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Studies on Lipid Changes During the Development of Frost Hardiness in *Chlorella ellipsoidea**

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Chlorella ellipsoidea cells at an intermediate stage in the ripening phase of the cell cycle were hardened at 3°C for 48 hr. Lipid changes during the development of frost hardiness were measured. Phospholipids and glycolipids increased from 0.4 μmoles to 1.2 μmoles/10^9 cells and from 0.9 μmoles to 1.6 μmoles/10^9 cells, respectively. The ratio of lipid (mg)/protein (mg) in hardened cells was about 1.4 times that in unhardened cells. These results suggest that the algal cells change into more lipid-enriched state during the development of frost hardiness and that considerable change in lipid metabolism is involved in the development of the algal hardiness. Moreover, the ratios of individual phospholipids to monogalactosyl diglyceride tended to increase during the hardening process. Non-polar lipid which remarkably increased with an increase in frost hardiness was detected. According to studies on inhibitory effect of oligomycin or 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea on the increase of frost hardiness, the non-polar lipid was always synthesized whenever the algal cells developed the frost hardiness. These results suggest the existence of an intimate correlation between the content of the non-polar lipid and frost hardiness and the importance of the non-polar lipid in lipid metabolism associated with the hardiness change in *Chlorella*.

INTRODUCTION

Many biochemical studies have indicated that various cells in higher plants undergo profound lipid changes associated with the frost hardiness. Quantity of phospholipids (Siminovitch *et* al., 1968; Yoshida and Sakai, 1973) and increase in unsaturation of fatty acids (Kuiper, 1970; de la Roche *et al.*, 1972) during hardening have been studied intensively. However, little information is available

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ABBREVIATIONS

MGDG : monogalactosyl diglyceride ; DGDG: digalactosyl diglyceride ; PE: phosphatidyl ethanolamine ; PG: phosphatidyl glycerol; PC: phosphatidyl choline ; SL sulfolipid ; TG: triglyceride; DG: diglyceride; MG: monoglyceride ; FFA: free fatty acid; UK: unknown non-polar lipid; β-Car.: p-carotene; Phe.: pheophytin ; Chl. A: chlorophyll a; Chl. B: chlorophyll b; Lut.: lutein ; OGM: oligomycin; DCMU: 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea

on the transition of lipid metabolism during hardening.

Previous studies have demonstrated that *Chlorella ellipsoidea* Gerneck (IAM C-27) is a hardy plant which can be acclimated by exposure to low temperature (Hatano *et al., 1976* a, b). It has been suggested that the hardening mechanism in the algal cells is similar to that in higher plants (Hatano et al., 1976 b).The unicellular green alga is a convenient material to study the lipid metabolism associated with the hardening process. We first measured changes of lipid classes associated with frost hardiness.

This paper describes changes in amounts of glycolipids and phospholipids, and that of non-polar lipid which remarkably increases with increase of the algal hardiness.

MATERIALS AND METHODS

Plant material

The strain of *Chlorella ellipsoidea* Gerneck used was the same as that used in the preceding study (Hatano et *al., 1976* a). Methods of the culture (at 25°C, under 9-10 kilolux, with 1 % CO_2–air) and synchronization of algal cells (under a 28–hr light — 14-hr dark regime) have been described previously (Hatano *et al.,* 1976a). Since the cells were most hardened at the L_2 stage (an intermediate stage in the ripening phase of the cell cycle), L_2 cells were used in this study.

Hardening

Algal cells synchronized at 25°C were directly hardened at 3°C for *48* hr. During treatment, the culture was kept under a light intensity of 9-10 kilolux and aerated with air containing about 1 % CO_2, unless otherwise stated. The inhibitors or glucose added to the culture during hardening were removed prior to freezing by repeated centrifugations as reported previously (Hatano *et al.,* 1976b). The control culture, without inhibitors or glucose, was subjected to the same procedures.

Freezing and thawing

Freeze-thawing of the cells was performed as described previously (Hatano et al., **1976** a). Five milliliters of the culture in the sterilized test tube, which was set aslant to decrease supercooling, was cooled in an air blast freezer at -20°C for 20 hr. The frozen specimen was thawed in a bath kept at 25°C. The cooling and thawing rates, represented by the time required to change the temperature between 10°C and −10°C, were about 41 min and 57 sec, respectively.

Determination of viability

The viability of algal cells was determined with the growth curve on the basis of A_{420}. Previous study (Hatano *et al.,* 1976 a, b) has demonstrated that the viability determined with the growth curve coincided with that determined by both colony count and packed cell volume.

Lipid extraction and analysis

Lipids were extracted from *Chlorella* cells as outlined by Bligh and Dyer (1959).

The cells harvested by centrifugation were added to hot methanol and boiled for 5 min (Paula and Heath, 1970). This extraction was repeated once more. After cooling and centrifuging, the residue was reextracted with chloroform-methanol (2: 1, v/v). After centrifuging, the combined lipid extracts were washed by water to remove non-lipid contaminants. The chloroform fraction was concentrated by use of a rotary evaporator. Thin-layer chromatography (TLC) plates were prepared with Silica Gel H and activated for 2 hr at 110°C. The plates were chromatographed successively according to the method of Hirayama and Matsuda (1972) with two different solvent systems. The first solvent was a mixture of chloroform/methanol/acetic acid/water (70/20/2/2, v/v). The second solvent was a mixture of n-hexane/diethyl ether/acetic acid (90/10/l, v/v). The band lipids were identified by spray of various color reagents; α-naphtol-5 % H_2SO_4 for monogalactosyl diglyceride (MGDG) and digalactosyl diglyceride (DGDG), ninhydrin for phosphatidyl ethanolamine (PE), Dragendorff's reagent for phosphatidyl choline (PC), Dittmer reagent for phoshatidyl glycerol (PG) and 50 % H_2SO_4 for all lipid components. Furthermore, the band lipids were identified by comparison with standard lipids. Pigments were separated with n-hexane/diethyl ether/acetone (60/30/20, v/v) as a developing solvent and identified by the methods of Hirayama and Suzuki (1968). The quantification of lipid classes was achieved by TLC scanner of Shimadzu CS-900 type. Phospholipids and glycolipids were determined according to the method of Shibuya et *al.* (1967) and that of Dubois et *al.* (1956), respectively.

Packed cell volume was measured with a hematocrit and dry weight was measured with lyophilized cells.

RESULTS AND DISCUSSION

Changes in packed cell volume and dry weight during the hardening process at 3°C for 48 hr were determined. As Fig. 1-A shows, packed cell volume increased from 0.42 ml to 0.64 ml/liter during the hardening process. Similarly dry weight increased from 17.5 mg to 26 mg/10^9 cells. Fig. 1-B shows that protein content in dry weight changed from 29 % to 31% during the hardening process while lipid content in dry weight changed from 9 % to 13. 6 %. Therefore, the ratio of lipid (mg)/protein (mg) in hardened cells is about 1.4 times that in unhardened cells. This result suggests that the algal cells change into more lipid-enriched state during the development of frost hardiness and that considarable change in lipid metabolism is involved in the development of the algal hardiness.

Total lipids and pigments extracted from the algal cells at L_2 stage were separated by TLC and identified (Fig. 2). B-carotene (P-Car.), chlorophyll a (Chl. A), chlorophyll b (Chl. B), pheophytin (Phe.) and lutein (Lut.) were identified as pigment components. MGDG, DGDG, PE, PG, PC and sulfolipid

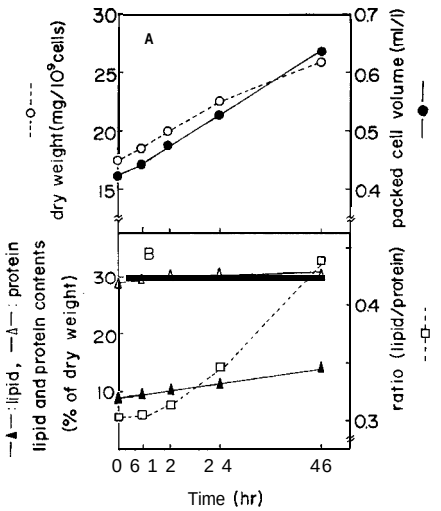

Fig. 1. Changes in packed cell volume, dry weight, lipid and protein contents during the hardening process.

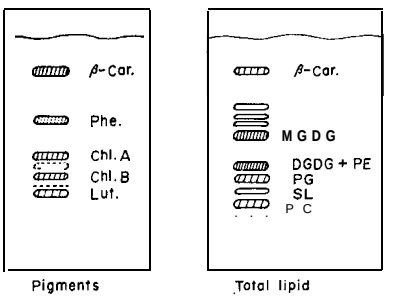

Fig. 2. Thin-layer chromatograms of total lipids and pigments extracted from ***Chlorella ellipsoidea.***
Total lipids were separated on the basis of two different developing solvent systems. The first solvent was a mixture of chloroform/methanol/acetic acid/water (70/20/2/2, v/v>. The second solvent was a mixture of n-hexane/ether/acetic acid (90/10/1, v/v). Pigments were separated with n-hexane/ether/acetone (60/30/20, v/v) as a developing solvent.

(SL) were identified as main lipid components. Yoshida and Suzuki (1973) have suggested that an interconversion between phospholipids and triglyceride was necessary to develop the frost hardiness in cortical cells from poplar stem. However, triglyceride was not identified in the algal cells.

Changes in amounts of phospholipids and glycolipids were measured during the hardening process. As shown in Table 1, glycolipids and phospholipids increased from 0.9 μmoles to 1.6 μmoles/10⁹cells and from 0.4 μmoles to 1.2 μmoles/10⁹cells, respectively. Several papers have reported the augmentation of phospholipids associated with the development of frost hardiness in higher plants. The augmentation of phospholipids during hardening was appar-

ent in ***Chlorella*** cells (Table 1). Siminovitch et ***al.*** (1968) and de la Roche et al. (1972) concluded, from the results in black locust bark and winter wheat, respectively, that there was the augmentation of phospholipids during frost hardiness but not any preferential synthesis of individual phospholipids. Yoshida and Sakai (1973) have reported that the augmentation of phospholipids during frost hardening of poplar bark was due to an increase in PC and PE. Therefore, changes of lipid composition during the hardening process were determined in ***Chlorella*** (Table 2). The ratios of individual lipid components to MGDG increased during the hardening process. Especially, the ratio of the unknown non-polar lipid (UK), which was indicated on TLC of Fig. 3, to MGDG increased among the lipids tested. Therefore, we studied the inhibitory effects of various antimetabolites on the increase of frost hardiness in order to reveal an interrelation between the unknown non-polar lipid content and the hardiness increase.,

Table 1. Changes in amount of glycolipids and phospholipids during the hardening process.

	Hardening time (hr)				
	0	6	12	24	48
Glycolipids (μmoles/10^9 cells)	0.9	1.0	1.1	1.2	1.6
Phospholipids (μmoles/10^9 cells)	0.4	0.5	0.6	0.7	1.2

Table 2. Changes in lipid composition during the hardening process.

Lipid component	Hardening time (hr)				
	0	6	12	24	48
PC	2.8	2.8	3.5 0.9	2.9	4.4
SL	1.2	0.8	5.4	1.0	2.4
PG	2.8	3.6 6.7	6.8	5.4	5.5
DGDG+PE				7	11
MGDG	10	10	10'	10	10
UK	4.7	5.3	10	11	19

The value shows the ratio of lipid component to monogalactosyl diglyceride calculated as 10.

Fig. 3 shows TLC of non-polar lipids extracted from the algal cells treated with oligomycin (OGM) or 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). A previous study (Hatano ***et al.,*** 1978) revealed that OGM and DCMU remarkably inhibited the development of frost hardiness. It has been reported that OGM and DCMU specifically inhibit energy coupling mechanism in mitochondria (Lardy et al., 1964) and the electron transport system in chloroplasts (Izawa and Good., 1965), respectively. The viability of the algal cells was determined with the growth curve on the basis of A,,,. The algal hardiness did not develop under the conditions of light-glucose-DCMU (under light with glucose and DCMU) and dark-glucose-OGM, while developed under the conditions of dark-

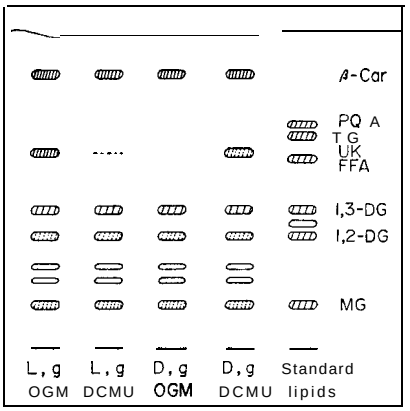

Fig. 3 Thin-layer chromatogram of non-polar lipids extracted from *Chlorella ellipsoidea* treated with OGM or DCMU.
Non-polar lipids were separated with n-hexane/ether/acetic acid (90/10/1, v/v). The algal cells were hardened in presence of OGM (5×10^{-5}M) or DCMU (5×10^{-6}M) in the light (L) or dark (D) with 0.1% glucose (g).

glucose-DCMLJ and light-glucose-OGM. The unknown non-polar lipid was not synthesized whenever the algal cells did not develop the frost hardiness. These results suggest that the unknown non-polar lipid is very important in the lipid metabolism associated with the hardening process.

Furthermore, the augmentation of lipid-body-like particles were observed with electron microscope in the algal cells during the hardening process (Hatano, 1978). Pomeroy and Siminovitch. (1971) have indicated that seasonal change of lipid bodies were related to the seasonal cycle of frost resistance. A parallel relationship existed between the increase of lipid-body-like particles in electronograph and the unknown non-polar lipid in TLC during the hardening process. We infer, therefore, that the unknown non-polar lipid playes a very important role in lipid metabolism associated with the hardiness change in *Chlorella*.

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