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LIPID COMPOSITION AND BIOSYNTHESIS IN THE MARINE DINOFLAGELLATE *CRYPTHECODINIUM COHNII*

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Key Word Index—*Crypthecodinium cohnii*; dinoflagellate; lipid composition; fatty acid biosynthesis.

Abstract—Triacyglycerols were the predominant lipid of the non-photosynthetic marine dinoflagellate *Crypthecodinium cohnii* grown heterotrophically for six days. Phosphatidylcholine was the major polar lipid. Triacylglycerol fatty acids were predominantly saturated, whereas 57% of the fatty acids in phosphatidylcholine were polyunsaturated and consisted almost exclusively of 22:6 (*n*-3); (*n*-6) polyunsaturated fatty acids were minor components. Radioactivity incorporated into lipid by the dinoflagellate grown in the presence of ^{14}C-acetate was recovered largely in phosphatidylcholine and triacylglycerols. Saturated fatty acids contained most of the radioactivity incorporated into both polar lipids and triacylglycerols. In polar lipids the relative specific activities of trienoic and particularly dienoic fatty acids were high in comparison with those of saturates, monoenes and fatty acids containing four or more double bonds. In triacylglycerols the differences in relative specific activities between groups of fatty acids were less obvious. The role of phospholipids as substrates for the desaturation steps in the formation of 22:6 (*n*-3) in *C. cohnii* is discussed.

INTRODUCTION

Although the lipids of marine organisms in general are characterized by long chain (*n*-3) polyunsaturated fatty acids (PUFA) [1], the ability to synthesize fatty acids *de novo* resides primarily in the phytoplankton. Little is known, however, of the mechanisms by which PUFA are formed in marine micro algae.

Studies with higher plants have shown that *sn*-1-acyl-2-oleoyl phosphatidylcholine is a substrate for the formation of 18:2 (*n*-6) from 18:1 (*n*-9) and that the further desaturation of 18:2 (*n*-6) to 18:3 (*n*-3) involves the glycolipid monogalactosyldiacylglycerol [2–4]. Since monogalactosyldiacylglycerol is associated with thylakoid membranes and phosphatidylcholine with endoplasmic reticulum, the formation of 18:3 (*n*-3) in higher plants involves co-operation between subcellular organelles. It remains to be established whether similar pathways operate in the formation of C_{16}–C_{22} PUFA which occur in marine microalgae [1].

Crypthecodinium cohnii is a non-photosynthetic, marine dinoflagellate in which *ca* 30% of the constituent fatty acids are 22:6 (*n*-3) and no other polyunsaturated fatty acids are present in excess of 1% [5, 6]. The absence of chloroplasts in *C. cohnii* means that it offers a system for the study of the synthesis of long chain PUFA without the involvement of chloroplast glycolipids. Previous studies have shown that fatty acid synthetase purified from *C. cohnii* produces mainly 16:0 and 14:0 [7] and that saturated C_{12}–C_{18} and unsaturated C_{18} fatty acids are not converted to 22:6 (*n*-3) when presented exogenously [8]. Consequently, the pathways of PUFA synthesis in this alga are still unclear. Although (*n*-6) PUFA are usually present in at least trace amounts in the total lipid of marine algae [9, 10], previous analyses of the lipids of *C. cohnii* have not reported the presence of (*n*-6) PUFA [5, 6].

The present study was undertaken to establish whether (*n*-6) PUFA do occur in the lipids of *C. cohnii* and to examine the relationship between newly synthesized fatty acids and their distribution in lipid classes.

RESULTS AND DISCUSSION

Lipid composition

Lipid accounted for 24.6% of the dry wt of *C. cohnii* cultured for six days to stationary phase. This lipid was mostly neutal lipid of which triacylglycerols were the major component (Table 1). Free fatty acids and sterols were also present in the neutral lipid fraction but in smaller proportions. Phosphatidylcholine (PC) was the major phospholipid present. Phosphatidylethanolamine (PE) was the second most abundant phospholipid although it was present in very much smaller amount than PC. Significant amounts of phosphatidylinositol (PI), phosphatidylglycerol (PG) and cardiolipin (CL) were present as were three unknowns which were visible on the chromatogram. PG is characteristic of chloroplast lipid in higher plants and occurs universally in photosynthetic algae [11]. However, its presence in *C. cohnii* can be attributed to its being a component of mitochondrial rather than photosynthetic membranes [12]. Under the conditions employed unknown **1** was evident in the region of phosphatidic acid, unknown **2** occurred in the region of CL, PE, and PG, and unknown **3** was visible between PE and PC. Complete identification of the three unknown polar lipids was not attempted.

More than 80% of the fatty acids in the total lipid in *C.*

Table 1. Lipid class composition (wt%) of *C. cohnii*

% Neutral lipid	71.5
% Polar lipid	28.5
Neutral lipid composition (% neutral lipid)	
Triacylglycerols	76.4
Free fatty acids	15.2
Sterols	8.4
Steryl esters	tr*
Polar lipid composition (% polar lipid)	
Phosphatidylcholine	63.6
Phosphatidylethanolamine	7.5
Phosphatidylserine	4.3
Phosphatidylinositol	6.7
Lysophosphatidylcholine	3.5
Sphingomyelin	—
Phosphatidylglycerol	3.6
Cardiolipin	4.6
Unknown 1	1.3
Unknown 2	0.8
Unknown 3	4.1

* tr; trace.

cohnii were saturated, with 14:0 accounting for more than half of these fatty acids (Table 2). Only 9.2% of the total lipid fatty acids were polyunsaturated. The fatty acid composition of the total lipid was similar to that of triacylglycerols, the major lipid class. In contrast to triacylglycerols, the phospholipids contained high proportions of polyunsaturated fatty acids. In agreement with previous published analyses [6] 22:6 (*n*-3) accounted for over 50% of the fatty acids in PC, the principal phospholipid. Only 38.4% of the fatty acids in this phospholipid were saturated, with 16:0 and 14:0 being present in almost equal proportions. PE and PS resembled each other in having PUFA contents of *ca.* 45%. However, PE contained high (43.8%) and low (10.5%) levels of monoenes and saturates, respectively, whereas only 4.7% of the fatty acids in PS were monoenes with 49% being saturated. All the phospholipids analysed contained only trace amounts of (*n*-6) PUFA with the exception of PI in which 18:2 (*n*-6) and 20:4 (*n*-6) accounted for 0.5% and 1.1%, respectively. Previous analyses of *C. cohnii* lipids have not included the fatty acid composition of all the component phospholipid classes and have reported the identified polyunsaturated fatty acids to be exclusively of the (*n*-3) series [5, 6]. Marine algae generally contain 18:2 (*n*-6) [11] and, more specifically, other dinoflagellates contain 18:2 (*n*-6) at 1–3% and 20:4 (*n*-6) in at least trace amounts [13]. The specific location of 20:4 (*n*-6) in PI in *C. cohnii* is in keeping with the situation in several tissues from a wide range of organisms including marine benthic invertebrates [14], teleost and elasmobranch fish [15, 16] and terrestrial animals [17]. However, the 20:4 (*n*-6) content of PI is much less than the 10–28% reported for the above species. The fatty acid composition of PI in micro algae has rarely been studied but 20:4 (*n*-6) does not appear to be specifically concentrated in PI in the few genera in which it has been studied [18]. Plants contain PI in chloroplast membranes although to a lesser degree than in other cellular membranes [12]. The association of 20:4 (*n*-6) with PI in an alga devoid of chloroplasts, such as *C. cohnii*, suggests that the association could be unique to non-chloroplast membranes. However, PI in the non-photosynthetic *N. alba* is devoid of 20:4 (*n*-6) although its other component lipids do contain small amounts [19].

^{14}C-*Acetate incorporation studies*

The amount of radioactivity from ^{14}C-acetate incorporated into lipid by *C. cohnii* over 6 hr increased with increasing cell number (Fig. 1). Triacylglycerols and PC together accounted for most of the radioactivity incorporated into lipid. The rate of incorporation into triacylglycerols was linear over 6 hr whereas that into PC exhibited a biphasic rate with an apparent decline occurring after 3 hr. More than 96% of the radioactivity incorporated into triacylglycerols was recovered in the fatty acid moieties at all times. Of the radioactivity present in total polar lipid 15 min after the addition of ^{14}C-labelled acetate, 74% was present in the fatty acid portion. After 6 hr this proportion had increased to 90.3%. Free fatty acids never comprised more than 2.0% of the radioactivity present in total lipid suggesting that newly-synthesized fatty acids were rapidly esterified. Likewise sterols and steryl esters contained less than 2.0% of the acetate-derived radioactivity. Although PE contained some 11% of the radioactivity present in the total lipid after 15 min the proportion decreased with time to reach 3.1% after 6 hr. Other polar lipid classes (PG, CL, SM, PI, PS and LPC) never contained more than 3% of the incorporated acetate recovered in the lipid. Two dimensional TLC of the total lipid isolated after 3 hr followed by autoradiography showed that of the unknowns described in Table 1, only unknown **3** contained radioactivity. It was, however, weakly labelled in comparison with the other phospholipids.

The incorporation of fatty acids into triacylglycerols and PC is in keeping with these lipids being the main neutral and polar lipids, respectively. Triacylglycerols are usually more abundant in microalgae during the resting phase associated with nitrate limitation or aged cultures [20]. The incorporation of newly-synthesized fatty-acids into triacylglycerols suggests that this neutral lipid is formed in *C. cohnii* even during periods of cell division. Triacylglycerols were obvious in extracts of lipid at all times separated by TLC in this study, and a previous study [6] also showed triacylglycerols to occur at all stages of the growth cycle.

Within the triacylglycerols, the amount of radioactivity increased linearly in all groups of fatty acids based on the degree of unsaturation, with saturates containing most radioactivity followed by monoenes (Fig. 2). PUFA were labelled to lesser extents. In the polar lipid, saturates also accumulated radioactivity to the greatest extent. Monoenes, dienes and fatty acids containing four or more double bonds were labelled to similar but lesser extents, with monoenes only being the highest labelled after 6 hr. Within the polar lipid trienoic fatty acids were least labelled from ^{14}C-acetate and the amount of radioactivity in these fatty acids did not increase to any great extent over the time studied.

The observed pattern of labelling is consistent with saturated fatty acids being the product of the fatty acid synthetase in *C. cohnii* [7] and reflects the desaturation of saturates to monoenes which in turn are further desaturated to PUFA. The synthesis of PUFA is indicated by the gradual incorporation of radioactivity into fatty

Table 2. Fatty acid composition (wt%) of total lipid, triacylglycerols and principal phospholipids of *C. cohnii*

	Total lipid	TAG	PC	PE	PS	PI
12:0	15.7	22.3	1.3	0.5	tr	0.6
14:0	46.9	49.4	17.5	3.3	3.4	7.8
15:0	tr	tr	tr	tr	tr	tr
16:0	18.6	11.7	18.0	4.5	44.1	29.4
16:1 (*n*-7)	1.1	1.1	tr	1.1	0.5	2.3
16:2	tr	—	tr	—	—	tr
16:3+17:0	tr	tr	tr	tr	tr	0.7
16:4	tr	tr	tr	—	—	tr
18:0	1.5	1.4	1.4	1.6	0.8	2.5
18:1 (*n*-9)	4.9	2.2	2.7	42.2	3.8	16.1
18:2 (*n*-6)	tr	tr	—	tr	—	0.5
18:3 (*n*-3)	—	1.1	tr	tr	tr	tr
18:4 (*n*-3)	—	tr	0.1	0.7	0.9	1.1
20:0	tr	tr	tr	tr	tr	tr
20:1 (*n*-9)	tr	tr	—	tr	tr	2.2
20:2 (*n*-6)	—	—	—	—	—	tr
20:4 (*n*-6)	—	tr	tr	tr	tr	1.1
20:4 (*n*-3)	—	tr	tr	tr	tr	tr
20:5 (*n*-3)	—	0.7	tr	1.0	tr	3.6
22:0	tr	tr	—	tr	tr	tr
22:2	—	—	—	tr	0.5	0.5
22:4	tr	—	tr	tr	—	tr
22:5 (*n*-3)	—	tr	tr	tr	—	0.9
22:6 (*n*-3)	8.8	6.9	57.2	42.1	43.5	23.1
24:0	tr	tr	—	tr	—	0.5
24:1 (*n*-9)	tr	tr	tr	tr	tr	tr
Unknowns	1.1	1.6	0.5	0.6	0.9	4.4
Total sats.	83.4	85.5	38.4	10.5	49.0	41.5
Total monos.	6.3	3.5	3.0	43.8	4.7	21.0
Total PUFA	9.2	9.4	57.6	45.1	45.4	33.1

tr, <0.5%. TAG, triacylglycerols; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine: PI, phosphatidylinositol. sats, saturates; monos., monoenes.

acids having four or more double bonds. Since acetate is known to be incorporated into 22:6 (*n*-3) by *C. cohnii* over a complete growth cycle [8], the low incorporation of radioactivity into PUFA including 22:6 (*n*-3) observed in this study is presumably a consequence of the short time course employed.

At all times 16:0 was the most labelled saturate in triacylglycerols, containing 55–64% of the total radioactivity in the triacylglycerol fatty acids; 14:0 contained 30–37% and 12:0 7–8%. 18:0 contained radioactivity (7%) only after 3 hr. 18:1 was the only menoene to contain acetate-derived radioactivity in both triacylglycerols and total polar lipid. Within the polar lipid fraction, 16:0 was always the most highly labelled saturated fatty acid (67–71%) but unlike the situation in triacylglycerols the amount of radioactivity in 18:0 (12–22%) exceeded that in 14:0 (6–12%). Radioactivity (12%) was only detected in the 12:0 of polar lipid from the 1 hr sample. Insufficient radioactive material prevented the analysis of the PUFA groups by radio-HPLC.

The saturates in both polar lipid and triacylglycerols, especially the latter, had low relative specific activities (Table 3) suggesting that the distribution of radioactivity within these fatty acids was *ca* proportional to their mass in the cells. In contrast, the dienoic fatty acids of the polar lipid fraction had very high relative specific activities. Thus, although dienes accounted for no more than 0.5% of the fatty acids in any phospholipid class analysed (Table 2), these fatty acids contained a very high percentage of the radioactivity derived from ^{14}C-acetate. The relative specific activities of trienes in the polar lipid were substantially lower than those of the dienes but were still higher than those of saturates and monoenes. With the exception of 12:0 the relative specific activities of fatty acids in triacylglycerols were lower than those in total polar lipid. Fatty acids containing four or more double

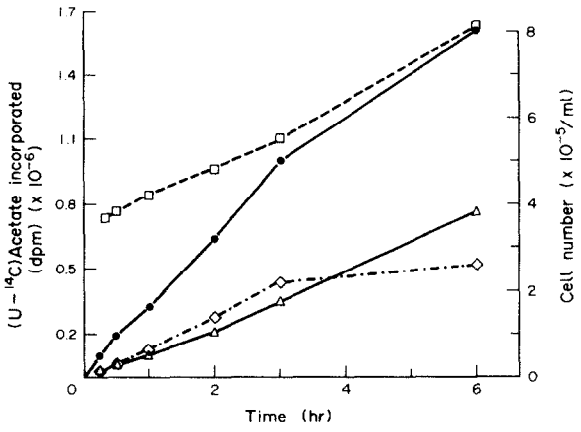

Fig. 1. The incorporation of radioactivity into lipids by *Crypthecodinium cohnii* grown in the presence of [U-^{1,4}C]acetate. Values are for 50 ml of culture. □---□, cell number; ●——●, total lipid; △——△, triacylglycerols; ◇-·-◇, phosphatidylcholine.

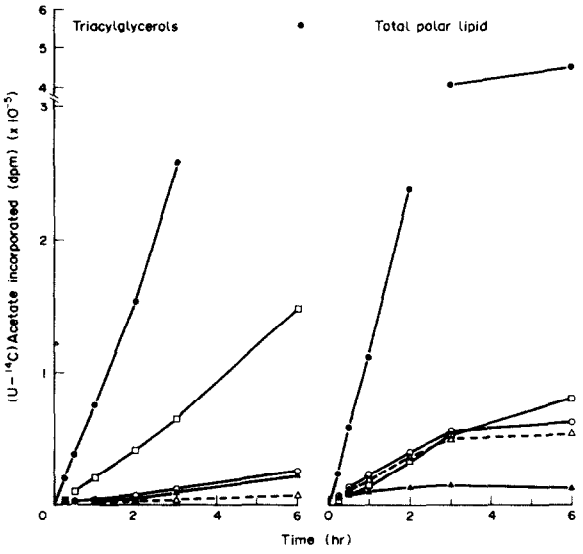

Fig. 2. The incorporation of radioactivity into triacylglycerol and polar lipid fatty acids of different degrees of unsaturation by *Crypthecodinium cohnii* grown in the presence of [U-^{14}C]acetate. Values are for 50 ml of culture. ●——●, saturates; □——□, monoenes; △--△, dienes; ▲——▲, trienes; ○——○, four or more double bonds.

bonds had very low relative specific activities in both polar lipids and triacylglycerols. Consequently it can be concluded that only small amounts of these PUFA, including 22:6 (*n*-3), had been synthesized *de novo* from acetate during the period of study.

In plants 18:2 (*n*-6) is considered to originate from 18:1 (*n*-9) esterified in PC with the subsequent conversion of 18:2 (*n*-6) to 18:3 (*n*-3) occuring in the monogalactosyldiacylglycerol of the chloroplast membrane [2–4]. Although the present results do not establish whether the

Table 3. Relative specific activities (% dpm/% wt) of fatty acids in triacylglycerols and phospholipid of *C. cohnii* grown in the presence of [U-^{14}C]acetate

	Time (hr)		
	1	3	6
Phospholipid			
14:0	0.8	2.1	1.6
16:0	1.7	2.1	2.1
18:0	2.7	4.1	3.0
18:1	1.4	1.4	1.7
Dienes	52.9	47.5	85.1
Trienes	15.1	5.6	19.9
4,5,6-double bonds	0.3	0.2	0.2
Triacylglycerols			
12:0	2.2	5.9	5.1
14:0	1.3	1.5	1.3
16:0	1.7	1.4	1.5
18:0	0	1.5	1.7
18:1	2.5	2.1	1.4
Dienes	1.6	1.8	1.6
Trienes	3.1	3.1	5.2
4,5,6-double bonds	0.2	0.1	0.2

desaturation of 18:1 (*n*-9) to 18:2 (*n*-6) utilizes PC as substrate, they do imply that in *C. cohnii* the 18:2 (*n*-6) of polar lipids, and presumably PC, is a possible substrate for 18:3 (*n*-3) formation. In this non-photosynthetic microorganism the Δ^{15} desaturase is perhaps capable of utilizing PC in the absence of chloroplast glycolipids. The direct desaturation of 20:3 (*n*-6) in PC to 20:4 (*n*-6) has been demonstrated in rat liver microsomes [21]. Studies are continuing to examine the extent to which phospholipids are involved in the desaturation steps leading to the formation of 22:6 (*n*-3) in *C. cohnii*.

EXPERIMENTAL

Crypthecodinium cohnii (Seligo) Javornicky [23], strain WH-d, was obtained from Prof. Carl Beam (Brooklyn College, City University of New York, U.S.A.). Stock cultures were maintained axenically at 27° in the dark on MLH medium [23] solidified with 1.5% agar. For lipid analysis, cells were cultivated in 3.5l of acetate-free MLH medium in the dark with gentle aeration and were harvested during the stationary phase after 6 days. For the study of lipid biosynthesis 250 μCi Na (U-^{14}C)acetate (56 mCi/mmol) dissolved in a small vol of sterile medium was passed through a 0.45 μm filter into a culture of *C. cohnii* of total vol. 400 ml and cell density 10^5/ml. When required, 50 ml portions were removed from the culture and extd for lipid as described below. Cell density was measured using a Coulter counter.

The dry wt of the algae was determined by a modification of the method of ref. [24] in which cells harvested by centrifugation (12 000 *g* min) were rinsed with 0.4 M NaCl containing 8 mM MES buffer, pH 6.6 [23] followed by centrifugation and resuspension in 0.4 M ammonium formate. Portions of this suspension were filtered through preweighed glass fibre filters, dried to constant wt at 95° and weighed using a microbalance.

Lipids were extd from the suspension of cells in ammonium formate or portions of culture with four vols of ice-cold $CHCl_3$–MeOH (2:1). After removal of solvents, lipid extracts

were dessicated overnight *in vacuo* before being weighed and redissolved in $CHCl_3$–MeOH (2:1). Lipid samples were stored at −20° under N_2 prior to analysis.

For compositional analysis, 100 mg total lipid was sepd into neutral and polar lipid fractions by TLC using several silica gel G plates and hexane–Et_2O–HOAc (40:10:1) as developing solvent. To visualize lipid classes, developed chromatograms were sprayed lightly with 0.1% (w/v). 2, 7-Dichlorofluorescein in 95% MeOH and viewed under UV light. The adsorbent from just above the origin up to and including the solvent front was scraped from the plates, transferred to a glass chromatography column equipped with a glass sinter and eluted with several vols of $CHCl_3$–MeOH (2:1). The ext. obtained upon removal of solvent by evapn was weighed after overnight dessication *in vacuo* and termed total neutral lipid fraction. The band of material visible at the origin was eluted from the adsorbent with 10 vols of $CHCl_3$–MeOH–H_2O (10:10:1). The solvent was evapd to yield an ext. of polar lipids which was weighed after dessication overnight under vacuum.

The composition of the neutral lipid fraction was determined by TLC-FID as described in ref. [25]. Triacylglycerols were separated by TLC and transesterified directly on the adsorbent [26]. The polar lipid fraction was subjected to 2D-TLC using the solvent systems of ref. [27] and the separated classes visualized with 2,7-dichlorofluorescein. Zones containing individual components were transmethylated after the addition of 19:0 as int std to permit quantitation by GC. Each polar lipid class was assumed to contain two fatty acids per molecule. Analyses of fatty acid Me esters were carried out using capillary GC as described elsewhere [16] using authentic standards for the identification of septd components. When necessary the unsaturated nature of component peaks was confirmed by re-analysis after hydrogenation of the sample over PtO_2 as catalyst.

In the expt. with [U-^{14}C]acetate, a portion of the total lipid ext. was measured after the removal of solvent for radioactivity by standard LSC techniques. Further portions were subjected to TLC using hexane–Et_2O–HOAc (40:10:1) or $CHCl_3$–MeOH–H_2O (25:10:1) as developing solvents for the sepn of neutral and polar lipid classes, respectively. Developed chromatograms were subjected to autoradiography for 5 days and subsequently stained with I_2 vapour. Bands of adsorbent containing the sepd lipids were measured for radioactivity. The fatty acid compositions of the triacylglycerols and total polar lipid from samples taken at different times in incorporation experiments were determined using the methods described above. For the determination of the distribution of radioactivity in fatty acid and glycerol portions of triacylglycerols and total polar lipid, the organic phase of the methylation extraction was taken as the fatty acid portion and the aqueous as the glycerol moiety. $AgNO_3$ chromatography of fatty acid Me esters was performed using the system of ref. [28] with 2, 7-dichlorofluorescein as the visualizing reagent. Sepd components were eluted from the relevant bands of adsorbent with hexane–Et_2O (1:1) and washed consecutively with 20% (w/v) NaCl and 2% (w/v) $KHCO_3$. After evapn of solvent the samples were redissolved in a known vol of hexane and a portion taken for measurement of radioactivity. The remainder of each sample was subjected to HPLC analysis [29] using a Spherisorb S5 ODS column 25cm × 4.9 mm id. The amounts of radioactivity present in individual fatty acid Me ester components were determined using a radioactivity detector.

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