Arch. Microbiol. 119, 153–156 (1978)

Archives of
Microbiology

The Cryopreservation of *Chlorella*

4. Accumulation of Lipid as a Protective Factor

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Abstract. Following growth under sub-optimal concentrations of nutrients, cells of *Chlorella emersonii* accumulated lipid and became more resistant to the damage caused by freezing and thawing. These results suggest that the factor responsible for the cold hardening of some *Chlorella* spp is not the effect of low temperatures per se but simply that of the reduced metabolic rate. Evidence is given that the post-thaw injury observed following rapid rates of cooling is associated with the vacuole.

Key words: *Chlorella* – Cryopreservation – Fatty acids analysis.

Following a period of cold acclimatization the recovery from −196° C of *Chlorella emersonii* (Morris, 1976b) and *C. ellipsoidea* (Hatano et al., 1976a, b) was significantly increased. It has been suggested that the increase in the degree of unsaturation of the membrane fatty acids induced by low temperatures is the factor responsible for the cold hardening of plant cells (Gerloff et al., 1966; Siminovitch et al., 1968), but preliminary experiments with *C. emersonii* indicate that for this organism it is not the effect of low temperatures per se which are important but simply that of reduced growth rate (Morris and Clarke, 1977). With a number of Chlorococcales it has been found that in the late stationary phase of culture, where growth is restricted by a number of physical and chemical factors, especially nitrogen limitation (Fogg, 1959; McLean, 1968), cells are more resistant to freezing injury than those from younger cultures (Morris, 1978). In the present study, the effect on the cellular freezing tolerance when a reduction in growth rate is induced by a limitation of nutrients at 20° C is investigated. The results obtained are compared with alterations in the phospholipid fatty acid composition and the total lipid content.

Abbreviations. TLC = Thin layer chromatography; fatty acids are noted by two numbers, the first of which give the number of carbon atoms and the second the number of double bonds

Methods

Cells. All strains of *Chlorella* were obtained from the Culture Centre of Algae and Protozoa, whose system of numbering and nomenclature is employed (George, 1976). *Chlorella* 211/8h was used in the majority of experiments; this strain was named *C. fusca* var. *vacuolata* by Shirira and Krauss (1965). However, as the epithet *fusca* is invalid for a *Chlorella* species the oldest valid name *emersonii* is now used (Fott et al., 1975). Cells were grown in Bolds medium (Bischoff and Bold, 1963) at 20° C though in some experiments the concentration of a single nutrient was reduced to 1 % of the control value. Unless otherwise stated experimental cells were from 14 day old cultures and were used without further preparation.

Freezing and Thawing. Cells were cooled at different rates to −196° C and the viability upon thawing assayed by colony formation in agar (Morris, 1976a). The response of the cells to hypertonic solutions of sodium chloride was determined as described previously (Morris et al., 1977). In each experiment there were three replicates for each treatment.

Lipid Extraction and Analysis. Lipid was extracted from cells and stored as previously described (Morris et al., 1977). Total phospholipids (including non-phosphorus containing glycolipids) were isolated by thin layer chromatography (TLC) on precoated silica gel H plates (Merck, 0.25 mm, 20 × 20 cm) in petroleum spirit (40° – 60° C)/diethyl ether/acetic acid, 80/20/1, v/v, scraped off and eluted with methanol/chloroform, 4/1.

Fatty acid esters were prepared from phospholipids by mild alkaline deacylation to avoid production of aldehyde dimethyl acetals from plasmalogens (Clarke, 1977). Fatty acid methyl esters were analysed by gas chromatography using a Pye GCD fitted with a 25 metre glass capillary coated with SP 1000 (column Technology, Newcastle), helium carrier gas at 0.6 ml min^{-1}, an all-glass inlet splitter set at 18:1 and flame ionisation detector with 12 ml min^{-1} helium make up gas. Identification of fatty acid methyl esters was by co-injection with standards (where available), semilog plot graphical techniques and hydrogenation; identification is therefore not definitive. Fatty acid data are expressed as percentage total uncorrected recorder response; peaks being quantified by the peak height × retention

0302-8933/78/0119/0153/$01.00

method. In all cases were the column separated structural isomers (mainly acids 16:1, 18:1 and 20:1), these were summed to give total value.

Dry weight determinations of the cells and extracted lipids are as described by Kates (1972).

Results and Discussion

The response of *Chlorella emersonii* to the stresses of freezing and thawing is partly determined by the growth rate before freezing (Table 1). Suboptimal concentrations of nitrate, phosphate and calcium in the growth medium reduced the cell yield and increased the recovery rate upon thawing. When cell growth was limited by magnesium there was no observed increase in tolerance to freezing injury. In parallel with the induction of freezing resistance, there was an increase in the proportion of cellular lipid as expressed on a dry weight basis. TLC of total extracted lipid indicated that this was due to an accumulation of polarlipid (Fig. 1).

The phospholipid fatty acid composition of control cells is presented in Table 2. The average number of double bonds per fatty acid molecule was 2.22. Incubation at 4° C was not found to alter significantly the mean molecular weight, though the average number of double bonds increased to a value of 2.44 per molecule. Following cold acclimatization there were alterations to the relative proportions of all fatty acids, but the most significant effect was an increase in the values of 16:4 and 18:3 acids. With cells grown under limiting concentrations of nitrate or from the late stationary phase of culture, the degree of fatty acid unsaturation increased to mean values of 2.29 and 2.42 double bonds per fatty acid molecule. This was again due to increase in the 16:4 and 18:3 fatty acid concentrations. A similar increase in the degree of unsaturation of membrane fatty acids following reduced growth rates has been reported for *Pseudomonas fluorescens* (Gill, 1975).

Following freezing and thawing of control cells (recovery 0.1%) the composition of the phospholipid fatty acids was modified, with an increase in the degree of saturation to 1.95 double bonds per molecule. A release of free fatty acids was also observed following freezing and thawing (Fig. 1). These findings are consistent with the activation of intracellular phospholipases as has been described following freezing and thawing of higher plant cells (Yoshida, 1974; Yoshida and Sakai, 1974).

With cells grown under sub-optimal conditions the maximal recovery following freezing to and thawing from −196° C was observed at rates of cooling faster than 10° C min^{-1} (Fig. 2). At slower rates the recovery approaches that of control cells. A similar relationship between cell survival and the rate of cooling has been observed for *C. emersonii* following cold acclimatization (Morris, 1976b). According to the 'two-factor'

Table 1. Effect of nutrient limitation on the response of *Chlorella emersonii* to rapid cooling (212° C min^{-1}) to −196° C

Limiting nutrient[a]	No cells ml^{-1} [b]	Recovery following freezing and thawing (%)	Dry weight lipid (%)
Control	6.4×10^6	<0.1	11.8
Nitrate	7.2×10^5	54.9	58.4
Phosphate	2.4×10^5	34.3	31.2
Calcium	3.2×10^5	3.7	12.1
Magnesium	2.6×10^5	<0.1	9.4

[a] Nutritional factors were at a concentration of 1% of control values
[b] After 14 days at 20° C. The initial cell concentration was 6.8×10^3 ml^{-1}

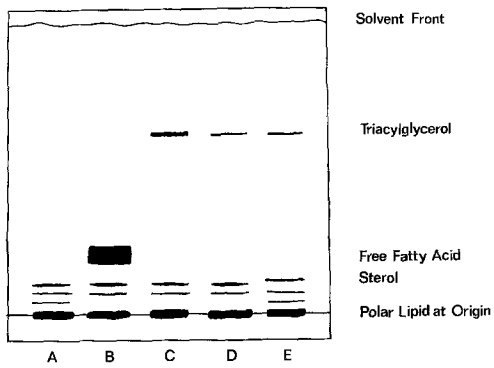

Fig. 1 A–E. Depiction of TLC of *Chlorella emersonii* lipids (150 µg) as described in the methods. Lipid extracts were from **A** control, **B** control frozen to and thawed from −196° C, **C** nitrate limited culture, **D** senescent culture (35 days at 20° C) or **E** cold acclimatized culture (21 days at 4° C)

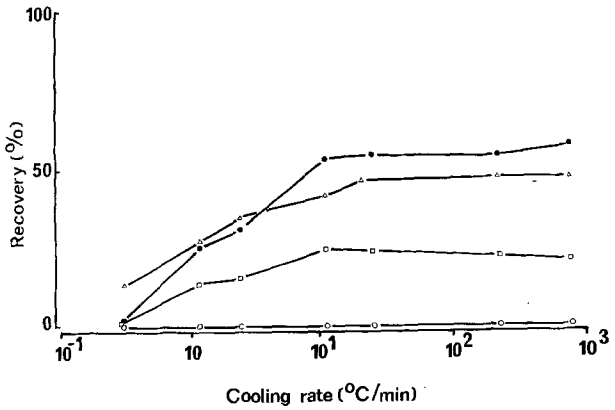

Fig. 2. Recovery (%) of *Chlorella emersonii* after cooling at different rates to −196° C. Cells were from cultures grown in Bolds medium for 14 days (○) or 35 days (●) or for 14 days in phosphate limited (□) or nitrate limited (△) Bolds medium

Table 2. Phospholipid fatty acid composition (% total uncorrected recorder response) of *C. emersonii* following different treatments

Acid	a	b	c	d	e
12:0	–	0.28	–	–	–
13:0	–	0.06	–	–	–
14:0	0.28	0.89	0.12	0.19	0.13
15:0	0.20	0.54	0.33	0.12	0.16
16:0	15.24	15.20	11.79	16.37	15.53
16:1	2.19	3.26	1.71	1.71	0.85
16:2	3.70	3.69	2.50	1.91	0.57
16:3	11.79	8.81	5.66	7.31	6.21
16:4	13.86	10.93	17.51	17.32	20.64
17:0	0.56	0.65	1.04	0.72	0.66
18:0	0.29	1.23	0.13	0.62	0.55
18:1	10.38	11.86	8.52	9.24	9.45
18:2	11.63	10.23	13.16	9.71	6.16
18:3	19.11	13.93	23.63	23.16	31.11
18:4	6.86	6.96	9.78	7.58	5.60
19:0	1.27	2.19	1.52	0.74	0.68
20:0	1.73	4.05	1.95	1.85	1.07
20:1	0.27	1.88	0.33	0.41	–
20:2	0.33	0.45	–	0.52	–
20:4	–	1.09	–	–	–
Unknowns	0.31	1.82	0.52	0.32	0.63
Mean mol. wt.	265.50	267.68	267.32	265.97	265.60
db/mol	2.22	1.95	2.42	2.29	2.44

Cells were from cultures maintained at 20° C for a) 14 days, b) 14 days then frozen to and thawed from −196° C, c) 35 days, d) in nitrate limited medium for 14 days, and e) at 4° C for 14 days; db/mol = mean number of double bonds per molecule of fatty acid; – = not detected

Table 3. Response of 60 strains of *Chlorella* to freezing and thawing

Species[a]	Recovery following freezing and thawing (%)		Presence of a vacuole[b]
	<10%	>60%	
C. emersonii	8	0	+
C. luteoviridis	9	0	+
C. kessleri	2	0	+
C. saccharophila	7	0	+
C. vulgaris f. *tertia*	3	1	∓
C. vulgaris var *vulgaris*	5	10	∓
C. protothecoides	0	15	–

[a] According to Kessler (1976)
[b] The presence of a large vacuole, visible by light microscopy, was taken from Fott and Nováková (1969)

theory of freezing injury (reviewed by Mazur et al., 1972), cells are damaged-at slow rates of cooling by alterations in the properties of solutions induced by extracellular ice formation, whilst at rapid cooling rates, cells are killed by intracellular ice formation and the effects of its subsequent recrystalization during warming. The cellular adaptions associated with reduced growth rates thus do not increase the resistance to the mechanism(s) of injury occuring at slow cooling rates but either reduce the probability of intracellular ice formation or render the cell less susceptable to its potentially damaging effects.

With the four species of *Chlorella* that contain a large vacuole the recovery following rapid cooling to −196° C was less than 10% and in most cases below 0.1% (Table 3). The presence of a vacuole in *C. vulgaris* is strain specific (Fott and Nováková, 1969) and some strains (11/19) survived freezing and thawing with a recovery greater than 60%. *C. protothecoides* does not have a prominent vacuole and all strains examined (15) were resistant to freezing injury. From these results it appears that the response of *Chlorella* to the stress of rapid cooling is determined by the presence or absence of a large vacuole. This is supported by the findings that the formation of a vacuole within *C. protothecoides* (Morris et al., 1977) and plant suspension cultures (Nag and Street, 1975) is correlated with a reduction in cellular freezing tolerance. During a damaging freeze-thaw cycle there is an activation of phospholipases (Yoshida, 1974; Yoshida and Sakai, 1974), and the localization of these enzymes within plant vacuoles (Matile, 1975) indicates that a loss of vacuole integrity is a primary effect of freezing injury. The mechanism of this injury would be associated with the high probability of ice formation in a large aqueous vacuole and the effects of its subsequent recrystalization and melting during warming.

Following growth in Bold's medium, cells from young cultures of *C. emersonii* have a large vacuole and are sensitive to freezing injury. However, following a period of reduced metabolism induced by limitation of nutrients or senescence an accumulation of lipid occurs and the cells become more freezing tolerant (Table 1). In other strains of algae lipid storage induced by nitrate limitation (Guerin-Dumartrait et al., 1970; Pyliotis et al., 1975) and senescence (McLean, 1968) reduces the size of the vacuole. It is therefore suggested that the cryoprotective effects of reduced metabolic rates are due to an accumulation of lipid resulting in a reduction of vacuole size, rather than specific alterations in the degree of unsaturation of the membrane fatty acids. This is supported by the fact that there is a direct correlation between the cellular lipid content and freezing tolerance, whereas no such relationship exists between cell survival and fatty acid composition. Limitation of nitrate induced the highest degree of freezing tolerance and lipid accumulation, with only minor alterations in the degree of unsaturation of the membrane fatty acids. It is also suggested that the role of low temperatures in inducing freezing tolerance in *Chlorella* is an effect of reduced metabolic rate resulting

in lipid accumulation rather than an increase in the degree of unsaturation of the membrane fatty acids induced by low temperatures. Other workers (Singh et al., 1975) have also suggested that the factor responsible for cold hardening of plant cells is accumulation of lipids rather than specific alterations in the composition of the membrane fatty acids.

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Received May 16, 1978