The Effect of Growth Temperature on Wax Ester Content and Composition of *Euglena gracilis*

By AKIKO KAWABATA* AND MUTSUKO KANEYAMA

Department of Natural Science, Osaka Women's University, Sakai, Osaka 590, Japan

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A streptomycin-bleached mutant of *Euglena gracilis* strain Z, a non-photosynthetic mutant, was grown aerobically in the dark at 33, 26 and 15 °C respectively. The content of wax esters was greater at higher growth temperatures, whereas the content of paramylon was greater at lower temperatures. The highest temperature (33 °C) caused the greatest accumulation of wax esters. Wax esters synthesized at the highest temperature had carbon chain lengths of C24–32 and the predominant chain lengths were C27 and C28. The constituent fatty acids and alcohols ranged from C11 to C18 with myristic acid and myristyl alcohol being the main components. At the lowest temperature (15 °C) the mean chain length of wax esters decreased. The most abundant wax ester was C26, and the most abundant fatty acids and fatty alcohols of wax esters were C13. A considerable amount (44–48% of the total) of odd-carbon-numbered wax esters were present. The effect of temperature on odd-carbon-numbered wax formation is discussed in relation to possible sources of propionate or methylmalonate units.

INTRODUCTION

The unicellular photosynthetic protist *Euglena gracilis* has a multifunctional fatty acid synthetase in the cytoplasm (Delo *et al.*, 1971; Goldberg & Bloch, 1972), two acyl-carrier-protein-dependent systems in the chloroplasts (one of the two systems is a chain elongation system) (Goldberg & Bloch, 1972), a fatty acid synthetase in the microsomes (Nagai & Bloch, 1965) and a novel malonyl-CoA-independent fatty acid synthetase system in the mitochondria (Inui *et al.*, 1984). Differences in the illumination regime during growth of *E. gracilis* can cause changes in both the chain length (Rosenberg, 1963; Rosenberg & Pecker, 1964; Rosenberg *et al.*, 1965) and the degree of unsaturation (Hulanicka *et al.*, 1964; Rosenberg & Gouaux, 1967; Ernst-Fonberg & Bloch, 1971; Pohl, 1973) of fatty acids. Inui *et al.* (1982) demonstrated a direct correlation between anaerobiosis and concomitant wax ester synthesis. However, the effect of environmental temperature on the regulation of lipid synthesis in *Euglena* has not been studied. The present experiments were planned to investigate the effect of temperature on the content and composition of wax esters, as part of a study of the effects of temperature on fatty acid metabolism in *E. gracilis*.

METHODS

Chemicals. Fatty acid methyl ester standards and alcohol standards were purchased from Wako Pure Chemical Industries, Tokyo Kasei and Sigma. Wax ester standards were synthesized by heating overnight equimolar mixtures of fatty acid methyl esters and fatty alcohols in n-hexane (Hashimoto *et al.*, 1967). They were purified by column chromatography on silica gel, eluted with n-hexane, followed by hexane/benzene (1:1, v/v) and methanol in succession. The hexane/benzene fraction contained the wax esters.

Organism and culture. To eliminate the effect of light, a streptomycin-bleached mutant of *E. gracilis* strain Z (Oda *et al.*, 1982) was used. As described in a previous paper (Kawabata *et al.*, 1982), the cells were adapted to 33, 26, or 15 °C by successive cultivation at these temperatures. Cells were grown in 150 ml of Koren–Hutner medium (Koren & Hutner, 1967) in 500 ml flat-shouldered flasks on a shaker (120 strokes min⁻¹) in the dark at the above

temperatures. Growth was determined by cell counts using a haemocytometer. Dissolved oxygen in the culture medium was determined using a Clark-type oxygen electrode.

Preparation and analysis of lipids. The cells were collected by centrifugation at 3000 r.p.m. for 2 min and washed twice with 1% (w/v) NaCl. Lipids were extracted from the cells by the method of Bligh & Dyer (1959). Lipid extracts were dried under nitrogen. Wax esters were purified from the lipid extract by thin-layer chromatography (TLC) using silica gel (Merck) plates with a solvent system containing n-hexane/diethyl ether (3:2, v/v) or n-hexane/diethyl ether/acetic acid (80:20:1, by vol.). The wax-ester-containing spots (R_F values 0.8 and 0.7 respectively in the two solvent systems) were scraped off the TLC plates and extracted three times with diethyl ether. The wax esters were dried under nitrogen, dissolved in n-hexane, and samples analysed by gas-liquid chromatography (GLC) on 5% (w/v) Thermon-1000 packed in a 3×225 mm (int. diam.) glass column operated at 240°C in a Yanako model G-80 chromatograph equipped with a hydrogen detector. The carrier gas was N_2 , used at a flow rate of 20 ml min^{-1} . The injection temperature was 280°C and the detector temperature 320°C . Decyl decanoate, octyl myristate, stearyl stearate and myristyl myristate, prepared as described above, were used as internal standards. The purified wax ester mixture was saponified with 0.5 M-KOH in 95% (v/v) aqueous methanol at $70\text{--}75^\circ\text{C}$ for 4 h, then dried under nitrogen, after which water and n-hexane were added. After shaking, the n-hexane layer was collected and used as the fatty alcohol fraction. The aqueous layer was acidified to pH 1.6 with 3 M-HCl and the wax fatty acids were extracted three times with n-hexane. The free alcohols were converted to their acetates with acetic anhydride (Farquhar, 1962). The fatty acids were methylated with 5% (w/v) methanolic hydrochloric acid solution at $80\text{--}90^\circ\text{C}$ for 1 h (Stoffel *et al.*, 1959). The wax fatty acid esters and fatty alcohol acetates were separated and identified by GLC on 10% (w/v) DEGS and 5% (w/v) Advance DS (Shimadzu) packed in a 3×150 mm (int. diam.) glass column. The column temperature was maintained at 120°C for the first 1 min and then was increased from 120 to 210°C at 4°C min^{-1} . The carrier gas flow rate, the injection temperature and the detector temperature were as in the GLC of wax esters.

Wax esters, fatty acid esters and fatty alcohol acetates were hydrogenated with palladium oxide as the catalyst in 2–3 ml ethanol at room temperature for 1 h until the iodine value was zero.

Paramylon estimation. Paramylon was measured using the method described in Kawabata *et al.* (1982).

RESULTS

Effects of temperature on growth and cellular constituents

Growth curves of the bleached mutant of *E. gracilis* strain Z cultured at 33, 26 and 15°C in the dark with shaking are shown in Fig. 1(a). The generation times were 14.8, 15.7 and 35 h for the cells grown at 33, 26 and 15°C , respectively. Cultures grown at 15°C completed the exponential phase after about 18 d, those at 26°C after about 6 d and at 33°C after about 5 d. The cell number of the cultures grown at 15°C was slightly higher than that of the cultures grown at 26°C . The temperature affected only the growth rate and the generation time but not cell yield, since weight and size of the cells grown at 33°C were twice those of the cells grown at 15 or 26°C . The concentrations of wax esters and paramylon in cultures grown at 33, 26 and 15°C are shown in Fig. 1(b). The paramylon content was highest in the cells grown at 15°C and lowest in those grown at 33°C , which contained about 50% of the paramylon present in the cells grown at 15°C . In contrast, the contents of the total lipids and wax esters showed a reverse relationship, being higher at 33°C and lower at 15°C ; the wax ester content of cells grown at 33°C was seven times as much as those grown at 15°C . However, the content of the polar lipids was almost constant in comparison with the increased contents of total lipids and wax esters.

Effect of temperature on the content and composition of wax esters

The wax ester contents of bleached cells of *E. gracilis* grown at 33, 26 and 15°C were 108, 23 and $14\text{ }\mu\text{g}$ per 10^6 cells, respectively.

The analysis by GLC of wax esters formed by *E. gracilis* grown at different temperatures is shown in Table 1. The wax esters in cells grown at the lowest temperature (15°C) were composed mainly of C26 and C27 esters (52% of the total), and the percentage of C28 esters was less than in the cells grown at 26 or 33°C . The cells grown at 26 or 33°C contained mainly C27 and C28 esters (49 and 55%, respectively, of the total). The mean carbon chain-lengths of the wax esters of cells grown at 15, 26 and 33°C were 26.5, 27.5 and 27.6, respectively, indicating that the wax esters at the lowest temperature were shorter at the 2% significance level than those from cells grown at the highest temperature. At 15°C approximately 50% of the wax esters had odd carbon-numbers.

Fig. 1. (a) Growth curves of the bleached mutant of *E. gracilis* cultured at different temperatures. ----, 15 °C; - · - ·, 26 °C; —, 33 °C. (b) Effect of temperature on the contents of total lipids (----), polar lipids (Δ), wax esters (○) and paramylon (◇) in *E. gracilis*.

Table 1. *Effect of temperature on chain-length of wax esters in E. gracilis*

Each value is the mean of five experiments (SD ≤ 12%). tr., Trace.

Carbon-chain length	Culture conditions . . .	Wax ester composition (weight%)			
		15 °C	26 °C	33 °C	27 °C (anaerobiosis)*
23		2.1	0.7	0.8	tr.
24		6.3	2.3	1.8	0.1
25		15.4	6.6	4.9	0.8
26		27.4	19.0	15.3	6.1
27		24.3	24.0	26.2	17.2
28		15.1	25.0	29.0	36.0
29		5.5	10.5	11.5	19.0
30		3.2	7.3	7.0	15.2
31		0.7	2.8	2.3	3.7
32		tr.	1.8	1.2	1.9
33		tr.	tr.	tr.	tr.
Total odd-numbered		48.0	44.6	45.7	40.7
Mean chain-length		26.5	27.5	27.6	28.4

* Cited from Inui *et al.* (1983).

After saponification, the component fatty acids and alcohols were identified by GLC (Table 2). The constituent fatty acids and alcohols ranged from C11 to C18. In the cells grown at 26 and 33 °C the main components were myristic acid and myristyl alcohol (42% of the total). At 15 °C the predominant fatty acids and alcohols were C13, (40 and 43% of the total). Approximately 50% of the fatty acids and 54% of the fatty alcohols of cells grown at 15 °C had odd carbon-numbers. As the temperature increased, the ratios of odd- to even-carbon-number fatty acids and fatty alcohols in the wax esters decreased at the 5% significance level. However, the ratio of odd- to even-number carbon chains in the polar lipids was little changed by growth temperature.

Table 2. *Effect of temperature on fatty acid and fatty alcohol composition of E. gracilis wax esters*
 Each value is the mean of three experiments (SD $\leq 15\%$). tr., Trace.

Carbon-chain	Culture conditions . . .	Fatty acids (weight%)				Fatty alcohols (weight%)			
		15 °C	26 °C	33 °C	27 °C (anaerobiosis)*	15 °C	26 °C	33 °C	27 °C (anaerobiosis)*
10:0		—	—	0.4	0.4	—	tr.	—	tr.
11:0		0.8	2.3	0.7	2.2	4.0	0.7	0.4	0.9
12:0		9.3	6.4	4.7	10.3	23.3	11.0	6.0	2.7
13:0		40.3	29.7	28.1	19.5	43.2	29.1	19.3	12.6
14:0		29.2	42.7	42.5	44.4	21.4	41.6	42.0	46.8
15:0		5.5	8.3	12.5	12.7	6.2	11.9	19.0	19.3
16:0		3.2	6.5	7.1	9.2	1.5	4.9	12.0	16.2
17:0		1.4	2.6	1.7	1.3	0.4	0.8	1.3	1.5
18:0		1.4	1.6	0.1	tr.	tr.	tr.	tr.	tr.
Other†		8.8	—	0.6	—	—	—	—	—
Total		>48.0	42.9	43.0	35.7	53.8	42.5	40.0	34.3
Mean		13.5	13.9	13.7	13.9	13.1	13.7	13.9	14.4
chain length									

* Cited from Inui *et al.* (1983).

† Others include chain length of C9 saturated + unidentified fatty acids.

DISCUSSION

In many unicellular organisms and aquatic animals, the quality and quantity of the lipids depend on the external temperature. At lowered temperatures, the acyl groups of the phospholipids in membranes are more unsaturated in *Tetrahymena* (Nozawa, 1979), *Candida lipolytica* (Ferrante *et al.*, 1983) and carp liver (Wodtke, 1978). At elevated temperatures, the total fatty acid increases in *Brevibacterium ammoniagenes* (Kawaguchi *et al.*, 1979), whereas the ratio of C16 acid to C18 acid in yeast (Okuyama *et al.*, 1979) and the total polar lipid contents in *Neurospora crassa* decrease (Vokt & Brody, 1985).

Our observations show that growth at low temperature results in the presence of more unsaturated total lipids and polar lipids in *Euglena* (data not shown), whereas elevated temperatures cause an increase in total lipid content, especially wax esters (Fig. 1b). *E. gracilis* accumulates wax esters when grown in a carbon-rich medium and under adverse conditions such as anoxia (Inui *et al.*, 1982), darkness (Rosenberg, 1967), lack of agitation (Nagai *et al.*, 1971), ageing (Marcenko, 1978) and thiamin deficiency (Inui *et al.*, 1988). In the present paper, we have shown that the highest growth temperature (33 °C) causes the greatest accumulation of wax esters in *E. gracilis*.

There are few reports of the temperature-dependent variation of wax ester content or composition in micro-organisms. Russell & Volkman (1980) reported the effect of growth temperature on wax ester composition in the psychrophilic bacterium *Micrococcus cryophilus*. At elevated temperature, the mean chain length of wax esters increased and their unsaturation decreased. Our present observations in *Euglena* agree with the data in *M. cryophilus*. The mean carbon-chain length of wax esters in *E. gracilis* grown anaerobically (Inui *et al.*, 1983) is higher at the 5% significance level than when the organism is grown aerobically, even at the highest temperature (Table 1). Inui *et al.* (1988) reported that thiamin deficiency in the cells results in the formation of wax esters with shorter carbon chains under anaerobic conditions. Wax ester formation occurs under anaerobic conditions, its rapid synthesis being initiated below an O₂ concentration of 10⁻⁶ M (Inui *et al.*, 1982). Hence, wax formation at a high temperature is not necessarily linked to anaerobiosis. In the present experiment, however, the cells were cultured at 33 °C for 5 d and the culture medium contained 100 µM dissolved oxygen, which is five times higher than that of the K_m of cytochrome oxidase (Barman, 1969). Hence, the dissolved oxygen concentration is sufficient to fulfill the respiratory capacity of *Euglena* cells.

The significant amounts of wax esters with odd carbon-chain lengths found in the present experiments could arise from α -oxidation of even-numbered precursors. However, this has been excluded in the case of *Euglena* by Schneider *et al.* (1984). Hence, the odd-numbered fatty acid and fatty alcohol components of wax esters accumulated in the cells are probably synthesized with propionyl-CoA as a primer. There are two main pathways for the synthesis of propionyl-CoA. One uses methylmalonyl-CoA as an intermediate, and another, less common, involves acrylyl-CoA. The latter pathway can be excluded for *Euglena*, since it does not explain the incorporation of CO₂ into odd-numbered chains (Schneider & Betz, 1985). The methylmalonyl-CoA pathway is the most probable, since its operation in the reverse direction, i.e. in propionate assimilation, has been demonstrated in *Euglena* by Yokota *et al.* (1982). Moreover, one additional ATP can be formed in the methylmalonyl pathway (Schneider & Betz, 1985); therefore propionyl-CoA formation is favourable for the cells. The amount of propionate supplied from branched amino acids or through unknown pathways may increase at higher temperatures. Catabolism of propionate in *Euglena* mitochondria releases CO₂ only in the dark, resulting in loss of carbon (Yokota *et al.*, 1982), but this is preferable to the accumulation of propionate which is toxic.

The increased formation of wax esters at higher temperatures is unique in that they have a relatively high molecular mass and are highly insoluble in water. They are sometimes called crystalloids (Marcenko, 1978), which are present as suspended solids in the cytosol, and apparently do not affect other pathways of metabolism. The wax ester is retained in cells with as little loss of carbon as possible, and when lower temperature conditions are restored the wax esters are rapidly converted back to paramylon (Kawabata *et al.*, 1982). *Euglena* is very sensitive to temperature (Cook, 1968) so it has developed unique strategies to cope with adverse conditions.

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