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LIPID COMPOSITION AND BIOSYNTHESIS IN AGEING CULTURES OF THE MARINE CRYPTOMONAD, *CHROOMONAS SALINA*

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Key Word Index—*Chroomonas salina*; cryptomonad; ageing cultures; lipid composition; lipid biosynthesis.

Abstract—Changes in the lipid composition of cultures of the marine cryptomonad *Chroomonas salina* allowed to age at 20 and 7° were examined. At both temperatures the proportion of wax esters present in lipid increased with culture age. After 14 weeks, 31% and 25% of the lipids from cultures maintained at 20 and 7° respectively were wax esters. The fatty acids of wax esters and triacylglycerols were predominantly saturated at both temperatures. The fatty alcohols of the wax esters were almost exclusively saturated and contained high proportions of 13:0 and 15:0. Polyunsaturated fatty acids (PUFA), particularly 18:4(*n*-3), 20:5(*n*-3) and 22:6(*n*-3), accounted for a higher proportion of the fatty acids in total polar lipid from cultures aged at 7° than at 20°. Cultures of the alga maintained under the same light intensity at 20 and 10° incorporated 11.8 and 22.9% respectively of total radioactivity assimilated from $^{14}CO_2$ into lipid. The amount of radioactivity from ^{14}C-labelled CO_2, acetate and 18:1(*n*-9) incorporated into triacylglycerols and wax esters increased with the age of cultures. The proportions of radioactivity from all three substrates recovered in trienoic and polyunsaturated fatty acids of total lipid were higher in cultures maintained at 10° than at 20°.

INTRODUCTION

Under optimum growth conditions algae are not considered to be rich in lipid and contain mostly the polar lipids associated with chloroplast and other membranes [1]. However, several species of microalgae, both freshwater and marine, are known to accumulate lipid up to 30–72% of their biomass when grown in medium of low nitrogen content [1–3]. Evidence also exists that the content of neutral lipid increases with age in green algae [4]. This accumulated lipid is usually neutral lipid in the form of triacylglycerols [3]. However, wax esters rather than triacylglycerols are the accumulated neutral lipid when the freshwater alga *Euglena gracilis* is grown under adverse conditions [1, 5]. The first report of wax ester synthesis by a marine alga was that of Antia *et al.* [6], who found that aged cultures of the cyrptomonad *Chroomonas salina* produced lipid which was 87% wax ester when grown photoheterotrophically on glycerol as a carbon source.

The wax esters produced by *C. salina* differ in reported composition [6] from those of the jojoba seed oil [7], the current commercial source of wax esters. Wax esters produced by microorganisms have been suggested [8] as being of potential use in the ongoing search for alternatives to sperm whale oil. In terms of useful lipid, algae such as *Spirulina platensis* and *Chlorella minutissima*, with their high contents of γ-linolenic acid 18:3(*n*-6) and 20:5 (*n*-3) respectively in cellular lipid, have been considered as a source of these polyunsaturated fatty acids for use in health foods and pharmaceuticals [9, 10].

Environmental conditions such as light intensity and temperature are known to influence the pattern of fatty acids synthesized by algae [1, 9]. Nothing is known of the biochemistry of wax ester synthesis in *C. salina* or whether the composition of wax esters in this algal species responds to changes in the environment. The present work was undertaken to study the production of lipids by *C. salina* grown and allowed to age at different temperatures.

RESULTS

Lipid composition

The lipid composition and cell densities of cultures of *C. salina* allowed to age at 20 and 7° are presented in Table 1. The growth rate of the culture maintained at 20° was initially greater than that maintained at 7° but after five weeks the cell density was similar in each culture. After 14 weeks, cultures at both temperatures showed extensive autolysis and the cell numbers could not be determined accurately. By this time both cultures were cream coloured. The lipid content of the 20° culture was apparently higher than that of the 7° culture over five weeks. However, after 14 weeks the lipid contents of both cultures were similar and were less than that of the initial starting culture. After five weeks lipid accounted for 25% and 17.9% of the dry weight of algae grown at 20° and 7° respectively.

Triacylglycerol was the principal lipid present in the cultures at the outset of the growth period, comprising 36% of the total lipid. Wax ester accounted for only 5% of the total lipid in the initial culture but its proportion increased with age in both cultures. The component corresponding to wax ester on the chromatograms of lipid from the five- and 14-week-old cultures did not exhibit the initial blue colour which is characteristic of cholesteryl esters under the staining technique used. For this reason the component was considered to be com-

Table 1. Changes in lipid composition of *C. salina* in relation to growth and ageing at different temperatures

Temp. (°)	Time (weeks)	Cell no $\times 10^{-6}$/ml	Lipid (mg/ml)	Lipids (% total lipid)						
				WE	TAG	FFA	FAl	St	Pig	PL
	0	1.52	0.56	5	36	15	7	5	17	15
	2	6.19	0.81	10	37	14	7	5	15	12
20	5	6.12	0.85	19	33	12	2	3	18	13
	14	N.D.	0.44	31	10	28	6	4	8	13
	2	2.46	0.57	4	44	17	7	3	14	11
7	5	6.79	0.64	7	26	21	2	5	22	17
	14	N.D.	0.48	25	37	21	1	4	6	6

WE, wax esters; TAG, triacylglycerols; FFA, free fatty acids; FAl, free fatty alcohols; St, sterols; Pig., pigments; PL, polar lipids, N.D., not determined.

Table 2. Fatty acid/alcohol composition (weight %) of lipids from cultures of *C. salina* maintained for five weeks at different temperatures

	20°				7°			
	Wax ester				Wax ester			
	Acid	Alc.	TAG	PL	Acid	Alc.	TAG	PL
12:0	27.5	0.8	4.4	1.6	22.9	6.8	3.9	0.5
13:0	—	9.2	—	—	—	10.6	—	—
14:0	25.0	36.5	32.8	15.6	15.5	27.1	27.3	8.0
15:0	7.4	28.5	6.0	4.2	6.1	24.1	3.9	1.1
16:0	13.2	18.6	19.2	17.7	18.7	26.6	14.9	1.3
16:1 (*n*-7/9)	0.5	—	1.2	1.1	2.0	—	3.0	8.9
16:2	—	—	—	—	—	—	—	1.1
17:0	—	1.9	0.7	—	—	—	0.5	—
18:0	2.3	1.0	2.1	1.2	3.1	2.1	1.4	—
18:1 (*n*-9)	5.1	3.5	14.6	8.8	11.0	2.6	16.4	1.7
18:1 (*n*-7)	0.6	—	0.7	3.5	1.6	—	1.8	3.4
18:2 (*n*-6)	9.4	—	8.3	11.0	6.1	—	5.2	1.9
18:3 (*n*-6)	—	—	—	0.9	—	—	—	—
18:3 (*n*-3)	2.2	—	8.2	12.9	3.3	—	10.0	10.8
18:4 (*n*-3)	0.7	—	1.1	4.2	3.3	—	6.2	26.7
20:1 (*n*-9)	—	—	—	—	—	—	0.7	3.7
20:4 (*n*-6)	—	—	—	1.7	1.7	—	—	0.7
20:5 (*n*-3)	0.7	—	0.7	6.1	—	—	2.1	16.5
22:6 (*n*-3)	4.5	—	—	7.7	1.0	—	2.1	11.7
Total sats	75.4	96.5	65.2	40.3	66.3	97.3	51.9	10.9
Total monos	6.2	3.5	16.5	13.4	14.6	2.6	21.9	17.8
Total PUFA	17.5	0.0	18.3	44.5	15.4	0.0	25.6	69.4

Alc., fatty alcohol; TAG, triacylglycerols; PL, polar lipids; sats., saturates; monos., monoenes; PUFA, polyunsaturates.

posed predominantly of wax esters. In the culture aged for 14 weeks at 20° wax ester was the most abundant lipid class accounting for 31% of the total lipid. Triacylglycerols were still the major lipid in the culture aged at 7° although wax esters comprised 25% of the total lipid. Free fatty acids were present in appreciable amounts in both cultures and were particularly notable in the culture aged at 20°. The level of pigments in the lipid extracts of both cultures was less than that observed in starting cultures.

Saturates made up the bulk of wax ester fatty acids in cultures aged for five weeks at both 20 and 7° (Table 2). 12:0, 14:0 and 16:0 were the major fatty acids in each case. The fatty alcohol component of the wax ester was made up almost entirely of saturated moieties having chain lengths of 12 to 16 carbons. 14:0 was the most abundant fatty alcohol at both temperatures. The odd numbered chain fatty alcohols, 13:0 and 15:0, were present in the wax esters of cultures aged at both temperatures. 15:0 was particularly abundant in the wax esters of

Table 3. Fatty acid/alcohol composition (weight %) of lipids from cultures of *C. salina* maintained for 14 weeks at different temperatures

	20°				7°			
	Wax ester				Wax ester			
	Acid	Alc.	TAG	PL	Acid	Alc.	TAG	PL
12:0	16.3	0.6	8.3	5.5	28.0	0.8	11.3	4.8
13:0	—	10.7	—	—	—	12.0	—	—
14:0	25.7	28.7	36.2	26.7	20.7	31.4	33.0	16.9
15:0	8.9	34.6	10.4	7.6	7.3	29.6	10.0	5.7
16:0	14.3	18.4	17.0	24.4	13.3	19.5	15.0	20.4
16:1 (*n*-7/9)	1.7	—	1.1	1.6	1.2	—	0.8	1.4
16:2	—	—	—	—	—	—	—	—
17:0	1.2	2.7	1.0	1.0	1.2	2.5	0.9	0.8
18:0	2.5	1.4	2.6	3.3	2.3	1.5	1.7	3.4
18:1 (*n*-9)	10.6	2.8	12.0	9.1	7.4	2.5	10.5	8.7
18:1 (*n*-7)	4.8	—	1.8	4.9	2.1	—	1.4	11.1
18:2 (*n*-6)	4.8	—	4.0	3.3	6.2	—	4.7	7.4
18:3 (*n*-6)	—	—	—	0.5	0.5	—	—	0.7
18:3 (*n*-3)	1.9	—	2.6	2.5	3.0	—	4.2	5.6
18:4 (*n*-3)	0.6	—	0.7	1.8	1.4	—	0.6	2.4
20:1 (*n*-9)	1.8	—	0.2	2.0	1.3	—	—	—
20:4 (*n*-6)	0.7	—	—	0.5	0.6	—	—	1.6
20:5 (*n*-3)	1.9	—	0.7	2.1	1.8	—	0.8	4.1
22:6 (*n*-3)	1.0	—	0.8	2.4	1.2	—	4.6	4.9
Total sats	68.9	97.1	75.5	68.5	72.8	97.3	71.9	52.0
Total monos	18.9	2.8	15.1	17.6	12.0	2.5	12.7	21.2
Total PUFA	10.9	0.0	8.8	13.1	14.7	0.0	14.9	26.7

Alc., fatty alcohol; TAG, triacylglycerols; PL, polar lipids; sats., saturates; monos., monoenes; PUFA, polyunsaturates.

the 20° culture, accounting for 28.5% of the fatty alcohols. Regardless of temperature of ageing, 14:0 was the principal fatty acid of triacylglycerols. Monoenes, particularly 18:1 (*n*-9), were present in higher proportions in the triacylglycerols from cultures aged at 7° than at 20°. Polyunsaturated fatty acids (PUFA) comprised 18.3 and 25.6% of the fatty acids in TAG from 20 and 7° cultures respectively. It was notable that the percentages of all the (*n*-3) PUFA were higher in triacylglycerols at 7 than at 20°. In contrast, the proportion of 18:2($n-6$) was lower at 7°. At both temperatures examined, the polar lipid fraction contained a lower proportion of saturated fatty acids, and conversely a higher proportion of PUFA, than wax esters or triacylglycerols. PUFA comprised 44.5% of polar lipid fatty acids in the 20° culture and 69.4% in the 7° culture. This difference was mainly attributable to the level of 18:4(*n*-3) which was present at a level of only 4.2% at 20° but 26.7% at 7°. Smaller increases occurred in the proportions of 20:5(*n*-3) and 22:6(*n*-3). The (*n*-6) PUFA, 18:2 and 20:4, were present in higher proportions in the polar lipid from the 20° culture than in that from the culture aged at 7°.

Ageing for a longer period influenced the fatty acid composition of the algal lipids. The fatty acid composition of lipids extracted from cultures aged for 14 weeks (Table 3) differed from that from cultures at five weeks in that saturates represented higher proportions of fatty acids in the triacylglycerols and polar lipids. The fatty acid composition of wax esters was less influenced by the age of the cultures than that of triacylglycerols and polar lipids. Regardless of temperature, the PUFA contents of triacylglycerols and polar lipids had decreased substantially as the cultures aged between five and 14 weeks. Polar lipids still contained the highest proportions of PUFA; 26.7% in polar lipid from the 7° culture and 13.1% in that from the 20° culture. Similarly PUFA were present in higher proportions in triacylglycerols from cultures aged at 7° than at 20°. The proportion of odd chain fatty alcohols in wax esters increased with the age of the cultures, from 37.7 to 45.3% and from 34.7 to 41.6% of total alcohols between five and 14 weeks in 20 and 7° cultures respectively.

Lipid biosynthesis

The proportion of total fixed carbon that was incorporated into lipid by *C. salina* was influenced by temperature and light intensity (Table 4). Algae grown in dark fixed less CO_2 than those grown in light. Less than 1% of the radioactivity incorporated by the algae into cellular material in the dark was located in lipid at both 20 and 10°. At an environmental temperature of 20°, doubling the light intensity from 1000 to 2000 lux resulted in an increase of 1.2 times in the amount of CO_2 fixed and a similar magnitude of increase in the percentage incorporated into lipid. In cultures maintained at 10° under light intensity of 1000 lux, the amount of $^{14}CO_2$ fixed was 1.2 times that observed with cultures maintained at 20°

Table 4. Effect of temperature and light intensity on the incorporation of $^{14}CO_2$ into lipid by *C. salina*

Temp. (°)	Light intensity (lux)	Total dpm × 10^{-3} fixed/10^6 cells	% total fixed ^{14}C in lipid
20	0	18.99 ± 0.88	0.9 ± 0.1
	1000	68.72 ± 5.18	11.8 ± 0.4
	2000	82.64 ± 5.45	13.9 ± 0.2
10	0	15.71 ± 5.17	0.4 ± 0.1
	1000	82.59 ± 4.12	22.9 ± 0.9

Values are means ± s.d. obtained with three separate cultures.

under the same light intensity. The proportion of total fixed radioactivity recovered in the lipid of cultures maintained at 7° (22.9%) was approximately double that found for cultures maintained at 20° under the same light intensity.

The incorporation of radioactivity from ^{14}C-labelled CO_2, acetate and 18:1(*n*-9) into lipid fractions by *C. salina* cultured at 20° under light intensities of either 1000 or 2000 lux, and at 10° under light intensity of 1000 lux is shown in Table 5. At light intensity of 1000 lux, the amount of radioactivity from $^{14}CO_2$ incorporated into lipid at 10° was considerably greater than at 20°. This difference was not so obvious or consistent with ^{14}C-labelled acetate or 18:1(*n*-9). Neutral lipids, particularly triacylglycerols, were heavily labelled from all three substrates under all growth conditions examined. Relative to other lipid fractions, the most extensive labelling of wax esters at all times examined was observed with *C. salina* incubated with $^{14}CO_2$ at 20° under light intensity of 2000 lux. Under these conditions 28.8% of the label incorporated into lipid was recovered in wax esters after 24 hr. With all three labelled substrates the percentage of incorporated radioactivity recovered in wax esters increased with the age of the culture. The most heavily labelled wax esters were consistently found in aged cultures of five weeks. The relative incorporation of ^{14}C-18:1 (*n*-9) into wax esters over five weeks was less extensive than with $^{14}CO_2$ or ^{14}C-acetate. With all three radiolabelled substrates, the proportion of label recovered in polar lipids generally decreased as the cultures aged. After five weeks less than 5% of incorporated radioactivity from any substrate was located in polar lipids. Free fatty acids and

Table 5. Effect of temperature and light intensity on the incorporation of ^{14}C-labelled CO_2, acetate and oleic acid into lipids by *C. salina*

				Lipids (% total lipid)					
Temp (°)	I (lux)	Time (days)	dpm in lipid*	W.E.	TAG	FFA	FAl.	Ster. + Pigm	PL
$^{14}CO_2$									
20	1000	1	8.2	5.5	46.9	5.2	0.7	4.8	36.9
		7	14.9	15.0	64.7	1.0	0.5	3.4	15.4
	2000	1	9.2	28.8	22.5	4.9	0.8	5.0	38.0
		7	27.5	23.2	62.2	1.3	0.8	3.2	9.3
		35	73.5	54.8	34.5	4.6	0.7	3.7	1.7
10	1000	1	39.2	1.2	40.2	1.5	0.6	4.7	51.8
		7	76.4	2.3	74.2	1.0	1.9	4.6	16.0
		35	122.7	30.3	61.6	0.8	0.5	2.6	4.2
^{14}C-Acetate									
20	1000	1	1.0	2.9	45.3	8.3	0.5	6.0	37.0
		7	6.1	3.4	65.3	1.3	0.4	2.6	27.0
	2000	1	0.5	6.5	31.5	12.7	0.0	9.8	39.5
		7	3.3	6.8	69.6	2.7	0.5	4.5	15.9
		35	1.6	24.0	56.6	6.7	1.1	8.3	3.3
10	1000	1	0.6	8.3	47.9	5.8	0.0	3.5	34.5
		7	3.9	2.2	73.9	1.7	1.0	3.2	16.7
		35	4.5	13.6	75.0	3.7	0.5	4.3	2.9
^{14}C-18:1 (*n*-9)									
20	1000	1	76.7	1.3	18.5	65.3	0.7	2.3	11.9
		7	41.2	5.8	58.1	3.3	0.7	2.5	29.6
	2000	1	73.3	2.0	44.1	1.0	0.9	4.0	48.0
		7	71.0	2.9	68.7	1.8	0.8	4.1	21.7
		35	81.7	6.4	34.0	51.4	1.0	3.5	3.7
10	1000	1	52.1	2.0	66.7	3.2	0.6	3.5	23.9
		7	67.6	2.4	77.2	1.1	0.6	2.5	16.2
		35	40.5	7.4	81.6	3.1	0.7	3.4	3.8

* × 10^{-3}/ml culture; W.E., wax esters; TAG, triacylglycerols; FFA, free fatty acids; Falc., free fatty alcohol; Ster., sterols; Pigm., pigments; PL, polar lipids.

alcohols generally contained a smaller proportion of the radioactivity present in lipid than sterols and pigments.

Radioactivity incorporated from ^{14}C-labelled acetate and CO_2 was almost equally distributed in the fatty acid and fatty alcohol moieties of wax esters formed by *C. salina* under all conditions examined (data not shown). About 85% of radioactivity incorporated from ^{14}C-18:1(*n*-9) was located in the fatty acid moiety with the remaining 15% in the alcohol portion. Regardless of labelled substrate, more than 90% of the radioactivity incorporated into triacylglycerols was present in the fatty acid portion.

Silver nitrate chromatography demonstrated that for cultures grown under light intensity of 1000 lux, the distribution pattern of radioactivity from labelled substrates in fatty acids of total lipid was influenced by temperature (Table 6). At all times a substantially higher proportion of radioactivity incorporated from $^{14}CO_2$ was recovered in trienes of *C. salina* grown at 10° than at 20°. After five weeks, 44.1% of radioactivity derived from $^{14}CO_2$ was located in fatty acids having two or more double bonds in the culture maintained at 20° compared with a corresponding value of 17.1% for the culture maintained at 10°. With ^{14}C-acetate as substrate, the dienes of the 20° culture always contained higher proportions of incorporated radioactivity than those of the 10° culture. However, after seven and 35 days the combined unsaturated fatty acids, particularly the trienes, of the algae maintained at 10° were relatively more labelled than those at 20°. As with ^{14}C-acetate, higher proportions of radioactivity from incorporated ^{14}C-18:1(*n*-9) were recovered in dienes at 20° than at 10°. Only after five weeks were the proportions of label from ^{14}C-18:1(*n*-9) recovered in dienes, trienes and PUFA higher at 10° than 20°. Analysis of lipid extracted from a culture of *C. salina* not incubated with radioactive precursors showed that the saturates, monoenes, dienes and trienes accounted for at least 98% of the fatty acids present in the fractions so termed. The fraction termed polyunsaturates contained 18:4(*n*-3), 20:4(*n*-3), 20:4(*n*-6), 20:5(*n*-3), and 22:6(*n*-3).

Table 6. Effect of temperature on the percentage distribution of radioactivity from ^{14}C-labelled CO_2, acetate and oleic acid in fatty acid classes of *C. salina* total lipid

	20°			10°		
Time (days)	1	7	35	1	7	35
$^{14}CO_2$						
Saturates	54.5	68.6	71.7	28.8	34.0	41.3
Monoenes	12.3	17.0	11.2	20.7	31.9	14.6
Dienes	16.3	9.3	8.4	9.2	8.2	6.5
Trienes	14.7	4.8	7.0	38.1	24.9	26.7
Polyunsats.	2.2	0.3	1.7	3.2	1.0	10.9
^{14}C-Acetate						
Saturates	27.0	34.9	40.5	68.8	25.4	26.6
Monoenes	21.2	43.0	37.1	17.0	47.1	33.0
Dienes	27.5	15.5	14.1	7.3	9.4	10.5
Trienes	17.0	6.1	6.3	5.5	17.0	21.9
Polyunsats.	7.3	0.5	2.0	1.4	1.1	8.0
^{14}C-18:1(*n*-9)						
Saturates	4.2	3.8	7.9	4.7	6.2	10.1
Monoenes	77.2	76.5	86.2	90.6	85.4	73.1
Dienes	16.3	16.5	4.6	3.1	3.9	5.9
Trienes	1.7	2.6	0.9	1.2	4.0	9.3
Polyunsats.	0.6	0.6	0.4	0.4	0.5	1.7

DISCUSSION

The present study demonstrates that the lipid associated with aged cultures of *C. salina* is predominantly neutral lipid of which wax esters can be the major component. The accumulation of wax esters during ageing sets *C. salina* apart from algae such as *Chlorella vulgaris* and *Scenedesmus obliquus* which produce triacylglycerols towards the end of their growth phase or when grown in medium having low N concentration [3]. In this respect *C. salina* is more akin to *Euglena gracilis* which can synthesize wax esters when grown under adverse conditions such as in the presence of low N levels [1]. As far as we are aware, the production of wax esters by species of marine algae other than *C. salina* has not been reported.

The predominance of saturated fatty acids in the triacylglycerols of *C. salina* cultures and the apparent increase in their proportion with culture age, is in keeping with the decreased unsaturation observed with the total lipid formed by *C. vulgaris* and *S. obliquus* as they near the end of their growth phase [4]. The high level of 14:0 in the triacylglycerols of *C. salina* is particularly notable and higher than that reported [1] for the total lipid of microalgae even when they are rich in neutral lipid [3]. Published data for the fatty acid composition of triacylglycerols from algae are rare, but high levels of 12:0 and 14:0 are present in the triacylglycerols produced by the marine dinoflagellate *C. cohnii* [11].

Although there are general similarities in the compositions of the wax esters of *C. salina* found in this study and those described previously by Antia *et al.* [6], there are also some notable differences. In particular, odd numbered fatty alcohols accounted for only 6% of the total fatty alcohols of wax esters in the previous study [6] whereas here they comprised 34.7 to 48% depending on the culture age and environmental temperature.

The fatty alcohols of the naturally occurring wax esters such as those found in marine zooplankton [12] and jojoba seed oil [13] are almost exclusively even numbered. However, anaerobic conditions are known to favour the synthesis of odd numbered fatty alcohols by *E. gracilis* [14]. Thirty-four per cent of the fatty alcohols of wax esters produced by *E. gracilis* grown anaerobically have been reported to be odd numbered [15]. Since the cultures of *C. salina* were left to age without aeration, it is likely that partially anaerobic conditions prevailed within the culture vessels. Under such conditions *E. gracilis* is known to utilise ^{14}C-propionate as a precursor of odd numbered fatty acids and alcohols [5]. The substrate for wax ester synthesis in *E. gracilis* grown anaerobically is acetyl-CoA derived from the degradation of its reserve polysaccharide paramylon [16]. Although the present study showed radioactivity from ^{14}C-labelled CO_2 and acetate to be incorporated into wax esters, it did not establish whether this radioactivity passes through non-lipid material prior to its incorporation into wax ester. The yield of wax esters produced by *E. gracilis* is dependent on which substance is employed as a carbon source in

the medium [15]. If wax esters are derived from polysaccharide reserves in *C. salina* then substances, such as glucose, with the potential to encourage the production of these polysaccharides may enhance the production of wax esters by ageing *C. salina* when employed as a carbon source. The biochemistry of wax ester synthesis in *C. salina* remains to be examined. The lower yield of wax esters obtained from the aged *C. salina* cultures examined in this study in comparison with that of Antia *et al.* [6] may be due to differences in culture conditions and cell densities.

The observation that a higher proportion of the total carbon fixed by *C. salina* was incorporated into lipid at low temperature is in keeping with what has been reported for measurements carried out with phytoplankton sampled in Arctic waters [17, 18]. The proportion of total carbon incorporated into lipid by *C. salina* at 10° was also similar to that observed with phytoplankton taken directly from the sea [17, 18]. Therefore, information gained from studies with cultures of *C. salina* may be applicable to microalgae in the marine environment, particularly in polar waters where lipid is an important product of photosynthesis [19].

It is well established that poikilothermic fish [20] and invertebrates [21] respond to decreases in their environmental temperature by increasing the degree of unsaturation of the fatty acids in their membrane phospholipids. The present study suggests that the same response extends to unicellular algae since the polar lipids of *C. salina* grown at 7° had notably higher contents of PUFA than those from algae grown at 20°. Likewise, the recovery of increased proportions of radioactivity from labelled precursors in the more unsaturated groups of fatty acids confirms an increased fatty acid desaturase activity in *C. salina* as a response to low temperature. From the results of the few studies which have been carried out on algal lipid composition in relation to growth temperature [10, 22–24], it is difficult to conclude whether an inverse relationship between the PUFA content of lipid and environmental temperature is ubiquitous in algae. Previous studies have not employed temperatures as low as the 7° examined in this study. The ability of *C. salina* to adapt its polar lipid fatty acid composition may allow the alga to grow at the relatively low temperature of 7°. Algal species which exhibit narrow temperature ranges for growth may be less capable of increasing the PUFA content of their polar lipids.

The specific increases and decreases in the proportions of (*n*-3) and (*n*-6) PUFA respectively over the first five weeks of ageing at low temperature implies that the activity of the Δ_{15} desaturase responsible for the conversion of 18:2(*n*-6) to 18:3(*n*-3) increased in response to adaptation to growth at a low temperature. It is notable that in the total lipid of two *Scenedesmus* species the proportion of 18:2(*n*-6) was greater than that of 18:3(*n*-3) when grown at 35° whereas the situation was reversed at 20° [9].

The results of the present study suggest that environmental temperature does not influence the composition of the wax esters produced by ageing cultures of *C. salina*. This contrasts with the increased degree of unsaturation noted in the wax esters produced by the bacterium *Acinetobacter* species when its growth temperature is reduced [7]. The saturated nature of the wax esters and triacylglycerols in *C. salina* is consistent with their being formed late in the growth phase when growth has stopped. At this stage the synthesis of unsaturated fatty acids for incorporation into new membrane phospholipids can be expected to be minimal. Consequently, the saturated products of fatty acid synthetase can be incorporated directly into neutral lipids without further desaturation or elongation.

From their fatty acid and fatty alcohol composition, it can be deduced that the bulk of the wax esters produced by *C. salina* are shorter in chain length than those of sperm whale, jojoba oil or bacteria [7, 25]. In view of this compositional difference and the apparent inability to alter the composition by simple environmental changes it is unlikely that wax esters derived from *C. salina* can replace jojoba oil as a commercial substitute for sperm whale oil.

However, in addition to being a useful system for studying the biochemistry of wax ester synthesis, the ability of *C. salina* to grow at low temperature makes it suitable for the study of PUFA biosynthesis in photosynthetic microalgae in relation to environmental temperature. Studies are continuing using this microalga to examine the effect of temperature on the synthesis of PUFA and their incorporation into specific phospholipids and glycolipids.

EXPERIMENTAL

Alga and culture conditions. Chroomonas salina (Wislouch) was obtained from the Scottish Marine Biological Association (Oban, Scotland). The basal seawater medium used for the culture of the algae was that described in ref. [26] with the addition of 0.25 M glycerol. Stock cultures were maintained axenically in an environmental cabinet at 20° with continuous aeration under continuous white light of intensity 2000 lux. Sterility checks were carried out routinely by plating small samples of culture on nutrient blood agar. Cell numbers were determined using an improved Neubauer counting chamber.

To study the effects of temperature on lipid composition, two cultures were set up simultaneously using inocula from the stock culture. One culture was maintained at 20° and the other at 7°, both under light intensity of 2000 lux. Both cultures were aerated with sterile air at a flow rate of 150 ml/min. When the cultures changed colour from brown to green, additional vitamins, nitrate and phosphate were added to offset the depletion of nutrients and encourage further growth. This occurred 7 and 15 days after initiating the cultures at 20 and 7° respectively. The cultures were then allowed to age without the further addition of nutrients, the culture vessels only being opened to sample for analysis. Aeration was discontinued 4 weeks after initiation of the cultures. When required for determination of dry wt, algal cells were harvested by centrifugation (15000 *g* min), washed with 0.4 M ammonium formate [27] and freeze-dried.

The effects of temperature and light intensity on the fixation of $^{14}CO_2$ into lipid was studied in triplicate using glass vials containing 10 ml of culture of cell concentration 0.91×10^6/ml taken from an actively growing stock culture. The cultures were preincubated under the required conditions for 2 hr before 17 μCi Na $H^{14}CO_3$ (55 mCi/mmol) were added to each vial. The vials were immediately sealed and incubated under the relevant conditions for 24 hr. 0.2 ml 6 M HCl was then added to each vial. After 30 min, 3 ml of each culture were neutralized and measured for content of radioactivity to give a value for total fixed ^{14}C. Lipid was extracted from the remainder of the sample as described below and its content of radioactivity measured.

To study the incorporation of radioactively labelled substrates into lipids during ageing, 100 ml cultures of initial cell concentration of 3×10^6 cells/ml were incubated with either 70 μCi Na $H^{14}CO_3$, 25 μCi Na [U-^{14}C] acetate (56 mCi/mmol) or 25 μCi [1-^{14}C] oleic acid (57 mCi/mmol). Samples were taken at intervals for lipid analyses as described below. Cultures were aerated continuously with sterile air, with the exception of the cultures containing Na $H^{14}CO_3$ which were only aerated for 1 hr each day.

Lipid extraction. Lipids were extracted from aliquots of cultures or freeze-dried samples by homogenization in $CHCl_3$–*iso*-PrOH (2:1). After removal of solvents, lipid extracts were desiccated overnight *in vacuo* before being weighed and redissolved in $CHCl_3$–MeOH (2:1). Lipid samples were stored at −70° under N_2 prior to analysis.

Lipid analysis. For the estimation of lipid classes, total lipid was separated into component classes by TLC on glass plates coated with silica gel G using hexane–Et_2O–HOAc (85:15:1) as developing solvent. The developed chromatograms were stained with the copper acetate reagent of ref. [28]. Separated lipid classes were identified by comparison of their R_f values with authentic standards and quantitated using a Vitatron flying spot densitometer. The above solvent system was also used to separate lipid classes for measurement of their radioactivity content. Bands of adsorbent containing the lipids were visualised by exposure to I_2 vapour and scraped into scintillation vials and measured for radioactivity after addition of scintillation fluid.

For analysis, wax esters and triacylglycerols were separated by prep. TLC using the above solvent system. To visualize the lipid classes, developed chromatograms were sprayed lightly with 2′,7′-dichlorofluorescein and viewed under UV light. Wax esters and triacylglycerols were eluted from the adsorbent with hexane–Et_2O (1:1) and the eluates extracted with 2% (w/v) $KHCO_3$. After removal of solvent, wax esters were saponified using potassium *t*-butoxide [29]. Free fatty acids and fatty alcohols were recovered from the acidified reaction mixture by hexane extraction and purified by TLC using hexane–Et_2O–HOAc (70:30:1) as developing solvent. The free fatty acids were converted on the adsorbent to their Me esters [30]. Free fatty alcohols were eluted from the adsorbent with $CHCl_3$ and converted to their acetate derivatives using Ac_2O in pyridine [31]. Triacylglycerols were also transesterified directly on the silica adsorbent. Both fatty acid Me esters and fatty alcohol acetates were purified by TLC using hexane–Et_2O–HOAc (85:15:1) as developing solvent and recovered from the adsorbent with hexane–Et_2O (1:1).

To determine the radioactivity present in the fatty acid and fatty alcohol moieties, wax esters were purified and saponified with subsequent TLC as described above. Bands of adsorbent containing fatty acid and fatty alcohols were measured directly for radioactivity. Radioactivity present in the organic and aqueous phases of the triacylglycerol transesterification extraction was assumed to be located in the fatty acid and glycerol moieties respectively.

Purified fatty acid Me esters and fatty alcohol acetates were analysed by GC using either a 25 m × 0.32 mm internal diameter (i.d.) fused silica column coated with CP Wax 51 or a 30 m × 0.25 mm i.d. fused silica column coated with chemically bonded Supelcowax 10. Samples were applied by on-column injection and all chromatographic analyses were temperature programmed. Authentic standards were used for the identification of separated components.

For the separation of Me esters according to their degree of unsaturation, $AgNO_3$ TLC was performed using the system described elsewhere [32]. To confirm the composition of bands, fatty acid Me esters of total lipid extracted from *C. salina* were separated using the method described and subjected to GC analysis after recovery from the adsorbent.

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