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Alkenone and alkenoic acid compositions of the membrane fractions of *Emiliania huxleyi*

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Abstract

The lipid classes and unsaturation ratios of long-chain alkenones (nC$_{37}$–C$_{39}$), related alkyl alkenoate compounds (nC$_{37}$–C$_{38}$) and alkenoic acids (nC$_{14}$–C$_{22}$) were determined in isolated membrane and organelle fractions of *Emiliania huxleyi*. The percentage distribution of these compounds was predominantly high in the endoplasmic reticulum (ER) and coccolith-producing compartment (CPC)-rich membrane fraction, although alkenones and alkenoates could be detected in all membrane fractions. In particular, the alkenones were mainly located in CPC, since their distribution was closely correlated with that of uronic acids which are markers of CPC. In contrast, the alkenoic acids seemed to be mainly located in chloroplast (thylakoid)-rich fractions. The alkenone unsaturation ratio and the ratio of alkenoates to alkenones were similar in all fractions, while the unsaturation ratio of alkenoic acids in the thylakoid-rich and plasma membrane (PM)/Golgi body-rich fractions was overwhelmingly higher than that in the ER/CPC-rich fractions. Thus, alkenoic acids seemed to be typical membrane-bound lipids, and could be closely related to photosynthesis and involved in regulating membrane fluidity and rigidity in *E. huxleyi*. It is presumed from these results that the alkenones and alkenoates were membrane-unbound lipids that might be associated with the function of CPC.

Keywords: Coccolithophorid; *Emiliania huxleyi*; Haptophyceae; Alkenones; Alkenoic acids; Lipid biomarker; Membrane fractionation; Intracellular localization; Unsaturation index

1. Introduction

Long-chain alkenones (nC$_{37}$–C$_{39}$) are known as specific lipid biomarkers of the family Gephyrocapsaceae, which have recently been classified into the Noelaerhabdaceae (Jordan and Kleijne, 1994), and Isochrysidaceae of Haptophycean algae. These molecules have frequently been used for determining the paleotemperature in geochemical and geophysical sciences, since the number of these double bonds in the molecules is produced in response to the prevailing temperature during their growth (Brassell, 1993 and references therein). The unsaturation index of C$_{37}$ alkenones ($U^{k'}_{37}$), which is calculated as the ratio of (C$_{37:2}$ alkenone)/(C$_{37:2}$ + C$_{37:3}$ alkenones) has been used for estimating the surface water paleotemperature in marine and lacustrine environments, since $U^{k'}_{37}$ and temperature during the growth of algae are closely correlated (e.g. Prahl and Wakeham, 1987; Brassell, 1993; Conte and Eglinton, 1993; Rosell-Mele et al., 1995). These compounds are believed to be intracellular membrane-bound lipids and to function as regulators of membrane fluidity and rigidity (Prahl et al., 1988; Brassell, 1993). The double-bond configuration of fatty acid esters in such membrane-bound lipids as phospholipids and galactolipids is generally *cis*, while that of alkenones has been found to be *trans* (Rechka and Maxwell, 1988). This fact has indicated that the physiological and biochemical roles of alkenones and alkenoates are very different from those of fatty acid esters involved in membrane lipids. It has

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recently been suggested that alkenones are not membrane lipids, but served as metabolic storage lipids (Bell and Pond, 1996; Epstein et al., 2001). This hypothesis was derived from the fact that the cellular content of alkenones tended to increase throughout the logarithmic and stationary growth phases and that the pattern of variation resembled that of triglycerides. In addition, Fernandez et al. (1994) have suggested that the alkenones and the related compounds as alkenoates and alkenes might serve to control the buoyancy in *E. huxleyi* cells, since the cells become heavier when coccoliths are produced on the cell surface. The cellular density of such high lipid-containing cells was smaller than that in protein- and carbohydrate-rich cells. They therefore assumed that the increase in intercellular content of such lipids as alkenones would result in a decreased sinking rate of the cells and increased residence time of cells in the euphotic layer of the ocean. However, there has only been circumstantial evidence for this, and further evidence such as the intracellular distribution and the amount of these lipids is needed.

In the present study, we isolated the membrane fractions and cellular organelles by a liquid two-phase fractionation method and analyzed the distribution of lipid biomarker molecules in each fraction. We then determined the lipid class composition and examined the distribution of alkenones and alkenoates and their unsaturation ratio in the individual membrane and organelle fractions of *E. huxleyi* cell in order to understand the physiological role of these lipids in this organism.

2. Results and discussion

2.1. Separation of the membrane fractions

The microsomal fraction of *E. huxleyi* cells was subjected to sucrose density centrifugation and separated into three fractions (C1–3 and D1–3 in duplicate experiments). The purity of each fraction was checked by distribution of marker enzymes and marker compounds as shown in Table 1. In the lighter fraction C-1, the activity of NADPH:cyt *c* reductase, a marker of the endoplasmic reticulum (ER), and the concentration of uronic acids, marker compounds of the coccolith-producing compartment (CPC), were highest. However, the distribution of the ER and CPC markers was slightly different in the duplicated measurements (series D), since the homogenized sample formed a pellet by coagulation of membranes and organelles without any treatment. Concentration of chlorophylls *a* and *c* (markers for the thylakoid membranes of chloroplasts), latent IDPase (the Golgi marker) and vanadate (VO_4^{3-})-sensitive ATPase (the plasma membrane (PM) marker) were highest in the denser fractions C-3 and D-3, although VO_4^{3-}-sensitive ATPase was broadly distributed among all the fractions. We could thus obtain ER/CPC-condensing membranes and thylakoid/Golgi/PM-condensing membranes in fractions C-1 and C-3, respectively. Cyt *c* oxidase, a mitochondrial marker, was higher in fraction C-2 than in the other fractions, although being broadly distributed in all the fractions. Since fraction C-2 showed significant activities of VO_4^{3-}-sensitive ATPase and NADPH:cyt *c* reductase, it was presumed that the mitochondrial fraction was contaminated by some other organelles such as PM and ER.

Further purification was then undertaken, with fractions C-1 and C-3 being applied to Percoll density centrifugation and separated into eight (C-1-1 to 8) and six (C-3-1 to 6) fractions, respectively as shown in Table 1. The high activity of NADPH:cyt *c* reductase was separated into two bands, namely C-1-2 and C-1-3, and C-1-6 to C-1-8. Uronic acids were distributed among the low-density fractions (C-1-1 to C-1-4). The ER and CPC markers could therefore not be separated to isolate pure organelles. The chlorophyll concentration was maximized in the higher-density fractions (C-3-5 and C-3-6) and well isolated from the other organelles. Latent IDPase and VO_4^{3-}-sensitive ATPase showed a narrow distribution in two fractions (C-3-1 and C-3-3), suggesting that isolation of the thylakoids and Golgi/PM-rich fractions had been successful.

2.2. Lipid abundance and class distribution: intracellular distribution of unsaturated lipids

The total abundance and percentage distribution of the major lipids among the microsomal fractions are shown in Tables 1 and 2, respectively. Fig. 1 shows that the membrane- and organelle-rich fractions were C-1-4 and C-1-7 for ER/CPC, C-3-3 for Golgi/PM, C-3-4 for PM/thylakoids, and C-3-5 and C-3-6 for thylakoids. C_{37}–C_{39} Alkenones, C_{37}–C_{38} alkyl alkenoates, C_{14}–C_{18} alkanoic acids (saturated fatty acids) and C_{18}–C_{22} alkenoic acids (unsaturated fatty acids), C_{29}–C_{33} alkenes and C_{27}–C_{28} sterols were obtained in all fractions of series C and D, but with some variation depending on the compounds. Alkenoic acids were identified as homologues with even carbon number, while alkenes had only the odd-numbered homologues. The total abundance of alkenones (μg/fraction) was high in the ER/CPC-rich fraction (C-1), but low in the thylakoid/Golgi/PM-condensing fraction (C-3). However, this distribution was slightly different in series D depending on the separation of the membrane and organelles. The distribution pattern of the alkyl alkenoates was similar to that of the alkenones, suggesting that the alkenones and alkenoates may have been mainly located in ER/CPC. On the other hand, the total abundance of alkanoic and alkenoic acids with saturated and unsaturated fatty acids was higher in thylakoid-rich fractions (C-3 and D-3) than in the ER/CPC-rich fractions (C-1 and D-1) (Table 1). Therefore, these

Table 1
Distribution of enzyme activities and concentrations of chlorophyll, uronic acids and lipids among the separated membrane-vesicle and organelle fractions of *E. huxleyi*

Fraction No.	Enzyme activities					Concentrations of membrane compounds					
	Cyt *c* oxidase (pmol O_2/ml/h), mitochondria	NADPH:cyt *c* reductase (mmol cyt *c*/ml/h), ER	VO_4-sensitive ATPase (nmol Pi/ml/h), PM	Latent IDPase (nmol Pi/ml/h), Golgi	Chlorophyll *a* and *c* (μg/ml), chloroplast	Uronic acids (μg/ml), CPC	Alkenones (μg/fr.)	Alkenoates (μg/fr.)	Fatty acids (μg/fr.)	Alkenes (μg/fr.)	Sterols (μg/fr.)
C-1	813.6	199.5	22.2	18.3	2.83	3.63	383.70	5.19	29.96	8.32	16.42
C-2	1486.4	56.3	25.1	0.0	5.21	0.42	186.62	3.87	376.91	1.53	11.91
C-3	968.1	4.5	40.7	132.8	67.80	0.71	26.29	0.67	737.60	3.29	8.15
D-1	1021.2	103.2	33.5	18.5	4.83	0.38	142.31	2.72	9.30	2.92	1.42
D-2	2042.4	92.4	18.3	8.6	0.07	0.47	171.33	3.31	323.66	0.68	2.32
D-3	900.2	8.6	127.7	114.7	45.16	0.68	64.50	1.17	666.92	1.58	1.11
C-1-1	–	57.1	–	–	–	0.91	20.89	0.37	0.88	0.47	1.57
C-1-2	–	64.5	–	–	–	1.08	16.67	0.38	0.57	0.42	1.02
C-1-3	–	68.2	–	–	–	0.26	10.61	0.28	0.41	0.24	0.47
C-1-4	–	32.8	–	–	–	1.88	17.69	0.68	0.51	0.45	0.92
C-1-5	–	tr.	–	–	–	0.87	13.56	0.33	0.34	0.34	0.82
C-1-6	–	72.6	–	–	–	0.53	12.94	0.30	0.59	0.40	1.61
C-1-7	–	96.2	–	–	–	0.38	11.82	0.36	0.78	0.28	1.65
C-1-8	–	68.7	–	–	–	0.23	13.1	0.31	0.81	0.35	0.53
C-3-1	–	–	25.2	18.5	12.4	–	0.82	0.014	1.36	0.07	0.13
C-3-2	–	–	29.8	105.4	16.7	–	0.82	0.014	2.13	0.05	0.39
C-3-3	–	–	17.5	82.4	16.1	–	0.84	0.015	1.20	0.06	0.22
C-3-4	–	–	29.1	tr.	16.6	–	0.61	0.018	2.40	0.04	0.18
C-3-5	–	–	tr.	tr.	26.4	–	0.67	0.024	5.72	0.07	0.26
C-3-6	–	–	tr.	tr.	60.4	–	0.58	0.012	2.49	0.06	0.15

tr: Trace amount (<0.1); μg/fr.: μg per fraction.

Table 2
Percentage distribution of lipids among the separated membrane-vesicle and organelle fractions of *E. huxleyi*

Fraction no.	Alkenones (%)		Alkenoates (%)	Fatty acids (%)			Alkenes (%)				Sterols (%)		
	C_{37}	Total	Total	Saturated	Unsaturated	Total	C_{29}	C_{31}	C_{33}	Total	Cholest.	Brass.	Total
C-1	49.86	86.50	1.17	5.43	1.32	6.75	tr.	1.62	0.25	1.87	tr.	3.70	3.70
C-2	19.18	32.13	0.67	29.64	35.25	64.89	tr.	0.23	0.04	0.26	tr.	2.05	2.05
C-3	2.04	3.39	0.09	24.71	70.34	95.05	0.01	0.37	0.04	0.42	tr.	1.05	1.05
D-1	55.21	89.69	1.72	3.61	2.25	5.86	0.35	1.49	tr.	1.84	tr.	0.89	0.89
D-2	21.73	34.18	0.66	28.93	35.63	64.56	0.03	0.11	tr.	0.14	tr.	0.46	0.46
D-3	5.34	8.77	0.16	23.96	66.75	90.70	0.04	0.17	tr.	0.21	tr.	0.15	0.15
C-1-1	51.98	86.45	1.52	1.66	1.92	3.58	tr.	1.78	0.16	1.95	0.17	6.33	6.50
C-1-2	54.20	87.46	1.98	2.26	0.72	2.97	0.01	1.94	0.28	2.22	0.17	5.19	5.37
C-1-3	55.81	88.38	2.34	2.80	0.58	3.38	0.01	1.67	0.31	1.99	0.24	3.67	3.91
C-1-4	54.97	87.37	3.38	2.18	0.32	2.50	tr.	1.83	0.38	2.20	0.15	4.40	4.55
C-1-5	54.35	88.06	2.16	1.88	0.35	2.24	0.02	1.82	0.37	2.21	0.21	5.13	5.34
C-1-6	50.43	81.70	1.90	3.10	0.60	3.70	0.02	2.08	0.40	2.50	0.28	9.91	10.20
C-1-7	49.56	79.37	2.44	4.52	0.69	5.22	0.02	1.61	0.25	1.89	0.20	10.88	11.08
C-1-8	53.24	86.75	2.05	4.50	0.83	5.34	0.02	2.12	0.21	2.34	0.07	3.44	3.52
C-3-1	20.45	34.22	0.60	26.71	30.03	56.74	tr.	2.75	0.31	3.06	0.38	5.01	5.38
C-3-2	14.11	24.10	0.40	29.98	32.52	62.50	tr.	1.29	0.20	1.50	0.62	10.88	11.49
C-3-3	21.44	36.01	0.64	25.72	25.59	51.32	tr.	2.14	0.32	2.46	0.56	9.00	9.56
C-3-4	11.39	18.78	0.55	32.12	41.84	73.95	tr.	1.02	0.12	1.14	0.35	5.23	5.59
C-3-5	6.08	9.93	0.35	31.63	53.21	84.84	tr.	0.96	0.03	0.99	0.18	3.71	3.89
C-3-6	10.64	17.64	0.35	25.55	50.28	75.82	tr.	0.64	1.05	1.69	0.24	4.26	4.50

tr: Trace amount (<0.01%).

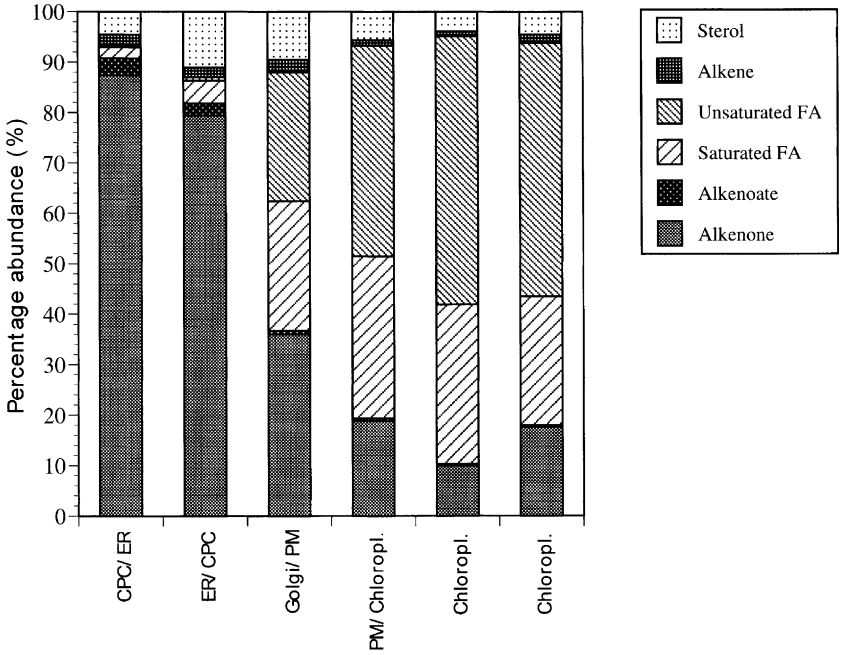

Fig. 1. Percentage distribution of lipids in each membrane-vesicle and organelle fraction of *E. huxleyi*. CPC-dominant CPC/ER: C-1-4; ER-dominant ER/CPC: C-1-7; Golgi/PM: C-3-3; PM/thylakoids: C-3-4; thylakoids: C-3-5 and C-3-6.

abundant fatty acids might have been mostly condensed in the thylakoid fraction. The fatty acids in *E. huxleyi* strain EH2 mainly comprise of tetradecanoic ($C_{14:0}$), hexadecanoic ($C_{16:0}$), octadecapentaenoic ($C_{18:5}$), octadecatetraenoic ($C_{18:4}$) and docosahexaenoic ($C_{22:6}$) acids. Octadecatrienoic acid ($C_{18:3}$) could not be found in this study, despite its detection in *E. huxleyi* in a previous study (Bell and Pond, 1996). The abundance of such polyunsaturated fatty acids as $C_{18:5}$, $C_{18:4}$ and $C_{22:6}$ was significantly high in the thylakoid-rich fractions (Table 3). This result is in accordance with that reported by Bell and Pond (1996) who have shown that the unsaturated

Table 3
Alkenone unsaturation index ($U^{k'}_{37}$), alkenoate/alkenone ratio (EE/K_{37}) and poly-/mono-unsaturated C_{18} alkenoic acid ratio (C_{18}PU/MU) in the separated membrane-vesicle and organelle fractions of *E. huxleyi*

Fraction no.	Alkenone index			Alkenoic acid ratio	
	$U^{k'}_{37}$	EE/K_{37}	K_{37}/K_{38}	C_{18} PU/MU	$C_{22:6}$%
C-1	0.870	0.020	1.431	0.89	0.36
C-2	0.865	0.031	1.483	13.12	4.39
C-3	0.855	0.030	1.532	23.79	3.31
D-1	0.827	0.031	1.601	1.22	0.31
D-2	0.887	0.030	1.776	14.25	3.82
D-3	0.853	0.030	1.575	22.24	3.89
C-1-1	0.913	0.019	1.520	7.61	4.89
C-1-2	0.828	0.037	1.706	3.87	1.56
C-1-3	0.824	0.037	1.721	4.86	0.63
C-1-4	0.825	0.039	1.714	0.80	0.45
C-1-5	0.829	0.040	1.700	2.02	0.30
C-1-6	0.829	0.038	1.699	1.99	0.30
C-1-7	0.825	0.041	1.670	1.11	0.42
C-1-8	0.828	0.039	1.697	2.16	0.33

fatty acids were dominant in glycolipids which are known to be exclusively present in the thylakoid membranes.

In those fractions separated by Percoll density centrifugation (C-1-1 to C-1-8), the total alkenone abundance was closely correlated with the concentration of uronic acids, except in the lightest fraction (C-1-1) (Fig. 2). The correlation coefficient (r^2) of abundance between the alkenone and uronic acids was high ($r^2 = 0.82$), except again in fraction C-1-1. The results suggest that these two compounds might be closely associated with each other in their intracellular distribution, and therefore implies that the alkenones were localized in the CPC rather than in the ER.

The results of this study show that the total abundance of alkenes was highest in the ER/CPC-rich fraction (C-1). The alkenes detected in *E. huxleyi* strain EH2 were nonacosene ($C_{29:1}$), hentriacontene ($C_{31:2}$ and $C_{31:3}$) and tritriacontene ($C_{33:2}$ and $C_{33:3}$). There were three kinds of isomers of the C_{31} and C_{33} alkenes, although the positions of the double bonds could not be determined. The C_{37} and C_{38} alkenes reported in a previous studies (Volkman et al., 1980; Conte and Eglinton, 1993) could not be detected in the present study.

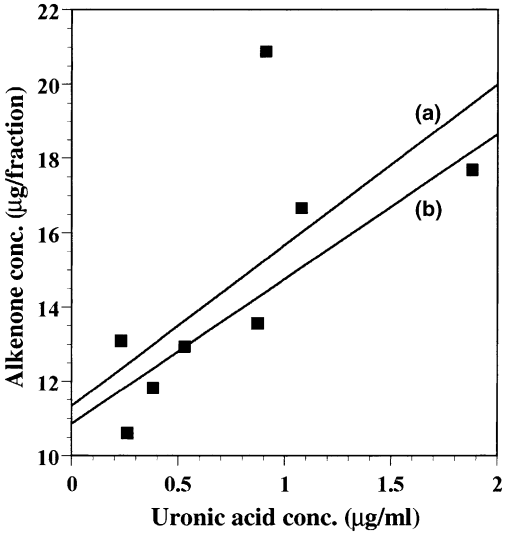

Fig. 2. Relationship between the concentrations of alkenones and uronic acids. Lines (a) and (b) represent linear fits with all plots and plots precluding sample C-1-1, respectively.

The sterols detected were brassicasterol (24-methylcholesta-5, 22*E*-dien-3*E*-ol) and cholesterol, which agrees with those previously reported (Volkman et al., 1981; Yamamoto et al., 2000). The sterol abundance was similar in all fractions, although slightly lower in the thylakoid-rich fractions.

More than 80% of the alkenones were distributed in the ER/CPC-condensing fractions (Table 2 and Fig. 1). On the other hand, in percentage composition, alkanoic (ca. 26%) and alkenoic acids (ca. 51%) were dominant in the thylakoid-rich fractions. The percentage of alkenones in the Golgi and PM-rich fractions was similar to those of the alkanoic and alkenoic acids. Alkenes were minor compounds (less than 3%) in all fractions, while the percentage abundance of sterols was significantly higher in the ER-rich fractions (ca. 10%).

The intracellular localization of alkenones in *E. huxleyi* has been studied by Conte and Eglinton (1993). Alkenones were not detected in the membranes from broken cells, indicating that they might not have been membrane-bound lipids. Mouzdahir et al. (2001) have suggested from the results of photodegradation experiments that the long-chain alkenones were much more recalcitrant to photochemical reactions than the unsaturated fatty acids in membranes, and therefore, that the intracellular localization might be different between these compounds. A similar result was found from the present study. In addition, Mouzdahir et al. (2001) have proposed that alkenones are not membrane-bound lipids, but presumably membrane-unbound lipids. Thus, Conte and Eglinton (1993) possibly failed to detect the alkenones from broken cells, due to the loss of these compounds as membrane-unbound lipids. It is therefore considered that the alkenones were mainly localized in ER and CPC, and particularly in the latter, as membrane-unbound lipids in the form of micelles, while alkanoic and alkenoic acids were incorporated as generally believed in the thylakoid membrane of chloroplasts as membrane-bound lipids.

2.3. Unsaturation ratios of alkenone, alkenoate and alkenoic acids

The alkenone unsaturation index ($U^{k'}_{37}$), the ratio of alkenoates (C_{36} fatty acid ethyl ester) to C_{37} alkenones (EE/K_{37}; Prahl and Wakeham, 1987) and the ratio of C_{37} alkenones to C_{38} alkenones (K_{37}/K_{38}; Prahl and Wakeham, 1987) in the membrane and microsome fractions are shown in Table 3. The $U^{k'}_{37}$ and EE/K_{37}

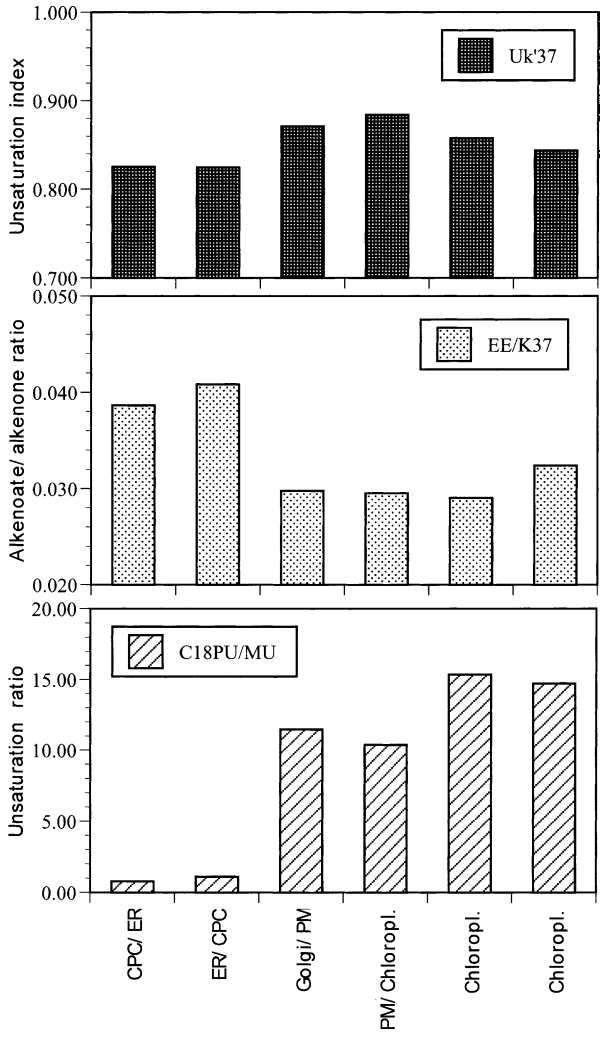

Fig. 3. Alkenone unsaturation index ($U^{k'}_{37}$), alkenoate/alkenone ratio (EE/K_{37}) and poly/mono-unsaturated C_{18} alkenoic acid ratio (C_{18} PU/MU) in each membrane and organelle fraction of *E. huxleyi*.

values are summarized in Fig. 3 according to their intracellular location. The $U^{k'}_{37}$ values were similar in all the fractions, although the values in the Golgi/PM- and PM/thylakoid-rich fractions were slightly higher than those in the other fractions. The EE/K_{37} and K_{37}/K_{38} ratios were also consistent in most of the fractions. However, the $U^{k'}_{37}$ value and EE/K_{37} ratio in the lightest fraction (C-1-1) of Percoll density centrifugation were significantly different from those in the other fractions, although the reason for this is currently unknown. Therefore, when the values for fraction C-1-1are not considered, the differences were insignificant ($U^{k'}_{37}$: 0.06; EE/K_{37}: 0.01; K_{37}/K_{38}: 0.2) among the ER/CPC-, Golgi/PM- and thylakoid-rich fractions (Table 3 and Fig. 3). These results suggest that the composition of alkenones and alkenoates might be similar in the individual organelles. These data show that the contamination by alkenones and alkenoates of the other fractions was negligible. The unsaturation ratio did not seem to be affected by the fractionation, although reexamination is required to confirm this.

The unsaturation index of alkenoic acids is represented by the ratio of C_{18} polyunsaturated fatty acids ($C_{18:4}$ and $C_{18:5}$ acids) to C_{18} monounsaturated fatty acids ($C_{18:1}$) (C_{18}PU/MU) and the percentage of $C_{22:6}$ acid in the total fatty acids ($C_{22:6}$%) (Table 3). The C_{18}PU/MU and $C_{22:6}$% values varied significantly throughout the separated fractions, in contrast to the alkenone ratios as $U^{k'}_{37}$. C_{18}PU/MU in the Golgi/PM (fraction C-3-3)- and thylakoid (C-3-5 and C-3-6)-rich fractions were significantly higher than those in the ER/CPC-rich fraction (C-1-7). Polar lipids in the biological membranes of microalgae are generally composed of more polyunsaturated fatty acids, in comparison with the membrane-unbound lipids as triglycerides that have proportionally more saturated and mono-unsaturated fatty acids (Piorreck et al., 1984; Dunstan et al., 1993). In addition, the $C_{18:5}$ and $C_{18:4}$ acids would be contained in glycolipids as components of the thylakoid membrane in *E. huxleyi* as already mentioned, suggesting a close relationship with the photosynthetic activity (Bell and Pond, 1996). It was therefore apparent that the typical membrane-bound lipids were located in the thylakoid-rich fractions. On the other hand, the lower C_{18}PU/MU values in the ER/CPC-rich fractions suggests that these fractions mainly contained membrane-unbound lipids rather than membrane-bound lipids. The percentage $C_{22:6}$ values were highest in the Golgi/PM- and PM/thylakoid-rich fractions (Table 3). This result concurs with that of Bell and Pond (1996), who have suggested that the phospholipids specifically localized in PM had proportionally more $C_{22:6}$ fatty acids in *E. huxleyi*. The $C_{22:6}$% values were much lower in the ER/CPC-condensing fractions. It was thus found that the main localization of the polyunsaturated fatty acids, which are generally associated with the regulation of membrane fluidity, was different from that of alkenone.

2.4. Possible physiological role of alkenones

We have commented that the alkenones and related compounds as alkenoates and C_{31}–C_{33} alkenes were mainly localized in the ER/CPC-rich fractions of *E. huxleyi*, but hardly contained in the thylakoid-rich fractions (Table 2). The function(s) of the alkenones and related compounds might be different from those of the unsaturated fatty acids that were mainly localized in the thylakoids of the chloroplasts. We presume that the membrane fluidity and rigidity might be regulated by phospholipids and glycolipids that contain polyunsaturated fatty acids rather than by alkenones in *E. huxleyi*. This presumption supports the recent proposals that alkenones are the metabolic storage lipids (Bell and Pond, 1996; Epstein et al., 2001). In addition, the result that the alkenones were predominantly distributed in

CPC is relevant for assessing the physiological role of the alkenones, and implies that the alkenones and alkenoates might also be closely associated with the formation of coccoliths. The alkenones possibly function as the buoyancy controllers for the heavy cells bearing coccoliths as reported by Fernandez et al. (1994), although Bell and Pond (1996) have suggested that the alkenones were unlikely to be involved in buoyancy, because the intracellular content of the alkenones in non-calcifying cells of *E. huxleyi* was similar to that of the calcifying cells. It thus seems that the biosynthesis of alkenones and related compounds is not simply correlated with the coccolith formation, and further investigation of the function of alkenones is required to elucidate this.

3. Experimental

3.1. Culturing and harvesting

Emiliania huxleyi strain EH2 (Haptophyceae) was collected from the Great Barrier Reef during the November 1990 expedition of Sohgen-Maru by the Marine Biotechnology Institute. This strain was grown as a stock culture in 100–200-ml Erlenmeyer flasks containing 50–100 ml of an ESM-enriched natural seawater medium at 23 °C under a 16h light/8 h dark regime, as described in our previous paper (Sekino and Shiraiwa, 1994). The culture was illuminated with cool white fluorescent lamps at an intensity of 3 klx (60 $\mu mol\,m^{-2}\,s^{-1}$). As a pre-culture, a small portion of the algal culture was inoculated into a 500-ml Mericron flask (Iwaki, Tokyo, Japan) containing 300 ml of Marine Art SF artificial seawater (MA: Senju Pharmaceutical Co., Japan) enriched with modified ESM (Danbara and Shiraiwa, 1999) under a 16h light/8h dark regime at an intensity of 3 klx (60 $\mu mol\,m^{-2}\,s^{-1}$). A 1.5-litre Sakaguchi flask containing 1 litre of the same medium was also used for culturing under continuous illumination at an intensity of 10 klx (200 $\mu mol\,m^{-2}\,s^{-1}$), the temperature being maintained at 23 °C.

As an experimental culture, the suspension of the preculture was transferred to large-scale 5–10-litre flasks respectively containing 3–8 litre of an MA-ESM medium at 23 °C under continuous illumination of 15–22 klx (300–440 $\mu mol\,m^{-2}\,s^{-1}$). The cells were harvested at the stationary phase and then centrifuged at 600*g* or 1200*g* for 10 min. The collected pellet was immediately used for fractionating the membranes and organelles, and the rest was stored at −80 °C for subsequent analyses.

3.2. Separation of the membrane fractions

About 10 g (wet weight) of the pellet of *E. huxleyi* cells was homogenized in 30 ml of buffer A (125 mM sucrose, 1 mM Na_2–EDTA, 1 mM dithiothreitol (DTT), and 50 mM Tris–MES, pH 7.5) to disrupt the cells in a Teflon homogenizer. After pre-centrifuging at a maximum of 1200*g* for 10 min, the precipitate was re-homogenized in 30 ml of buffer B (buffer A and PMSF). The resulting slurry was then passed through two layers of Miracloth (Calbiochem, USA) and centrifuged at 300*g* for 10 min. The supernatant was re-centrifuged at 89,000*g* for 30 min in an ultracentrifuge (L-60, Beckman, USA) and the resulting pellet was suspended in buffer B as a crude microsomal fraction.

The cellular membranes and microsomes were separated by liquid-two phase fractionation using both sucrose and Percoll density centrifugation. The microsomal fraction (1–2 ml) was layered onto a linear gradient of 15–45% (w/w) sucrose in buffer C composed of 50 mM Tris–MES (pH 7.5), 1 mM EDTA and 1 mM PMSF made up by an Auto Densi-flow® IIC fractionator (Labconco, Kansas City, MO). After centrifuging at 105,000*g* for 2 h with an SW41 rotor, L-60 ultracentrifuge (Beckman), the microsomes were separated into three visible color bands and a precipitate, these three bands being collected by pipettes (fractions C-1, C-2 and C-3 and D-1, D-2 and D-3 in duplicate experiments). The respective separated fractions of C-1 and C-3 (5.6 and 3 ml, respectively) were subsequently fractionated by Percoll gradient centrifugation. The microsomal fractions were mixed with 450 μl of a Percoll solution in buffer C, and then centrifuged at 50,000*g* for 30 min with an RA-3 rotor, KH-180 centrifuge (KUBOTA, Tokyo, Japan). The C-1 and C-3 fractions were further respectively fractionated into 8 fractions (C-1-1 to C-1-8) and 6 fractions (C-3-1 to C-3-6). The collected membrane fractions were diluted with buffer B and then centrifuged at 105,000*g* for 1 h in order to condense the respective microsomal fractions, namely the membranes vesicles and organelles. The purity of the membrane and organelle fractions was checked by measuring the activity of either marker enzymes or marker components that are specific to particular organelles, as described next.

3.3. Enzyme assay

The respective markers used for the plasma membrane (PM), endoplasmic reticulum (ER), mitochondria and Golgi body were vanadate (VO_4^{3-})-sensitive ATPase, NADPH:cyt *c* reductase, cytochrome (cyt) *c* oxidase and latent IDPase. The assays of the markers were performed as reported by Chanson et al. (1984). Briefly, the VO_4^{3-}-sensitive ATPase activity was determined by the difference between activities in the absence and presence of Na_3VO_4 in an assay solution that contained 30 mM Tris–MES, 3 mM $MgSO_4$, 50 mM KCl and 0.025% Triton X-100. The NADPH-dependent cyt *c* reductase activity was spectrophotometrically determined by monitoring the oxidation of NADPH in a phosphate buffer (pH 7.4)

containing 0.7 mg/ml of cyt *c*, 10 μM NaCN, 1 μM antimycin A, NADP and the sample. The cyt *c* oxidase activity was polarographically determined by monitoring the decrease in oxygen concentration resulting from the oxidation of cyt c in a 22 mM Tris–acetate buffer (pH 7.4) containing 216 mM sucrose, 6.5 mM isoascorbate, 13 μM EDTA, 0.65 mM N,N,N',N'-tetramethyl-*p*-phenylenediamine, and a sample. The latent IDPase activity was spectrophotometrically determined by monitoring the release of Pi from IDP in a 30 mM Tris–MES buffer (pH 7.5) containing 3 mM $MgSO_4$, 50 mM KCl and 3 mM IDP. The final activity was calculated from the difference in that between the presence and absence of 0.1% digitonin.

3.4. Analyses of chlorophylls and uronic acids

The respective marker compounds for thylakoid membranes of the chloroplasts and coccolith-producing compartment (CPC) were chlorophylls *a* and *c* and uronic acids. The concentrations of chlorophylls were determined according to the method of Jeffrey and Humphrey (1975) by spectrophotometric measurement of the absorbance of acetone extracts at 630, 664 and 750 nm. To determine uronic acids by the H_2SO_4-carbazole method (Knusten and Jeanes, 1968), CPC was washed twice with 3% NaCl by ultracentrifuging at 105,000*g* for 90 min and then suspending the resulting pellet in methanol (MeOH). After incubating overnight, the suspension was centrifuged, and the resulting precipitate was dissolved in distilled water. The supernatant was applied to the assay. Each sample was mixed with 1.5 ml of 25 mM $Na_2B_4O_7$ in H_2SO_4 at 100 °C. After cooling, 60 μl of 0.125% carbazole was added, and the mixture incubated at 100 °C for 15 min. The concentration of uronic acids was determined by measuring the absorbance at 530 nm. D-Glucuronolactone (Wako, Osaka, Japan) was used as a standard.

3.5. Lipid analyses

Lipids were extracted from the membrane fractions with dichloromethane and MeOH as described by Sawada et al. (1996). d_{62}-Triacontane and 15-hentriacontanone (synthesized by Prof. K. Ohta of Nagoya University) were respectively added prior to extraction as internal standards for quantifying alkenes, and alkenones and alkyl alkenoates. The lipids were separated by adding distilled water to the combined extracts, before the CH_2Cl_2 layer was siphoned off and passed through an anhydrous Na_2SO_4 column. The extract was dried in a rotary evaporator and then re-dissolved in hexane. The lipid-containing hexane extract was passed through a silica gel column (95% activated), and the hydrocarbon and ketone–ester fractions were respectively eluted with hexane and hexane–ethyl acetate (9:1 v/v). After this elution procedure, the polar lipid-containing residue was re-dissolved in ethyl acetate–MeOH (1:1 v/v) and then passed through the same silica gel column. This fraction was saponified with 1 M KOH in MeOH at 80 °C for 30 min. The non-saponifiable (neutral) lipids were extracted by partitioning with hexane–ethyl acetate (8:1 v/v), and the fatty acids were extracted in the same way after acidification to pH 2 with HCl. The fatty acids were esterified by using 14% BF_3 in MeOH at 60 °C for 30 min. After adding pure water, the fatty acid methyl esters (FAMEs) were extracted with hexane. Sterols in the neutral fraction were silylated by using bis(trimethylsilyl) trifluoroacetamide (BSTFA, Wako) at 60 °C for 1 h. These FAME and sterol trimethylsilyl ether fractions were analyzed by GC and GC/MS.

The lipids were identified by gas chromatography (GC)/mass spectrometry (MS) with a Hewlett Packard 6890 attached to a capillary GC (50 m × 0.32 mm i.d. CPSil5CB fused silica column, Chrompack) directly coupled to a Hewlett Packard MSD quadrupole mass spectrometer (electron voltage, 70 eV; emission current, 350 μA; mass range, m/z 50–600 in 1.3 s). The GC temperature was programmed as follows: 60 °C for 5 min, 60–120 °C at 4 °C/min, 260–320 °C at 3 °C/min and 320 °C for 25 min. The lipids were quantified with a Shimadzu GC-14A capillary gas chromatograph equipped with a flame-ionization detector (FID), the capillary column and temperature program used being the same as those used for GC/MS.

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