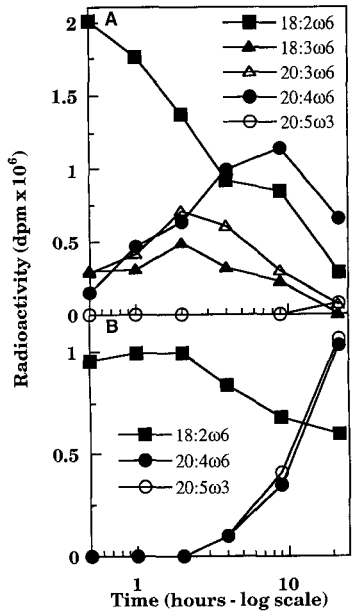


**Figure 2.** Redistribution of total radioactivity in fatty acids of PC (A) and MGDG (B) after labeling (see Fig. 1) with [1-$^{14}$C]linoleic acid. Lipids were separated by TLC and fatty acids were determined by radio HPLC.

### Incorporation of [1-$^{14}$C]Linoleic Acid

Following the pulse, PC and TAG were the highest-labeled lipids (Fig. 1); however, their label decreased rapidly. Other phospholipids were labeled to a much lower extent and remained so throughout the experiment. At the end of the pulse, chloroplastic lipids accounted for 15% of total counts, increasing to 77% after 22 h. The time-dependent increase of the label of chloroplastic lipids exceeded the decrease in PC during the same time period. The changes in the labeling pattern suggest a flux of fatty acids from cytoplasmic to chloroplastic lipids, mainly from PC and TAG to galactolipids, a characteristic of a eukaryotic pathway.

The fatty acid composition of total lipids included only ω6 fatty acids except for 20:5ω3 (data not shown). The labeling kinetics suggested a turnover of 18:2 through 18:3ω6 and 20:3ω6 to 20:4ω6 and possibly to 20:5ω3, according to Sequence III. In the first 2 h after the pulse, 18:2 was the only radiolabeled fatty acid in TAG, but between 4 and 9 h most of its label was lost (data not shown). The only other fatty acid to appear was 20:4ω6, which was detected after 4 h and increased slightly thereafter.

The labeling kinetics of the fatty acids in PC were similar to those of total lipids; however, 20:5 was only scarcely labeled and that late in the process (Fig. 2A). These findings suggest that, in PC, 18:2 is metabolized mainly via an ω6 pathway to 20:4ω6, according to Sequence II. Positional analysis of PC (Table I) has shown that the *sn*-2 position contained 94% of the label after the 1st h and 71% after 4 h. Since most of the label in the 1st h was found in $C_{18}$ fatty acids (Fig. 2A), we infer that initially the major radiolabeled molecular species of PC, 20:4/18:2* and 20:4/18:3ω6*,

were labeled only in the *sn*-2 position (Fig. 3). After 4 h the label in the *sn*-1 position increased to 29%, and $C_{20}$ fatty acids, predominantly 20:4ω6, constituted more than 90% of the label (data not shown). This indicates that 20:4ω6*/20:3ω6* (a mixture of both positional isomers) and 20:4ω6*/20:4ω6* were labeled in both positions (data not shown). Similar to PC of higher plants, some of the molecular species of PC in *P. cruentum* contained 16:0 at the *sn*-1 position. Thus, 16:0/18:2*, which was initially labeled, was gradually replaced by 16:0/18:3* and 16:0/20:4* (data not shown). The flow of radioactivity from 20:4/18:2* and 16:0/18:2* to 20:4*/20:4* and 16:0/20:4*, respectively, suggests the existence of a cytoplasmic ω6 pathway utilizing PC-bound fatty acids as its substrates, according to Sequence V. The labeling pattern of other phospholipids (phosphatidylinositol and phosphotidylethanolamine) was relatively low, and we could not observe any substantial changes in their label during the course of the experiment. Still, we cannot entirely exclude the possibility that PC rapidly exchanges acyl groups with other phospholipids that serve as substrates for the desaturations.

20:4ω6/18:2* → 20:4ω6/18:3ω6* → 20:4ω6/20:3ω6*
(16:0) (16:0) (16:0)

→ 20:4ω6*/20:4ω6*
(16:0)

**Sequence V**

In MGDG only 18:2 (initially) and 20:4ω6 and 20:5 (later) were significantly labeled (Fig. 2B). However, in contrast to PC, 18:2-MGDG does not seem to be a major source of 20:4ω6 and 20:5, since their increase was not accompanied with a compatible decrease in 18:2. HPLC analysis of MGDG has shown that 18:2*/16:0 (Fig. 4) and two other molecular species, 20:4ω6/18:2* and 20:5ω3/18:2* (data not shown), were responsible for all the counts immediately after the end of the pulse. The extent of label of 18:2*/16:0 and 20:5ω3/18:2* did not change much, but disappeared rapidly from 20:4/18:2*. Low labeling of two unidentified molecular species were also observed (data not shown). After 4 h two prokaryotic-like molecular species, 20:4ω6*/16:0 and 20:5ω3*/16:0, were observed. The first eukaryotic molecular species to appear was 20:4ω6*/20:4ω6* followed by its putative ω3 (Δ17) desaturation products 20:4ω6*/20:5ω3* and 20:5ω3*/20:5ω3*. We did not find any other molecular species that could have been considered as a precursor for either the prokaryotic or the eukaryotic $C_{20}$-containing species, suggesting that these are imported from the cytoplasm and that only the Δ17 (ω3) desaturation takes place in the chloroplast (Sequences VI and VII).

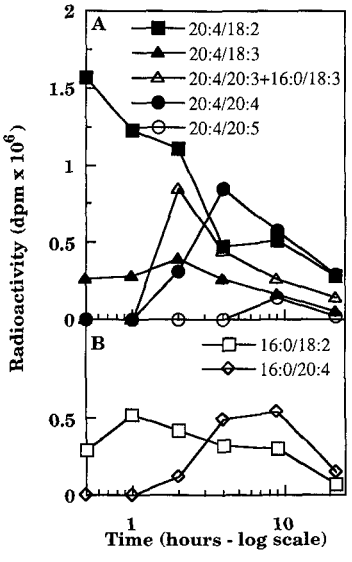


**Figure 3.** Redistribution of radioactivity in the molecular species of PC after labeling (see Fig. 1) with [1-$^{14}$C]linoleic acid. All of the fatty acids except for 20:5 are of the ω6 family. PC was separated by TLC and its molecular species composition was determined by radio HPLC.

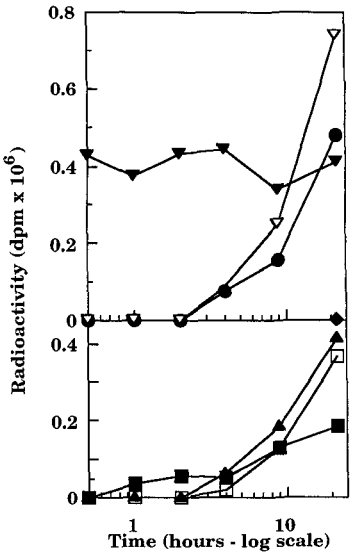


**Figure 4.** Redistribution of radioactivity in the molecular species of MGDG after labeling with [1-$^{14}$C]linoleic acid. ▼, 18:2/16:0; ◆, 20:4ω6/16:0; ▽, 20:5/16:0; ■, 20:4ω6/20:4ω6; ▲, 20:4ω6/20:5; □, 20:5/20:5.

20:4ω6/16:0 → 20:5ω3/16:0

**Sequence VI**

20:4ω6/20:4ω6 → 20:4ω6/20:5ω3 → 20:5ω3/20:5ω3

**Sequence VII**

### Labeling with [1-$^{14}$C]AA

Most of the early label resulting from the introduction of [$^{14}$C]AA evolved from PC (data not shown). In contrast to the [$^{14}$C]18:2 labeling experiments, the label in TAG was much lower and chloroplastic lipids were not significantly labeled until 4 h after the pulse. However, the latter became the most heavily labeled lipids toward the end of the experiment. This further supports the postulated existence of a eukaryotic pathway. Two unidentified lipids, which accounted for less than 5% of the counts each, were also observed. The radioactivity of digalactosyldiacylglycerol and sulfoquinovosyldiacylglycerol was significantly lower

**Table I.** *Positional distribution of label in PC of P. cruentum following the incorporation of radiolabeled fatty acid precursors*

Cultures of *P. cruentum* were pulse-labeled with various 1-$^{14}$C-radiolabeled fatty acid precursors for 30 min. The cultures were reconstituted in label-free medium and sampled periodically. Lipids were extracted and PC was isolated by TLC and hydrolyzed by phospholipase $A_2$ as described in "Materials and Methods". The radioactivity of the resulting fatty acids and lysoPC was measured by a scintillation counter.

| Time after Pulse | Radiolabeled Precursor | | | | | |
|---|---|---|---|---|---|---|
| | 18:2ω6 | | 18:3ω3 | | 20:4ω6 | |
| | Position | | | | | |
| | *sn*-1 | *sn*-2 | *sn*-1 | *sn*-2 | *sn*-1 | *sn*-2 |
| *h* | % of total counts | | | | | |
| 0 | 6.2 | 93.8 | 5.1 | 94.9 | 34.7 | 65.3 |
| 1 | 5.7 | 94.3 | 5.0 | 95.0 | | |
| 2 | 14.1 | 85.9 | | | | |
| 4 | 28.9 | 71.1 | 6.3 | 93.7 | 32.1 | 67.9 |
| 9 | | | | | 52.9 | 47.1 |
| 22 | | | 9.1 | 90.9 | 54.2 | 45.8 |
| 30 | | | | | 60.3 | 39.7 |

compared with the labeling with either [$^{14}$C]18:2 or [$^{14}$C]18:3ω3 (see below). It is possible that externally introduced AA was not readily incorporated into chloroplastic lipids.

The fatty acid analysis of total lipids showed that a low level of EPA appeared after 2 h, but EPA was significantly labeled only after 9 h (data not shown). In PC, however, AA that labeled both the *sn*-1 and *sn*-2 positions (Table I) remained the only labeled fatty acid even after 48 h (Fig. 5A). It appears, therefore, that although AA is the major product of fatty acid biosynthesis in PC, its further desaturation to EPA in the cytoplasm is either insignificant or nonexistent. In contrast, in both *Nannochloropsis* (Schneider and Roessler, 1994) and *P. tricornutum* (Arao et al., 1994; Arao and Yamada, 1994), AA is desaturated to EPA by cytoplasmic ω3 (Δ17) desaturases using phospholipid-bound AA as its substrates.

The predominant molecular species of PC were 20:4ω6*/20:4ω6* and 16:0/20:4ω6* (Fig. 6; Table I). We also observed the appearance of 20:4ω6*/18:2 and its downstream products along the ω6 pathway, 20:4ω6*/18:3 and 20:4ω6*/20:3ω6, as well as the EPA-containing 20:4ω6*/20:5ω3. In keeping with the postulated role of PC in the eukaryotic

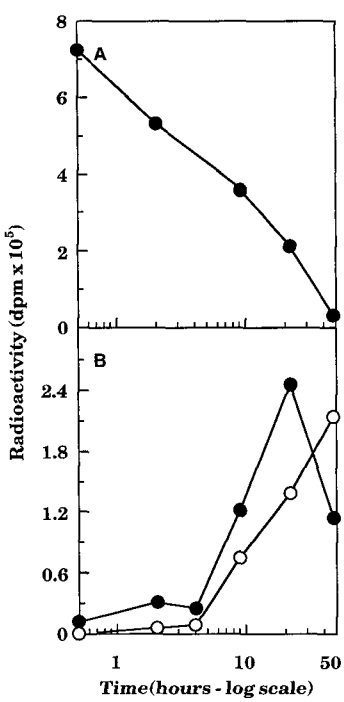


**Figure 5.** Redistribution of radioactivity in fatty acids of PC (A) and MGDG (B) after labeling with [1-$^{14}$C]AA. ●, 20:4ω6; ○, 20:5ω3.

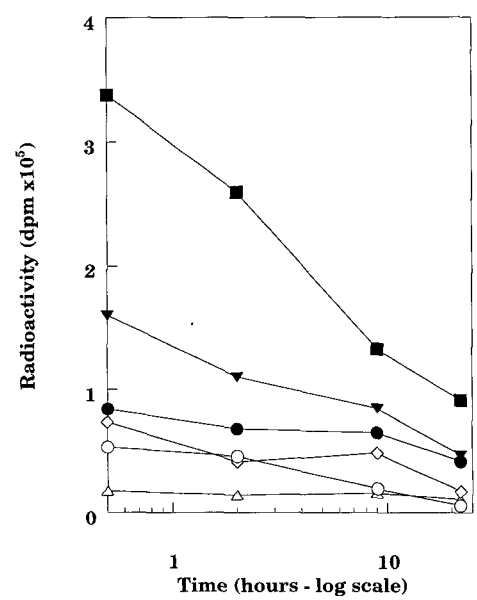


**Figure 6.** Redistribution of radioactivity in the molecular species of PC after labeling with [1-$^{14}$C]AA. ■, 20:4ω6/20:4ω6; ▼, 16:0/20:4ω6; ●, 20:4ω6/18:2; △, 20:4ω6/18:3ω6; ◇, 20:4ω6/20:3ω6; ○, 20:4ω6/20:5.

pathway, all PC molecular species eventually lost most of their label.

In MGDG labeling of EPA lagged behind that of AA initially; however, labeled AA started to decline after 22 h, and after 48 h that of EPA was much higher (Fig. 5B). The initial molecular species composition of MGDG was dominated by 20:4ω6*/16:0, 20:4ω6*/20:4ω6*, and 20:4ω6*/20:5* (Fig. 7), which were gradually replaced by 20:4ω6*/20:5* and 20:5*/20:5*. We interpret this as being further evidence of the existence of chloroplastic ω3 (Δ17) desaturase(s) capable of converting AA to EPA in both prokaryotic and eukaryotic species of MGDG, as shown in Sequences VI and VII, respectively.

### Incorporation of [1-$^{14}$C]α-Linolenic Acid

Similar to the 18:2 labeling experiments mentioned above, the initial label of [$^{14}$C]18:3ω3 was mainly incorporated into TAG and PC (data not shown) and was gradually turned over into chloroplastic lipids. The radiolabeled fatty acid of total lipids contained all the ω3 fatty acids depicted in Sequence IV (data not shown). A similar distribution was observed in PC (Fig. 8A); however, the proportion of total radioactivity of the intermediates 18:4ω3* and 20:4ω3* was higher than that in total lipids. The labeling kinetics of these fatty acids demonstrated a successive conversion of 18:3ω3 to EPA, according to sequence IV, suggesting that the enzymatic sequence necessary to convert 18:3ω3 to EPA, in PC, is the same one required for the production of AA, i.e. a Δ6 desaturase, an elongation system, and a Δ5 desaturase.

In MGDG, initially only 18:3ω3 was labeled; however, after 2 h labeled EPA rapidly accumulated and eventually became dominant (Fig. 8B). However, we could not detect any possible intermediate in either the fatty acid or the molecular species analysis (data not shown). These findings further support our hypothesis that all steps of EPA biosynthesis except the last one take place in the cytoplasm.

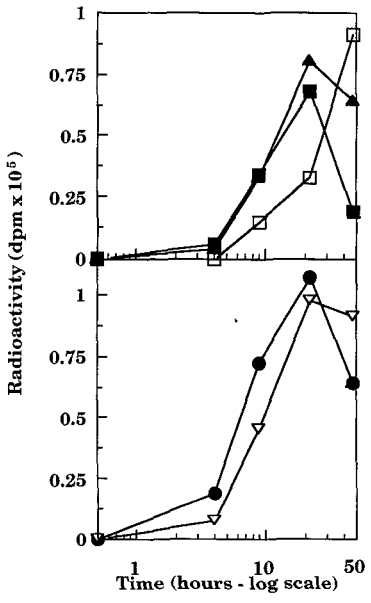


**Figure 7.** Redistribution of radioactivity in the eukaryotic (top) and prokaryotic (bottom) molecular species of MGDG after labeling with [1-$^{14}$C]AA. ■, 20:4ω6/20:4ω6; ▲, 20:4ω6/20:5; □, 20:5/20:5; ●, 20:4ω6/16:0; ▽, 20:5/16:0.

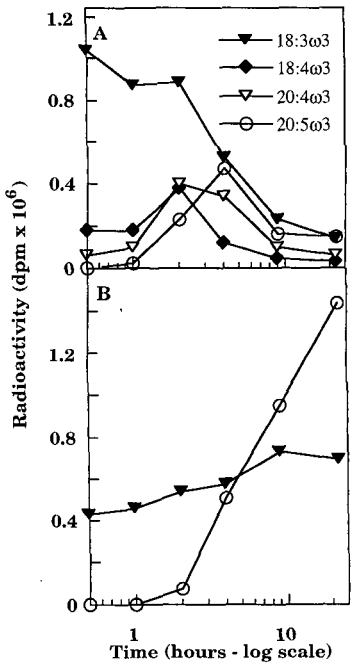


**Figure 8.** Redistribution of radioactivity in fatty acids of PC (A) and MGDG (B) after labeling with [1-$^{14}$C]α-linolenic acid.

## DISCUSSION

### EPA Biosynthesis

Based on the data presented in this paper, we have outlined the most likely biosynthetic routes of EPA in *P. cruentum* (Fig. 9). EPA is biosynthesized by two pathways originating at the level of 18:2, namely the ω6 and the ω3 pathways. In the ω6 pathway, 18:2-PC is sequentially converted in the cytoplasm to 20:4ω6/20:4ω6-PC (Fig. 3). In the ω3 pathway, 18:2-PC is first desaturated by a Δ15 (ω3) desaturase to 18:3ω3-PC, which is further converted, by the same enzymatic sequence utilized in the ω6 pathway, to 20:5ω3-PC (Fig. 8). The products of both pathways are exported, as their DAG moieties, to the chloroplast to be galactosylated into the respective MGDG molecular species. The AA in the molecular species that evolves from the ω6 pathway is further desaturated to EPA by a Δ17 (ω3) desaturase. Although we believe that Figure 9 indeed describes some of the most essential aspects of EPA biosynthesis in *P. cruentum*, our experimental data invoke several additional points of concern that deserve further discussion.

### Acylation at the *sn*-1 Position of PC

Labeled 18:2* was initially introduced only to the *sn*-2 position of PC (Fig. 2; Table I). However, once $C_{20}$ fatty acids were formed, radioactivity could be found in both the *sn*-1 and the *sn*-2 positions (Figs. 2 and 6; Table I). Indeed, the metabolism of lipids labeled from exogenously fed

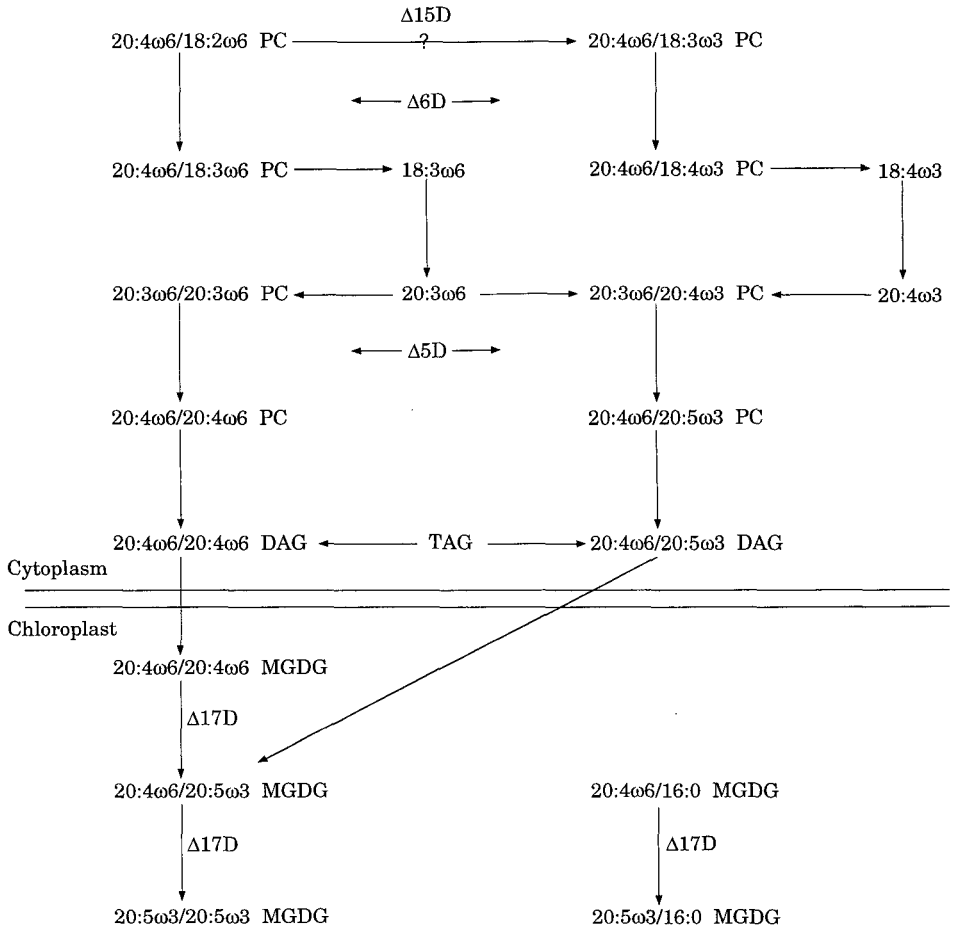


**Figure 9.** Suggested outline of EPA biosynthesis in *P. cruentum*.

radioactive fatty acids may not be the same as that of lipids assembled from fatty acids synthesized de novo. However, $C_{18}$ fatty acids in nonlabeled PC are also restricted to the *sn*-2 position (Table II). Although $C_{18}$ as well as $C_{20}$ fatty acids can be incorporated into the *sn*-2 position (Table I), the finding that $C_{20}$ but very little $C_{18}$ fatty acids are incorporated into the *sn*-1 position, even at time 0, raises the possibility that there must be another activity that is specific to the *sn*-1 position and excludes $C_{18}$ fatty acids. Similarly, Schneider and Roessler (1995) suggested the existence of an *sn*-1 acyltransferase specific to $C_{20}$ fatty acids in *Nannochloropsis*.

If $C_{18}$ fatty acids are indeed restricted to the *sn*-2 position, then what is the origin of $C_{20}$ fatty acids residing in the *sn*-1 position? Two possibilities could be put forward: (a) AA could be recycled from the *sn*-2 position of another molecular species of PC (or any another phospholipid); and (b) desaturation of $C_{18}$ fatty acids at the *sn*-1 position may take place in other phospholipids, and after a subsequent elongation to 20:3ω6, they could be rapidly exchanged with PC. One could also suggest a very rapid metabolism of $C_{18}$ fatty acids at the *sn*-1 position. However, the lack of any label in $C_{18}$ or $C_{20}$ at the *sn*-1 position in the 1st h after the pulse suggests otherwise.

**Table II.** *Molecular species composition of PC and MGDG in P. cruentum*

Lipids were extracted and PC and MGDG were separated by TLC. Molecular species analysis was performed by reverse-phase HPLC using a light-scattering detector.

| Molecular Species of PC | Percent of Total | Molecular Species of MGDG | Percent of Total |
|---|---|---|---|
| 20:4/20:5 | 8.4 | 20:5/20:5 | 42.0 |
| 20:4/20:4 | 65.0 | 20:5/20:4 | 2.8 |
| 20:4/20:3 + 16:0/18:3 | 0.8 | 20:4/20:4 | t[a] |
| 20:4/18:2 | 1.4 | 20:5/18:2 | t |
| 16:0/20:5 | 0.8 | 20:5/16:0 | 48.8 |
| 16:0/20:4 | 20.7 | 20:4/16:0 | 1.4 |
| 16:0/18:2 | t | 18:2/16:0 | 5.0 |

[a] t, Trace level.

## The Existence of the ω3 Pathway

We could demonstrate the appearance of EPA in PC by labeling with [$^{14}$C]18:2 (Fig. 2) but not with [$^{14}$C]AA (Fig. 5A). It thus follows that 18:2-PC can be converted to EPA in a sequence that is different from Sequence II. The finding that 18:3ω3-PC can utilize the same enzymatic sequence as 18:2-PC led us to suggest that the latter can be desaturated

to 18:3ω3-PC, and further converted to 20:5-PC utilizing Sequence IV. Supporting evidence for this claim was obtained by feeding unlabeled 18:2 to *P. cruentum*, which resulted in the production of 18:3ω3 as well as other ω3 intermediates depicted in Sequence IV (Shiran et al., 1996).

The identity of the desaturase that converts 18:2 to 18:3ω3 is still unknown. This desaturase could possibly convert 18:2-PC to 18:3ω3-PC. Such activity is likely to be a Δ15 rather than an ω3 desaturase, since it cannot desaturate any AA-containing molecular species of PC. Another possibility is that the chloroplastic ω3 desaturase that converts AA to EPA is responsible for this desaturation as well, using 18:2/16:0 galactolipids as its substrate. However, the extent of labeling of 18:2/16:0 did not change appreciably during the course of the experiment (Fig. 4). Therefore, the desaturation of AA to EPA is most likely carried on by a Δ17, rather than an ω3, desaturase.

### The Origin of AA in Prokaryotic Lipids

Both the eukaryotic and prokaryotic molecular species of galactolipids require AA. In analogy to higher plants eukaryotic species of MGDG import 20:4ω6/20:4ω6 from PC and probably also from TAGs (see below). However, in contrast to higher plants, in which the fatty acids to be incorporated into the *sn*-1 position of prokaryotic species are synthesized in the chloroplast, that of *P. cruentum* is apparently unable to produce AA for the *sn*-1 position (Fig. 4). Presumably, the AA of prokaryotic lipids originates in the cytoplasm. The prokaryotic lipids of *P. cruentum* are thus only structurally but not biosynthetically similar to prokaryotic species of higher plants.

### The Involvement of TAGs in EPA Biosynthesis

Most of the fed $C_{18}$ fatty acids were initially allocated into PC and TAGs (Fig. 1). During the chase, the label of these two lipids decreased, whereas that of chloroplastic lipids increased with no significant loss of total radioactivity. The substantial decrease in the label of TAGs and the concomitant increase in that of chloroplastic lipids suggest that the former, which are generally considered to be just reserve material, could actually participate in PUFA biosynthesis. TAGs are apparently in equilibrium with phospholipids, which convert 18:2 to AA that can be exported to the chloroplast. We have recently isolated a chill-sensitive mutant of *P. cruentum*. Radiolabeling studies have shown a lack of time-dependent decline of radioactivity of TAGs (Khozin et al., 1996). This mutant had also a lower-than-normal level of eukaryotic molecular species of MGDG. We have thus suggested (Khozin et al., 1996) that TAGs contribute DAG moieties to the eukaryotic pathway and are essential for the adaptation of this organism to suboptimal temperatures. The role of the eukaryotic molecular species and that of the TAGs with respect to environmental conditions will be discussed elsewhere.

### ACKNOWLEDGMENTS

We are indebted to an anonymous reviewer for helpful remarks. We are grateful to Ms. S. Didi for dedicated technical assistance.

Received October 28, 1996; accepted February 5, 1997.


### LITERATURE CITED

**Arao T, Sakaki T, Yamada M** (1994) Biosynthesis of polyunsaturated lipids in the diatom, *Phaeodactylum tricornutum*. Phytochemistry **36:** 629–635

**Arao T, Yamada M** (1994) Biosynthesis of polyunsaturated fatty acids in the marine diatom, *Phaeodactylum tricornutum*. Phytochemistry **35:** 1177–1181

**Bligh EG, Dyer WJ** (1959) A rapid method for total lipid extraction and purification. Can J Biochem Physiol **37:** 911–917

**Browse J, Somerville CR** (1991) Glycerolipid synthesis: biochemistry and regulation. Annu Rev Plant Physiol Plant Mol Biol **42:** 467–506

**Cohen Z, Didi S, Heimer YM** (1992) Overproduction of γ-linolenic and eicosapentenoic acids by algae. Plant Physiol **98:** 569–572

**Cohen Z, Norman HA, Heimer YM** (1995) Microalgae as a source of omega-3 fatty acids. World Rev Nutr Diet **77:** 1–31

**Cohen Z, Vonshak A, Richmond A** (1988) Effect of environmental conditions of fatty acid composition of the red alga *Porphyridium cruentum*: correlation to growth rate. J Phycol **24:** 328–332

**Demandre C, Tremolieres A, Justin AM, Mazliak P** (1985) Analysis of molecular species of plant polar lipids by high-performance and gas liquid chromatography. Phytochemistry **24:** 481–485

**Henderson JR, Mackinlay EE** (1991) Polyunsaturated fatty acid metabolism in the marine dinoflagellate *Crypthecodinium cohnii*. Phytochemistry **30:** 1781–1787

**Jones AL, Harwood JL** (1992) Lipid composition of the brown algae *Fucus vesiculosus* and *Ascophyllum nodosum*. Phytochemistry **31:** 397–403

**Jones RE, Speer L, Kury W** (1983) Studies on the growth of the red alga *Porphyridium cruentum*. Physiol Plant **16:** 636–643

**Khozin I, Adlerstein D, Bigogno C, Cohen Z** (1996) Elucidation of the biosynthesis of eicosapentaenoic acid (EPA) in the microalga *Porphyridium cruentum*. *In* JP Williams, MU Khan, NW Lem, eds, Physiology, Biochemistry, and Molecular Biology of Plant Lipids, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 93–95

**Khozin I, Cohen Z** (1996) Differential response of microalgae to the substituted pyridazinone Sandoz 9785 reveal different pathways in the biosynthesis of eicosapentaenoic acid (EPA). Phytochemistry **42:** 1025–1029

**Lynch DV, Gundersen RE, Thompson GA** (1983) Separation of galactolipid molecular species by high performance liquid chromatography. Plant Physiol **72:** 903–905

**Lynch DV, Thompson GA** (1986) Analysis of phospholipid molecular species by gas chromatography and coupled gas chromatography-mass spectrometry. *In* HF Linskens, JF Jackson, eds, Modern Methods of Plant Analysis (New Series), Vol 3: Gas Chromatography/Mass Spectrometry. Springer-Verlag, Berlin, pp 100–120

**Nichols BW, Appleby RS** (1969) The distribution of arachidonic acid in algae. Phytochemistry **8:** 1907–1915

**Norman HA, Smith LA, Lynch DV, Thompson GA** (1985) Effects of low-temperature stress on the metabolism of phosphatidylglycerol molecular species in *Dunaliella salina*. Arch Biochem Biophys **242:** 157–167

**Schneider JC, Roessler P** (1994) Radiolabeling studies of lipids and fatty acids in *Nannochloropsis* (Eustigmatophyceae), an oleaginous marine alga. J Phycol **30:** 594–598

**Schneider JC, Roessler P** (1995) A novel acyltransferase activity in an oleaginous alga. *In* JC Kader, P Mazliak, eds, Plant Lipid Metabolism. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 105–107

**Shiran D, Khozin I, Heimer YM, Cohen Z** (1996) Biosynthesis of eicosapentaenoic acid in the microalga *Porphyridium cruentum*. I. The use of externally supplied fatty acids. Lipids **31:** 1277–1282

**Simopoulos AP** (1991) ω-3 fatty acids in health and disease and in growth and development. Am J Clin Nutr **54:** 438–463