Molecular Species Composition of Phosphatidylcholine from *Crypthecodinium cohnii* in Relation to Growth Temperature

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The molecular species composition was determined for phosphatidylcholine (PC) isolated from the marine dinoflagellate *Crypthecodinium cohnii* grown at three different temperatures. At all three temperatures the didocosahexaenoyl species comprised about 25% of the PC with 14:0/22:6 and 16:0/22:6 also being of major importance; these three species comprised 75–82% of the total. Another 20 species were identified, including several short chain disaturated species. Only small differences in the composition of PC were found in response to growth at 16, 23 and 27°C. On dropping the growth temperature from 27°C to 16°C the largest changes were a decrease of 8.9% in saturated/saturated species and an increase of 5.3% in saturated/PUFA species; the 22:6/22:6 content only increased slightly (by 1.9% to 25.4%). This unusual molecular species composition is discussed.
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Crypthecodinium cohnii is a marine dinoflagellate in which ca. 30% of the constituent fatty acids are docosahexaenoic acid, 22:6(n-3), and other polyunsaturated fatty acids (PUFA) are usually present at <1% of the total (1,2). Since this alga is non-photosynthetic, the glycolipids characteristic of photosynthetic membranes are absent and phospholipids are the predominant polar lipids. Most of the 22:6(n-3) in *C. cohnii* is present in the phospholipids, particularly in phosphatidylcholine (PC), the major phospholipid class (64%), in which it comprises 50–60% by weight of the total fatty acids (3). The other major fatty acids present in PC are 14:0 and 16:0, with small amounts of 12:0, 18:0 and 18:1(n-9), and several minor components present at <0.5% (3).

The present study had two aims. First, to determine the molecular species present in PC derived from *C. cohnii,* since the unique fatty acid composition suggested the presence of unusual species such as 22:6/22:6. Second, to determine both whether and how the molecular species composition of PC altered in relation to the homeoviscous adaptation of membranes, which is characteristic of poikilotherms grown at different temperatures.

MATERIALS AND METHODS

Materials. Phospholipase C from *Bacillus cereus* (Sigma type XIII), butylated hydroxytoluene (BHT) and 3,5-dinitrobenzoylchloride were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Didocosahexaenoin was obtained from BAST of Copenhagen, Copenhagen V, Denmark.

Merck thin-layer chromatography (TLC) and high performance thin-layer chromatography (HPTLC) plates coated with silica gel 60, Analar grade glacial acetic acid, methyl acetate, propan-1-ol, propan-2-ol and pyridine were purchased from BDH Ltd. (Poole, Dorset, U.K.). All other solvents of high performance liquid chromatography (HPLC) grade were from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland).

Ultrasphere ODS and Ultrasphere C8 HPLC columns (25 × 0.46 cm, 5 micron particle size) were obtained from Altex/Beckman (Beckman Instruments U.K. Ltd., High Wycombe, Bucks, U.K.).

Growth of organism. Stock cultures of *Crypthecodinium cohnii* (Seligo) Javornicky (4), strain WH-d, were maintained axenically at 27°C in the dark on MLH medium (4) solidified with 1.5% (w/v) agar. Cells for lipid analysis were cultivated in 5 × 200 ml of MLH medium (in the dark) with gentle aeration at 16 ± 1°C, 23 ± 1°C and 27 ± 1°C. Cell density was measured using a haemocytometer, and cells were harvested at densities of 4.6×10^5/ml (13 days, 16°C), 8.2×10^5/ml (5 days, 23°C) and 6.8×10^5/ml (3 days, 27°C). Cells were collected by centrifugation at 7500 g for 15 min, and the resulting pellets freeze-dried.

Extraction and purification of lipids. Total lipid was extracted from the freeze-dried pellets essentially by the method of Folch *et al.* (5). Solvents routinely contained 0.01% (w/v) BHT, and between preparative procedures samples were stored at −20°C under N_2. Neutral lipids were separated from polar lipid by TLC using hexane/diethyl ether/acetic acid (70:30:1, v/v/v). The polar lipid remaining on the origin was eluted from the absorbent with chloroform/methanol/water (5:5:1, v/v/v), dried by rotary evaporation under vacuum at 30°C, and finally under N_2. PC was purified by TLC using methyl acetate/propan-2-ol/chloroform/methanol/0.25% (w/v) KCl (25:25:25:10:9, v/v/v/v/v/) (6) alongside an authentic PC standard. Lipids were visualized under UV light after spraying the chromatogram with 0.1% (w/v) 2,7-dichlorofluorescein in methanol containing 0.01% (w/v) BHT. PC was eluted from the silica gel as before. The purity of PC was checked by two-dimensional HPTLC in chloroform /methanol/water/0.88 ammonia (130:70:8:0.5, v/v/v/v), then in chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v/v/v/v) (7).

Preparation of 3,5-dinitrobenzoyl derivatives. PC was hydrolyzed with phospholipase C (from *Bacillus cereus*) and the resultant 1,2-diacylglycerols reacted with 3,5-dinitrobenzoylchloride to give the 3,5-dinitrobenzoyl derivatives (8).

Separation of molecular species. Molecular species were separated by HPLC on Beckman ODS and C8 columns (25 × 0.46 cm, 5 μ particle size) using three isocratic solvent systems: methanol/propan-2-ol 95:5, v/v; acetonitrile/propan-2-ol 80:20, v/v; methanol/water/acetonitrile 93:5:2, v/v/v (9). Peaks were detected at 254 nm, and peak areas were measured using a Shimadzu CR 3A (Shimadzu

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Abbreviations: BHT, butylated hydroxytoluene; HPLC, high performance liquid chromatography; HPTLC, high performance thin-layer chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; TLC, thin-layer chromatography; molecular species, e.g., 14:0/22:6, 1-myristoyl-2-docosahexaenoyl-*sn*-glycero-3-phospho-

TABLE 1

Molecular Species Composition of Phosphatidylcholine from *C. cohnii* Grown at Different Temperatures

	Mole %			Significance[a]
	16°C	23°C	27°C	16°C vs 27°C
12:0/12:0	0.5 ± 0.1	1.1 ± 0.1	0.6 ± 0.2	ns
12:0/14:0	0.9 ± 0.1	1.7 ± 0.4	2.4 ± 0.2	$p < 0.005$
14:0/12:0	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	ns
14:0/14:0 } 12:0/16:0	0.8 ± 0.1	2.4 ± 0.3	7.2 ± 0.2	$p < 0.0001$
14:0/16:0	0.3 ± 0.1	0.9 ± 0.1	1.0 ± 0.3	$P < 0.05$
16:0/16:0	0.2 ± 0.1	0.7 ± 0.2	0.4 ± 0.1	ns
Sat/sat	3.1	7.2	12.0	
12:0/16:1	0.4 ± 0.1	—	0.7 ± 0.2	ns
14:0/16:1	0.3 ± 0.1	—	0.7 ± 0.2	ns
14:0/18:1	4.9 ± 0.6	3.9 ± 0.5	2.8 ± 0.2	$p < 0.005$
16:0/18:1	1.7 ± 0.1	2.3 ± 0.5	2.8 ± 0.4	$p < 0.01$
Sat/monoene	7.2	6.2	7.0	
14:0/20:5	tr	—	—	
14:0/22.6	35.7 ± 1.0	29.9 ± 0.7	28.7 ± 0.6	$p < 0.001$
16:0/20:5	0.7 ± 0.1	0.9 ± 0.1	2.5 ± 0.5	$p < 0.005$
16:0/22:6	22.3 ± 1.1	24.3 ± 0.4	23.4 ± 1.2	ns
16:0/22.5	tr	0.3 ± 0.1	—	ns
18:0/22.6	0.3 ± 0.1	0.4 ± 0.1	0.8 ± 0.1	$p < 0.005$
18:0/22.5	1.5 ± 0.2	0.7 ± 0.1	—	$p < 0.01$
Sat/PUFA	60.5	56.5	55.2	
18:1/18:1	0.2 ± 0.1	0.7 ± 0.1	1.3 ± 0.3	$p < 0.005$
16:1/22:6	tr	0.2 ± 0.1	—	ns
18:1/20:5	—	—	0.4 ± 0.1	ns
18:1/22:6	3.5 ± 0.8	4.4 ± 0.4	0.8 ± 0.3	$p < 0.005$
18:1/22:5	tr	—	—	ns
Monoene/PUFA	3.5	4.6	1.2	
22:6/22:6	25.4 ± 0.2	24.3 ± 0.8	23.5 ± 1.0	$p < 0.05$

[a]The significance of the changes in composition between 16 and 27°C were calculated using an ANOVA t-test program. All values are the mean of three determinations ± 1 S.D. All compositions are corrected to 100%, tr < 0.1%. Molecular species are abbreviated as follows: e.g., 14:0/22:6 is 1-myristoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine. The most saturated fatty acid is assumed to be on position 1 of the glyceride.

times relative to 16:0/22:6 were calculated and peaks were assigned from plots of Log_{10} (relative retention time × 10) vs the effective carbon number on the C-1 position of the glyceride constructed from known standards (9).

RESULTS AND DISCUSSION

Three molecular species, 22:6/22:6, 14:0/22:6 and 16:0/22:6, made up 75–82% of the PC in *C. cohnii* grown at 16, 23 and 27°C (Table 1). Another 20 species were detected, of which only 14:0/12:0 + 12:0/14:0, 14:0/14:0 + 12:0/16:0, 14:0/18:1, 16:0/20:5 and 18:1/22:6 were present at levels of greater than 2.0% at any one of the temperatures. Typical HPLC separations are shown in Figure 1.

The large amount of the highly unsaturated 22:6/22:6

unexpected, since an earlier study found that docosahexaenoic acid comprised only ca. 55% of the total fatty acids present in PC from *C. cohnii* grown at 27°C (3), and this was also the case under the growth conditions used here (Table 2). The large amounts of didocosahexaenoate PC were balanced by the presence of disaturated species, and at 27°C these comprised 12.0% of the total the most important of which were 14:0/14:0 + 12:0/16:0 (unfortunately, none of the three solvent systems resolved these two species). Thus, in this particular case the fatty acid composition does not give a true indication of the range of molecular species present.

Dipolyunsaturated molecular species of phosphoglycerides are increasingly being found in a variety of tissues, including PC from fish muscle (10), PC and phosphatidylethanolamine (PE) from cod roe (9), PE and

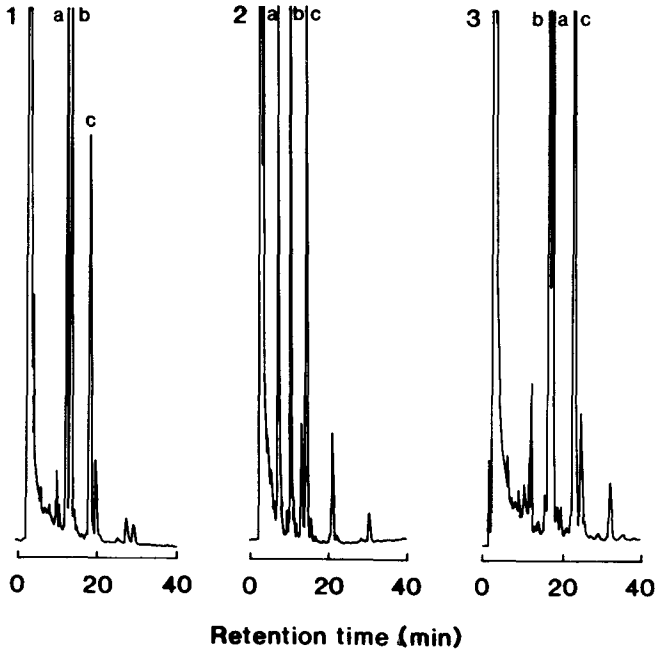

FIG. 1. Molecular species separations of the 3,5-dinitrobenzoyl derivatives of PC from *C. cohnii* grown at 16°C. The solvent systems are: (1) methanol/propan-2-ol, 95:5 (v/v), 1.0 ml/min; (2) acetonitrile/propan-2-ol, 80:20 (v/v), 1.0 ml/min; and (3) methanol/water/acetonitrile, 93:5:2 (v/v/v), 1.2 ml/min. The absorbance range at 254 nm was 0.04 units with ca. 20 μg of material injected. The three main molecular species are indicated: (a) 22:6–22:6, (b) 14:0–22:6, and (c) 16:0–22:6.

TABLE 2

The Fatty Acid Composition of Phosphatidylcholine from *C. cohnii* Grown at Different Temperatures Calculated from the Molecular Species Composition

	Mole %		
	16°C	23°C	27°C
12:0	2.1	2.7	3.8
14:0	21.3	18.8	21.4
16:0	12.8	15.2	17.0
16:1	0.4	0.1	0.3
18:0	0.9	1.2	0.4
18:1	5.2	5.8	4.7
20:5(n-3)	0.5	1.5	1.5
22:5(n-3)	0.9	0.5	—[a]
22:6(n-3)	55.9	53.1	50.0
Total saturates	37.1	37.9	42.6
Total monoenes	5.6	5.9	5.0
Total PUFA	57.3	55.1	51.5

[a]Not detected.

PE from rat testes (12) and especially in PC, PE and PS from mammalian retina (13) and fish brain and retina (14). The presence of such species is contrary to the earlier view of phosphoglyceride structure, which was that a saturated, or sometimes a monounsaturated, fatty acid is strongly preferred on the *sn*-1 position of the glyceride, with an unsaturated fatty acid strongly preferred on the

always the case, even when the overall fatty acid composition of the phosphoglyceride indicates no great excess of PUFA. The molecular species of PC from *C. cohnii* are particularly unusual in that large amounts of diPUFA, and disaturated species are present rather than the expected pairings of saturated with polyunsaturated fatty acids. The function of such diPUFA species in the biomembrane is presently unclear. However, it may be relevant that most galactolipid species in the chloroplast membranes of plants are also diPUFA (15).

The fatty acid and molecular species compositions of PC from *C. cohnii* are also unusual with respect to the length profile of the fatty acids. In mammals, C16 and C18 saturated and monounsaturated fatty acids, together with C20 and C22 PUFA, are the major constituents of phosphoglycerides. Shorter chain saturates like 12:0 and 14:0 are rarely found in phosphoglycerides, though some fish lipids contain a few percent of 14:0 (16). Computer modeling studies have shown that 22:6(n-3) has two energetically favored conformations, an "angle-iron" form and a helical form (17), both of which effectively shorten the fatty acid to approximately the same length as an extended 14:0 or 16:0 chain. Thus, it can be argued that only 4.1% of the fatty acids in PC from *C. cohnii* are effectively longer than 16:0. The phospholipid bilayer from the biomembrane of *C. cohnii* may therefore be narrower than "typical" mammalian cell membranes, and could be an interesting model system for studies on membrane function, e.g., it may have a higher curvature.

There was an increase of 5.5% in saturated fatty acids and a decrease of 5.8% in PUFA in PC between 16 and 27°C (Table 2). *C. cohnii* grows optimally at 27°C, yet even at this quite high temperature almost a quarter of the PC is the highly unsaturated di22:6 species. On decreasing the temperature to 16°C, the molecular species composition of PC, the major membrane phospholipid in this organism, is significantly, but not markedly, altered. Over the 11°C temperature decrease there is an increase of 1.9% in 22:6/22:6 content, a decrease of 8.9% in disaturated species and a decrease of 1.1% in 18:1/18:1. There is no change in the proportion of saturated/monounsaturated species. Saturated/PUFA species (mainly 14:0/22:6 and 18:0/22:5) increase by 5.3%, through 16:0/20:5 and 18:0/22:6 decrease by 1.8%, respectively (Table 1). Whether these changes alone are sufficient to bring about the alterations in membrane fluidity required to maintain membrane function at the lower temperature is not known, although the changes noted here are of a similar magnitude to those found in the membranes of trout on thermal acclimation from 20°C to 5°C (18). It is nonetheless surprising that the content of the major unsaturated species in the membrane, 22:6/22:6, is virtually unaltered by growth at the different temperatures. *C. cohnii* also contains an unusual sterol, dinosterol, which was found to be less soluble in model membranes than cholesterol (19), and this may be related to the high content of 22:6-rich phosphoglyceride species in *C. cohnii* membranes. Modulation of the amounts of sterols could also have a role in temperature adaptation.

In conclusion, PC from *C. cohnii* shows several interesting features. The molecular species composition is unusual, with didocosahexaenoate comprising a quarter of the total, while small amounts of disaturated species

changes in growth temperature was also unusual. Thermal acclimation was largely achieved by altering the proportion of disaturated and saturated/PUFA species, leaving the large amounts of 22:6/22:6 virtually unaltered. The role of this highly unsaturated species in biomembrane function remains to be clarified.

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