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Chilling-Susceptibility of the Blue-Green Alga *Anacystis nidulans*[1]

III. LIPID PHASE OF CYTOPLASMIC MEMBRANE

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ABSTRACT

The lipid phase of cytoplasmic membrane was studied by freeze-fracture electron microscopy in the chilling-susceptible blue-green alga, *Anacystis nidulans*. At growth temperatures, intramembrane particles were distributed at random in the fracture faces of cytoplasmic membrane, whereas, at chilling temperatures, the fracture faces were composed of particle-free and particle-containing regions. These findings indicate that lipids of the cytoplasmic membrane were in the liquid-crystalline state at the growth temperatures and in the phase-separation state at the chilling temperatures. Temperatures for the onset of phase separation were 5 and 16°C in cells grown at 28 and 38°C, respectively.

In comparison, another blue-green alga, *Anabaena variabilis*, which is not susceptible to chilling, was also examined by the freeze-fracture electron microscopy. The intramembrane particles were distributed at random in the fracture faces of cytoplasmic membrane at the growth, as well as at the chilling, temperatures.

The results in this and previous studies suggest that the chilling susceptibility of *A. nidulans* is a result of irreversible leakage of ions from the cytoplasm when the lipids of cytoplasmic membrane are in the phase-separation state at low temperatures.

In previous papers (11, 12), we described detailed studies on temperature dependence of chilling-induced phenomena in the blue-green alga (Cyanobacterium), *Anacystis nidulans*. Midpoint values for critical temperatures of the inactivation of photosynthesis were 4 and 12°C in cells grown at 28 and 38°C, respectively (11). Potassium ions and free amino acids leaked to the outer medium below these temperatures (12). Nevertheless, the temperatures were about 10°C lower than those for the onset of phase separation of lipids of the thylakoid membrane (7, 8, 10, 14). Based on these findings, we proposed a mechanism in which the lipid phase of the cytoplasmic membrane should be involved in the chilling susceptibility of this alga (12). To address this hypothesis experimentally, we need to know a precise value for temperature of the onset of phase separation of the cytoplasmic membrane.

In this respect, freeze-fracture electron microscopy is a powerful technique. It is established in various kinds of cellular membranes (5, 15) that, when the membrane lipids are in the liquid-crystalline state, the intramembrane particles are distributed at random in the fracture faces, and that, when the membrane lipids are in the phase-separation state, the particles are displaced from the domain of gel phase. This method was applied to *A. nidulans* by several investigators (1, 2, 4, 16) to supply information on the lipid phase of the cytoplasmic and thylakoid membranes. In these studies, however, growth conditions of the algal cells and temperatures of fixation were different depending on the investigators. Therefore, the temperature of the onset of phase separation was not definitely determined.

To investigate the relationship between the lipid phase of cytoplasmic membrane and the chilling-induced injury in *A. nidulans*, we fixed the cells at a number of temperatures and examined the lipid phase of the cytoplasmic membrane by means of freeze-fracture electron microscopy. The experimental result suggests that the phase-separation state of lipids of the cytoplasmic membrane induces the chilling susceptibility of this alga.

MATERIALS AND METHODS

A. nidulans and *Anabaena variabilis* strain M3 were obtained from the Algal Collection of the Institute of Applied Microbiology, University of Tokyo. The cells were photoautotrophically grown at 28 and 38°C in medium C of Kratz and Myers (6), as described previously (10, 11).

Cells at the late-logarithmic phase were harvested by centrifugation at 5,000*g* for 5 min at a temperature equal to the growth temperature. They were suspended in fresh culture medium, the temperature of which was the same as the growth temperature. The cell suspension in a test tube was immersed in a water bath of a designated temperature for 60 min. To the cell suspension was added an equal volume of 5% glutaraldehyde in 0.2 M K-phosphate (pH 7.4), the temperature of which had been adjusted to the designated temperature. In some experiments, to study reversibility of the lipid phase, the cells that were treated once at 0°C for 60 min were rewarmed to, and incubated at, the growth temperature for 30 min and then fixed as described above. The fixed cells were washed with 0.1 M K-phosphate (pH 7.4) and repeatedly suspended in increasing concentrations of glycerol solution (10, 20 and 30%, by volume). After standing for 12 h in the 30% glycerol solution, the samples were frozen by a slurry of liquid and solid Freon 22 and then transferred into liquid N_2.

The samples were fractured at −110°C with a Balzers BAF-301 (freeze-etching apparatus and examined in a Hitachi HS-9 or a JEM 100B electron microscope. For each specimen fixed at different temperatures, 100 to 150 fracture faces on five or six grids were investigated. The fracture-face nomenclature is according to Branton *et al.* (3).

The effect of chilling on photosynthesis in *Anabaena variabilis* was studied, according to the method described previously (11).

RESULTS

The distribution of intramembrane particles on the freeze-fracture faces of cytoplasmic membrane in *A. nidulans* was markedly

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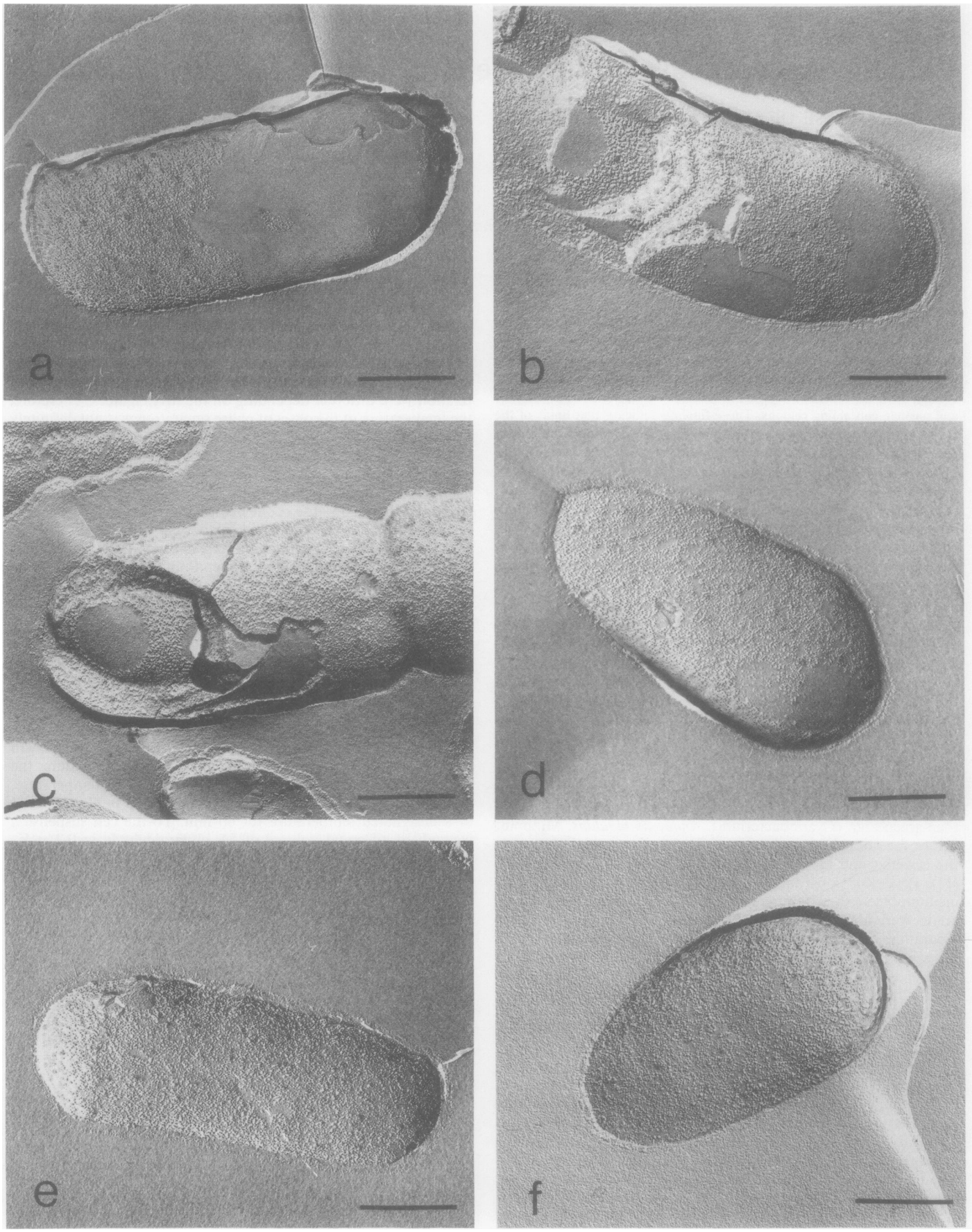

FIG. 1. Freeze-fracture electron micrographs of *A. nidulans*. Cells were grown at 38°C and fixed with glutaraldehyde at 0°C (a) 7°C (b), 13°C (c), 16°C (d), 19°C (e), and 38°C (f). Bars represent 0.5 μm.

altered depending on the temperature of fixation. Figure 1 shows the electron micrographs of fracture faces of cells grown at 38°C and fixed at various temperatures. Most of the fracture faces in the micrographs of this figure were the PF[4] of the cytoplasmic membrane. At 0 and 7°C (Fig. 1, A and B) particle-free regions were seen in almost all of the PF of the cytoplasmic membrane. This is in agreement with previous reports by other investigators (1, 2, 4, 16). At 13°C, the particle-free regions were small (Fig. 1C). At 16°C, a part of the fracture faces contained the particle-free regions (Fig. 1d), while the other part revealed a random distribution of particles. At temperatures from 19 to 38°C, there were no particle-free regions found in the PF of cytoplasmic membrane. Since the particle-free region in the fracture face is the gel phase domain (5, 15), these findings indicate that the cytoplasmic membrane is in the phase-separation state below 13°C and in the liquid-crystalline state above 19°C. In the temperature range between 13 and 19°C, the cytoplasmic membrane undergoes the phase change from the liquid-crystalline to the phase-separation state. A net-like distribution of particles, as reported by Furtado *et al.* (4), was not found at any temperature of fixation.

Under our experimental conditions, the cleavage tended to occur through the interior of the cytoplasmic membrane but not of the thylakoid membrane. Therefore, no clear result was obtained for the temperature dependence of lipid phase of the thylakoid membrane. At low temperatures, however, the thylakoid membrane was occasionally fractured, as seen in Figure 1C. It was obvious there that the thylakoid membrane was in the phase-separation state at 13°C.

The freeze-fracture faces of cytoplasmic membrane in cells grown at 28°C were similarly studied. In these cells, the particle-free regions were seen only when they were fixed below 8°C. This indicates that temperatures for the onset of phase separation are lower in cells grown at 28°C than they are in cells grown at 38°C.

The EF of the cytoplasmic membrane in *A. nidulans* were also studied. The particle density of EF was much lower than that of PF. This is consistent with previously reported results (1, 2). Particle-free regions were seen below 13°C, and the particles were distributed at random above 19°C in cells grown at 38°C.

In the previous paper (12), we proposed that the primary process of chilling susceptibility is an occurrence of the phase separation in the cytoplasmic membrane. By this mechanism, the formation of a domain of gel phase (*i.e.* one particle-free region) in the cytoplasmic membrane of a cell should be critical for the chilling susceptibility. We examined 100 to 150 fracture faces at each fixation temperature to obtain proportions of fracture faces having the particle-free regions and of fracture faces showing the random distribution of intramembrane particles. In this investigation, fracture faces equal to about 50% of the total area of the cytoplasmic membrane were subjected to the examination. In Figure 2, the proportion of the fracture faces having the particle-free region is plotted against the temperature of fixation. No distinct difference between the PF and EF was found in the occurrence of particle-free regions. This fact indicates that both of the inner and outer layers of the cytoplasmic membrane responded to the temperature in similar manners.

It should be pointed out that the proportion of fracture faces having particle-free regions, as presented in Figure 2, is not directly related to the temperature dependence of the lipid phase. Even if the particle-free region did not exist on the fracture face, it could occur on the nonfractured part of the cytoplasmic membrane. To obtain the temperature dependence of the lipid phase of cytoplasmic membrane, we assumed that the particle-free regions occurred in the fractured and in the nonfractured faces in the same probabilities. Thus, the proportion of cytoplasmic membrane in the liquid-crystalline state was calculated as the square

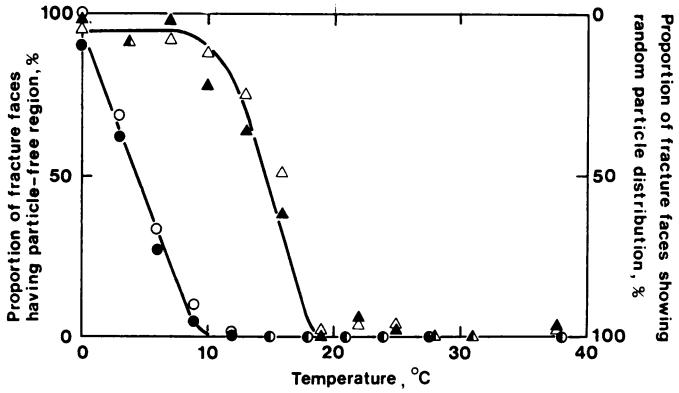

FIG. 2. Dependence on the fixation temperature of the proportion of fracture faces having intramembrane particle-free region in the cytoplasmic membrane in *A. nidulans*. PF of the cells grown at 28°C (○) and 38°C (△) and EF of the cells grown at 28°C (●) and 38°C (▲) are presented. Each point is a mean value of 100 to 150 fracture faces.

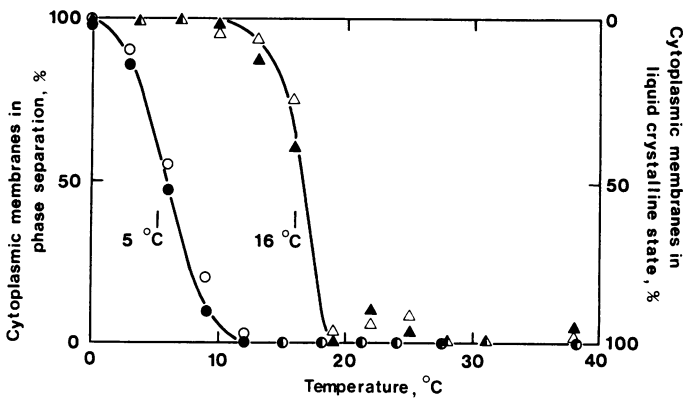

FIG. 3. Dependence on the fixation temperature of the proportion of the cytoplasmic membranes in the phase-separation state in *A. nidulans*. A proportion of liquid crystalline state was calculated as a square of the proportion of fracture faces showing random distribution of intramembrane particles. Symbols are the same as those in Figure 2.

of the proportion of fracture faces showing the random particle distribution. Figure 3 shows that the transition from the liquid-crystalline to the phase-separation state occurs in narrow temperature ranges. Temperatures for the transition midpoints were 5 and 16°C in cells grown at 28 and 38°C, respectively.

Reversibility of the particle distribution in the fracture face of the cytoplasmic membrane was studied in *A. nidulans*. When the cells grown at 38°C were first treated at 0°C for 60 min and then at 38°C for 30 min, the particle distribution was nearly random. This fact suggests that the cytoplasmic membrane, once in the phase-separation state at 0°C, reverses to the liquid-crystalline state at the high temperature. A similar reversibility of the particle distribution has been reported by Armond and Staehelin (1).

The freeze-fracture electron microscopy was applied to another blue-green alga, *Anabaena variabilis*. We first studied the effect of chilling on the activity of photosynthetic O_2 evolution in cells grown at 38°C. No chilling-induced damage was found after the algal cells were treated at 0°C for 60 min. This fact indicates that this alga is not susceptible to chilling. Figure 4 shows electron micrographs of PF of the cytoplasmic membrane of cells grown at 38°C and fixed at 0 and 38°C. The fracture faces revealed random distribution of the intramembrane particles, irrespective of the fixation temperature. This observation suggests that the cytoplasmic membrane of *Anabaena variabilis* is in the liquid-crystalline state at temperatures as low as 0°C.

[4] Abbreviations: PF, protoplasmic face(s); EF, exoplasmic face(s).

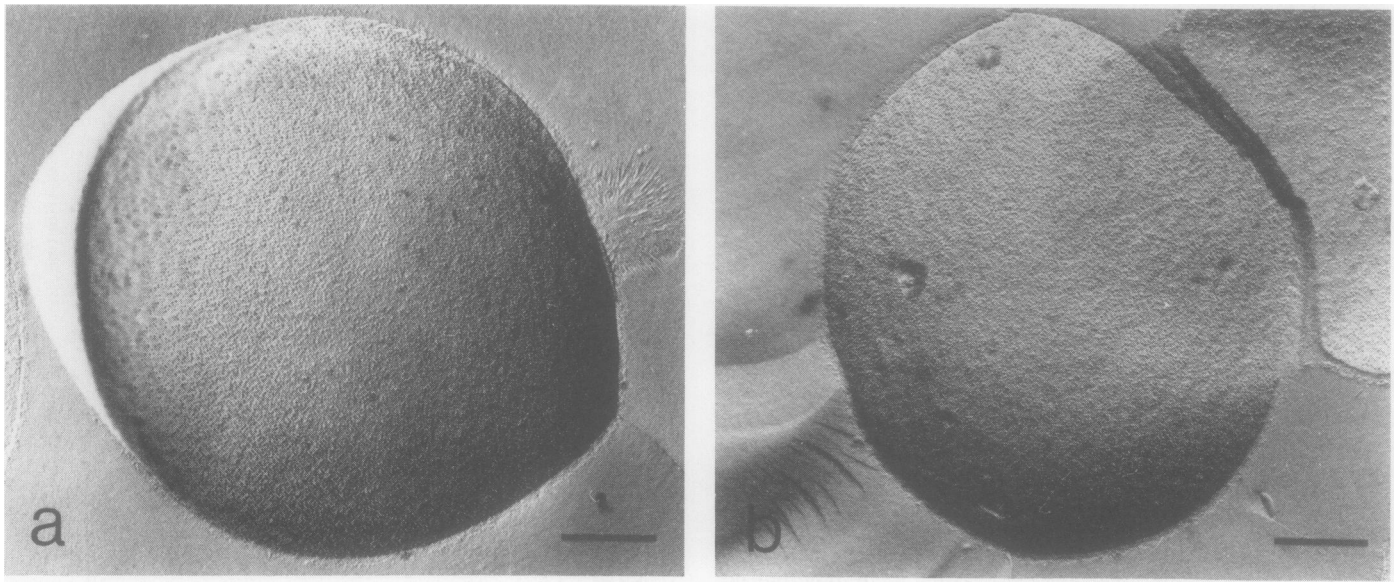

FIG. 4. Freeze-fracture electron micrographs of the cytoplasmic membrane of *Anabaena variabilis*. Cells grown at 38°C were fixed at 0°C (a) and 38°C (b). Bars represent 0.5 μm.

Table I. *Temperatures for the Onset of Phase Separation of the Cytoplasmic Membrane and for the Beginning of Chilling-Induced Phenomena in A. nidulans*

	Temperature	
	38°C-Grown	28°C-Grown
	°C	
Onset of phase separation of cytoplasmic membrane	16	5
Inactivation of photosynthetic O_2 evolution[a]	15	5
Inactivation of the Hill reaction[a]	15	5
Spectral shift of carotenoids[a]	15	5
K^+ leakage[b]	17	7
Amino acid leakage[b]	17	7

[a] Data taken from Reference 11.
[b] Data taken from Reference 12.

DISCUSSION

The study on the freeze-fracture electron microscopy indicates that the cytoplasmic membrane of *A. nidulans* undergoes the thermotrophic phase change from the liquid-crystalline to the phase-separation state at 5 and 16°C in cells grown at 28 and 38°C, respectively. In Table I, these temperatures are compared with temperatures for the beginning of the chilling-induced phenomena, which were investigated in the first two of this series of studies (11, 12). The inactivation of photosynthesis and the Hill reaction, the spectral shift of carotenoids, and the K^+ and amino-acid leakage all begin to appear at temperatures very close to the onset of phase separation of cytoplasmic membrane. The thylakoid membrane, on the other hand, undergoes the thermotrophic phase change at about a 10°C higher temperature than does the cytoplasmic membrane (7, 8, 10, 14). These findings suggest that the chilling-induced phenomena are closely related to the onset of phase separation of the cytoplasmic membrane but not of the thylakoid membrane. In summarizing the study in this series (including Refs. 11 and 12), we conclude that the chilling susceptibility of *A. nidulans* occurs in a mechanism as follows. First, the primary response which occurs at chilling temperatures is the formation of gel-phase domain in the cytoplasmic membrane, and, in the phase-separation state, the membrane becomes passively permeable to small molecules. Second, the ions and solutes having low mol wt leak from the cytoplasm to the outer medium. Finally, decreases in the concentrations of the ions and solutes in the cytoplasm inactivate the physiological activities of the algal cells. It should be noticed here that the ions and solutes are irreversibly lost from the cells, even if the cytoplasmic membrane regains the state of liquid crystal when the once-chilled cells are rewarmed. This should be the reason why the chilling-induced injury in *A. nidulans* is an irreversible process (11, 12).

In *Anabaena variabilis*, the temperature for the onset of phase separation of the thylakoid membrane lipids is 13°C when the cells are grown at 38°C (10). However, this alga is not susceptible to chilling. As shown in Figure 4, the cytoplasmic membrane was in the liquid-crystalline state at 0°C. This observation suggests that the phase separation of the cytoplasmic membrane, not of the thylakoid membrane, is responsible for the chilling susceptibility of the blue-green algal cells.

In a study by Armond and Staehelin (1) on freeze-fracture electron microscopy, the cytoplasmic membrane was in the phase-separation state at 21°C in *A. nidulans* grown at 38°C, indicating that the onset of phase separation should occur at a temperature higher than that in our case. The discrepancy probably originates from a difference in the growth conditions of the algal cells. In our recent thermal analysis data (T.-A. Ono, N. Murata, and T. Fujita, unpublished), the temperature for the onset of phase separation increased with aging of the culture. We suspect that Armond and Staehelin (1) used a relatively aged culture of *A. nidulans*.

The lipid phase of the thylakoid membrane could not be fully investigated by the freeze-fracture electron microscopy, since the membrane was not well fractured at high temperatures. However, judging from the fracture faces fixed at low temperatures (Fig. 1C), the temperature for the onset of phase separation is higher in the thylakoid membrane than it is in the cytoplasmic membrane. This is consistent with our findings that the temperatures for the onset of phase separation of the thylakoid membrane are about 15 and 25°C in cells grown at 28 and 38°C, respectively (7, 8, 10, 14).

As shown in the present study, the thermotrophic phase behavior of the cytoplasmic membrane depended on the growth temperature of the algal cells. Our previous study (9, 13) indicates that the content of saturated fatty acids decreases, and that of

unsaturated ones increases, when the growth temperature is lowered. It is known that introduction of double bonds in the fatty acids of lipids decreases the temperature of the thermotrophic phase transition of the membranes. Therefore, the effect of growth temperature on the phase behavior of the membrane lipids of *A. nidulans* can be explained by the variation of the fatty acid composition with the growth temperature.

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