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Cold Shock Syndrome in *Anacystis nidulans*[1]

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ABSTRACT

The phenomenon of cold shock in *Anacystis nidulans* has been explored further in terms of loss of viability and immediate and subsequent metabolic effects. Cold shock was observed also in two closely related strains in which unsaturated fatty acid contents are also known to be low and temperature-dependent. Loss of viability was maximum for cells grown at temperatures above 40 C ($<10^{-4}$ survivors after 5 min at 0 C) but became negligibly small for cells grown below 34 C. Development of the cold-sensitive condition after transfer 25 → 39 C was slow and comparable to rate of growth; development of the insensitive condition after transfer 39 → 25 C was rapid, implying rapid *in situ* alteration. An immediate metabolic effect, observed as a decrease in rate of photosynthetic O_2 evolution measured at growth temperature, was less severe than loss of viability. Continued light incubation under growth conditions led to slow decay in rate of O_2 evolution accompanied by loss of membrane chlorophyll. The multiple effects which comprise the cold shock syndrome appear to be membrane-related phenomena and thereby provide an experimental probe of normal membrane function.

Anacystis nidulans is a blue-green alga distinguished by wide use as an experimental organism. It is distinguished also by an unusual sensitivity to low temperature when it has been grown at higher temperatures. The sensitivity is manifest in reversible phase transitions attributed to its membrane lipids (13) and, more dramatically, in irreversible cellular damage following short exposures to low temperatures (0-10 C) (4, 8, 9).

The cold sensitivity is an impediment to preparation of active subcellular preparations. In fact, it was first recognized in early attempts to prepare cell fragments with Hill activity (4). However, the cold sensitivity also provides a unique experimental approach to the study of membrane function and already has been used as an experimental tool (13, 16). Three particular features of *A. nidulans* are pertinent. First, it (and closely related strains (18), contain zero or at most, very small amounts of polyunsaturated lipid. Second, storage lipid is not significant (3), and thylakoid lipid composition presumably is close to that of whole cells. Third, cold sensitivity is developed only at higher temperatures (>35 C) and is not evident in cells grown at 25 C which thereby provide internal controls. With these thoughts in mind, we have examined a number of the irreversible effects of low temperatures which, together with other reports (4, 8, 9), provide a description of the cold shock syndrome.

MATERIALS AND METHODS

The alga used was strain Tx20 of this laboratory, originally described as *A. nidulans* (11). It is taken to be identical to strain 6301 of the Berkeley collection in which it is redescribed as a *Synechococcus* (18). The control culture was grown in continuous culture under 240 w of tungsten illumination at 39 C with aeration by 1% CO_2 in air to maintain a cell concentration of about 2 μl/ml and specific growth rate of about 1.5 days^{-1}. The control medium was compounded from three solutions to give the final μM concentrations: major salts – 1,000 $MgSO_4$, 10,000 KNO_3, 106 $Ca(NO_3)_2$, 2,000 NaCl; buffer – 2,000 K-phosphate, 2,600 $KHCO_3$; microelements – 14.4 Fe N-hydroxyethylethylenediaminetetraacetate, 9.1 $MnCl_2$, 3.1 $ZnSO_4$, 4.2 MoO_3, 1.6 $CuSO_4$, 1.7 $Co(NO_3)_2$, 0.4 NH_4VO_3, 65 EDTA. The microelement solution was sterilized by filtration, the other solutions by autoclaving. Before compounding, the buffer solution was aerated with pure CO_2 to lower the pH. The final solution gave a pH of 7.1 under 1% CO_2 in air and pH of 7.4 in the steady-state culture. For smaller batch cultures, subjected to various temperatures, we used C_s medium in test tube cultures (19) held in water baths or in an aluminum temperature gradient block provided with windows; a standard illumination (about 1.5 mw/cm^2) was provided by warm white fluorescent lamps. Batch cultures were harvested during exponential growth as checked by monitoring optical density *versus* time (11). There were no differences in cold sensitivity attributable to the medium, culture configuration, or source of illumination used.

Exposure to low temperature was accomplished by a standard chilling procedure designed for rapidity and reproducibility. Harvested cell suspension in original medium contained in a 20 mm i.d. Pyrex test tube was aerated by 1% CO_2 in air via a 16 mm o.d. bubbling tube and immersed in a 4-liter darkened and stirred Dewar flask (water + ice for O C). With adequate stirring, cooling of the cell suspension was reproducible with $t_{0.5}$ = 22 sec and reached 0.1 C above bath temperature within 3 min.

Viable cell count was determined as number of colony-forming units by a procedure adapted from Van Baalen (22). Plates containing 20 ml of C_s medium + 1.5% agar were inoculated with aliquots of diluted cell suspension mixed with a 2-ml overlay of medium + 1% agar which had been held < 45 C. Plates were sealed with cellophane tape. Colonies were counted under a 10× microscope after 4 to 5 days incubation at 30 ± 2 C and 50 cm below banks of 40 w tungsten lamps.

We have also used a simpler method of observing loss of viability in terms of extent of growth delay. An exponential growth curve (log A *versus* time) for a culture containing 10% survivors shows a delay of log A = 1 in reaching some chosen value of A. The method is simple for semiquantitative estimation and can be made quantitative with sufficient attention to detail (17).

Metabolic O_2 exchange was measured by calibrated Clark-type electrodes. One arrangement was a YSI electrode in a 2-ml cuvette irradiated by tungsten illumination filtered by 2.5 cm of 0.02 M $CuSO_4$ to give 45 mw/cm^2. Other measurements were made with an electrode cuvette irradiated at various intensities of 620 nm light (14).

Chlorophyll was estimated in 80% acetone extracts using an absorption coefficient of 82 cm^2/mg at 663 nm. Whole cell

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absorption spectra (corrected for scattering at 720 nm) were obtained with a Cary 14 spectrophotometer using 3 mm Rohm and Haas No. 7328 translucent Plexiglas plates immersed in 10-mm cuvettes.

RESULTS

Viability. Typical killing curves were exponential with time at low temperature as shown in Figure 1 for 0 C. Further, the curves extrapolated to values close to 100% survivors at zero time except under extreme conditions. For the very steep curves observed for growth temperature > 40 → 0 C (Fig. 1), the time for cooling to 0 C becomes an appreciable error on the time axis. For killing curves of very low slope, as observed for growth temperature 39 → 12 C (not shown), the curves extrapolated to > 100% survivors. We take the time at low temperature as a dosage and the slope of the killing curve (K sec^{-1}) a measure of sensitivity as viewed by cell viability.

Figure 2A shows sensitivity (K) at 0 C for various growth temperatures. There are three apparent transitions. For growth temperatures 25 to 34 C, cells are insensitive by our procedure although actually this means less than 10% killing or $K < 2 \times 10^{-4}$ during 600 sec. Sensitivity increases monotonously from 34 to about 39 C, then sharply to about 41 C. Except to say that maximum sensitivity is reached at about 41 C, our data become uncertain; at $K = 8 \times 10^{-2}$ sec^{-1} we actually observe < 1% survivors at 60 sec, a time at which our standard cooling procedure has reached only about 4 C. For reference, Figure 2A also shows data on specific growth rate *versus* temperature under light saturation (11). Maximum cold sensitivity and maximum specific growth rate are reached at about the same temperature (41 C). However, the transitions at about 34 and 39 C are not evident in the growth *versus* temperature curve.

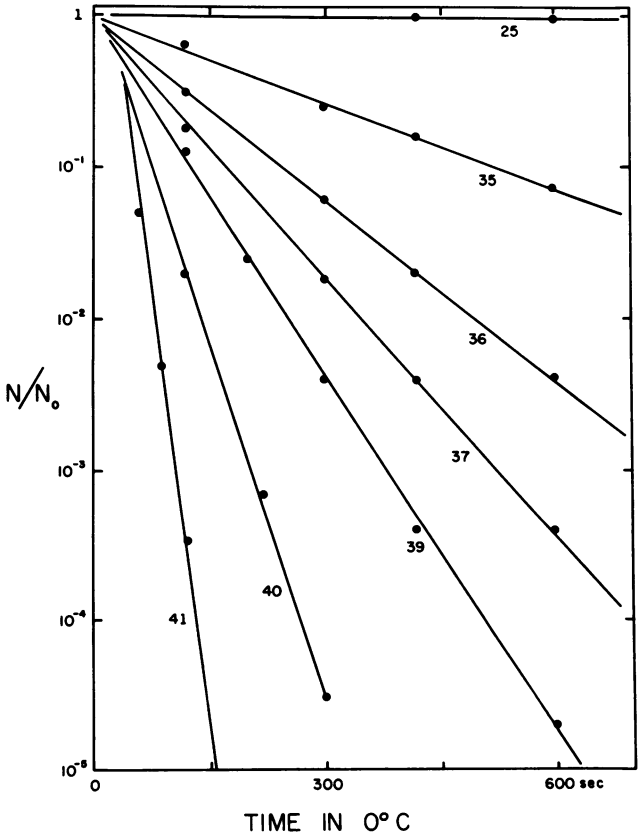

FIG. 1. Survival *versus* time in 0 C. The fraction of survivors (N/N_0) was estimated in terms of colony-forming units. Numbers on the curves describe the preceding temperature of culture.

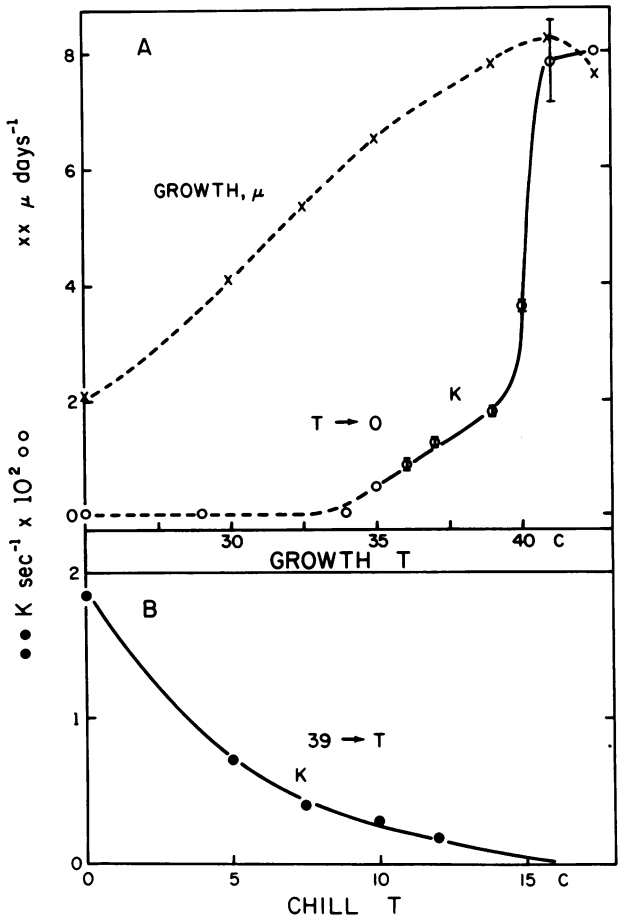

FIG. 2. Temperature effects involved in sensitivity to cold shock. Sensitivity, K sec^{-1}, is taken from survival curves as those of Figure 1. A: effects of preceding growth temperature on sensitivity to 0 C. Standard deviations are shown or are less than the size of the point except for the single datum at 42.5 C for which uncertainty is large. Also shown for reference is the specific growth rate (μ days^{-1}) at light saturation (11). B: sensitivity to chill at different temperatures for cells grown at 39 C.

Cold sensitivity is not peculiar to 0 C. Figure 2B shows that cells grown at 39 C have measurable cold sensitivity even at 12 C. To observe sensitivity >5 C it was necessary to use longer chill times to 30 min; killing at 10 min was negligible, and the killing curves extrapolated to >100% survivors at zero time.

Variations in growth temperature (Figs. 1 and 2A) necessarily included attendant variations in growth rate. We sought, therefore, to examine concomitant effects of light intensity and growth rate at one temperature (39 C). In addition to our control culture, other continuous cultures were managed at higher and lower light intensities. Sensitivity to cold shock was measured as in Figures 1 and 2A. The results (Table I) show that cells grown at high light intensity and growth rate are almost five times more sensitive than cells grown at low light intensity and growth rate. Ranges of the surviving ratio observed up to 600 sec at 0 C are given to show a peculiarity found only in cells grown at high light intensity: the killing curve was bimodal with a break to lower sensitivity observed at survival ratio 1×10^{-3}. This small heterogeneity, implying a lower sensitivity in 0.1% of the population, was the only such case found.

Figure 3 presents an experiment designed to explore the time course in transitions between the cold-sensitive condition at 39 C and the insensitive condition at 25 C. Sensitivity was estimated in terms of survival after 10 min at 0 C. At each time of observation, actual numbers of viable cells in the test tube cultures gave accompanying growth curves and indicate times at

which dilutions were made. Following transition t_1, 39 → 25 C, cold sensitivity was lost rapidly with a time constant about 2.8 hr^{-1}, about 50 times greater than the prevailing specific growth rate. Following the reverse transition t_2, 25 → 39 C, cold sensitivity was regained slowly with a time constant of about 0.18 hr^{-1}, close to that of the prevailing specific growth rate.

Immediate Metabolic Effects of Cold Shock. As previously noted (4, 9), an immediate effect of cold shock is a decrease in rate of photosynthetic O_2 evolution. Sensitivity observed in this way differed considerably from viability measured as colony-forming ability: apparent sensitivity was much lower, less consistent, and decay with chilling time was seldom logarithmic. Figure 4 shows a characteristic decay curve observed for cells in growth medium (as harvested) and observed at light saturation. Also shown is a still lower sensitivity when chilling is purposely done slowly. The per cent decrease in rate of net photosynthetic O_2 evolution was essentially independent of light intensity as observed in terms of complete light intensity curves and shown in Table IIA for two intensities.

Attempts at measurement of electron transport with substituted acceptors were only partially successful. Consistent results were obtained with quinone (Table IIB). The characteristically high quinone rate of *Anacystis* was always inhibited by cold shock but the inhibition was always somewhat less than that observed without quinone. A similar result of smaller inhibition by cold shock was seen also in some experiments measuring O_2 uptake with 2 mM methyl viologen plus 1 mM KCN (1). However, our results with methyl viologen were inconsistent, often giving rapidly decaying rates, and are not considered in detail.

Changes in Cellular and Metabolic Conditions Following Cold Shock. Cells grown at 39 C and cold-shocked for 10 min at 0 C show 30 to 50% of their photosynthetic O_2 evolution even though less than 10^{-4} of the cells can reproduce sufficiently to form colonies. Hence, a decay in metabolic rates with time is expected following cold shock. Some characteristics of the decay are shown in Figure 5. Cold shock cells held in darkness showed no decay in photosynthetic rate for up to 7 hr; for longer dark starvation there was a decay also in control cells. When challenged by light (intensity used for growth), a decay became evident only after 2 to 3 hr and proceeded slowly thereafter.

Table I. Sensitivity to Cold Shock (K) for Cells Grown at 39 C under Three Light Intensities and Specific Growth Rates (μ)

Illumination[a]	μ	K[b]	Range of N/N_o[b]
	day^{-1}	sec^{-1}	
320 W, 5 cm	3.6	0.037	$1 \rightarrow 10^{-3}$
		0.0074	$10^{-3} \rightarrow 10^{-4}$
240 W, 30 cm	1.5	0.018	$1 \rightarrow 10^{-5}$
120 W, 30 cm	0.5	0.0078	$1 \rightarrow 10^{-2}$

a) Illumination within white cabinets was multidirectional. Light intensity was not subject to inverse square law. An index of relative light intensity is provided by μ.

b) K was estimated from slopes of survival curves, log N/N_o vs time in 0 C, as in Fig. 1. Maximum time of exposure was 600 sec. Actual range of N/N_o over which K was evaluated is given.

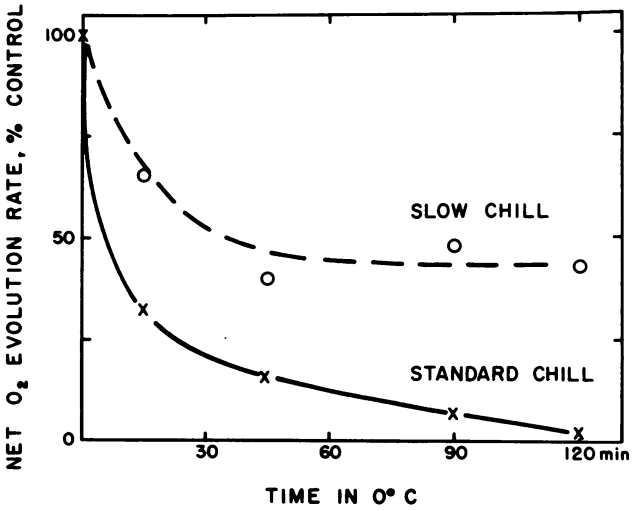

FIG. 4. Rate of photosynthesis as net O_2 evolution rate *versus* time in 0 C for cells grown and measured at 39 C. Also shown is the decay in rate resulting from a slow chill (about 1 C/min); for this curve, time is measured from time cells had reached <1 C. Intensity was 45 mw/cm² of filtered tungsten light.

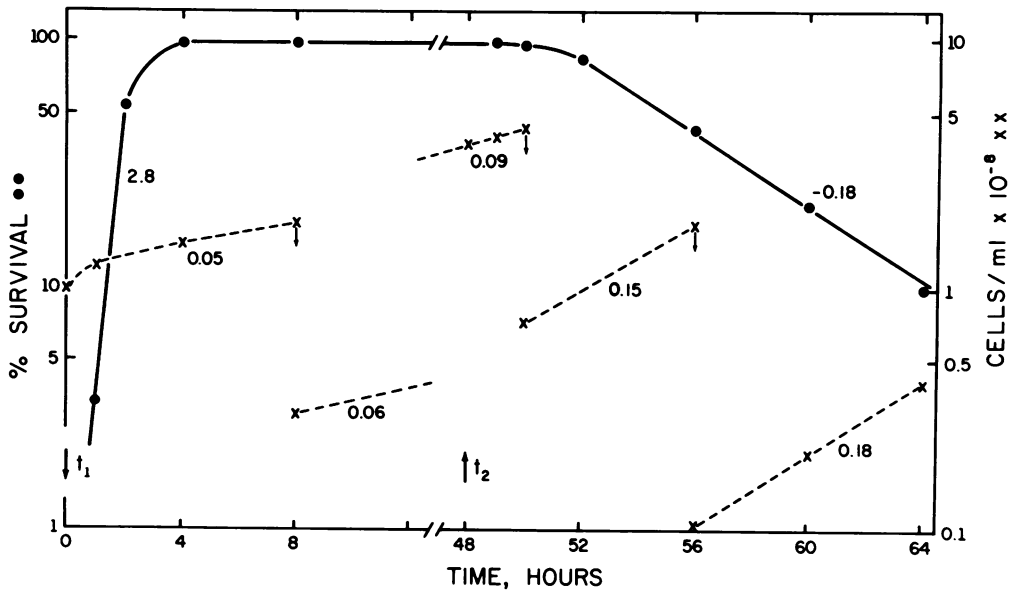

FIG. 3. Time courses for loss of cold sensitivity at 25 C and gain in cold sensitivity at 39 C. Sensitivity is measured as (decrease in) per cent survival (●●) after 10 min in 0 C. At t_1, the culture was transferred from 39 to 25 C; at t_2, it was transferred from 25 to 39 C. Cell concentrations (××) and culture dilutions (↓) are also shown. Numbers on all curves are rate constants (hr^{-1}).

Table II. Effects of Cold Shock on O_2 Evolution

For A the harvested suspension was diluted with fresh medium, 1% CO_2. For B cells were centrifuged and taken up in medium, but without trace elements or EDTA; pH 7.1 under 1% CO_2. Quinone (1 mM) was added together with 2 mM ferricyanide about 5 min before the rate measurement. Cold shock (15 min, 0 C) was given to the original harvested suspension. Rates, given as μ mols O_2/μ mol Chl·hr, were measured at 39 C.

	Addition	Lt. Int. mW/cm² 620 nm	O_2 Evolution Control Rate	Cold Shocked Rate	Cold Shocked % Control
A.	None	11.5	590	270	46
	None	1.3	340	160	47
B.	None	11.5	590	200	34
	Quinone	11.5	1330	670	50

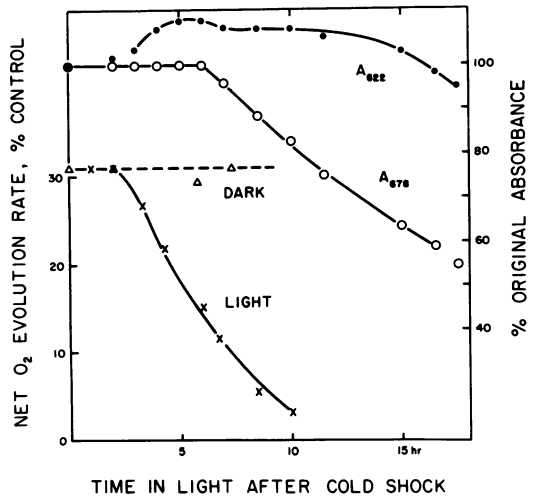

FIG. 5. Time courses of events in a culture after cold shock. A culture grown at 39 C was exposed to 0 C for 10 min and returned to 39 C in darkness or in the illuminated 39 C water bath used for growth. Rate of net O_2 evolution at 39 C (△, dark; ×, light) is given as per cent of that observed in the original culture (520 μmol O_2/μmol Chl·hr). Differential bleaching is shown in terms of A observed in whole cell spectra as per cent of those in the original culture for which $A_{622} = 0.735$ and $A_{678} = 0.535$.

Apparent O_2 evolution became negligible (< compensation) after 24 hr. In light, but not in darkness, there were attendant changes in pigmentation. After 3 to 4 hr in light, there was a characteristic differential bleaching seen first in terms of Chl (A_{678}) and only later in phycocyanin (A_{622}) in whole cell spectra. The differential bleaching was more rapid at higher light intensity: 30 min under 240 mw/cm² gave bleaching similar to that shown in Figure 5 at 18 hr.

The photochemically induced differential pigment bleaching is taken as evidence of membrane degradation in cold shock. A similar light-dependent differential bleaching (decay in Chl, followed by subsequent decay in phycocyanin) was induced also by chloramphenicol (100 μg/ml), by cetyltrimethylammonium bromide (Cetab, 5×10^{-5} M), or by longer exposure to very high light intensity (240 mw/cm²). Also occurring in all four treatments, usually before any evident loss in Chl, there was an increase in the *in vivo* fluorescence at 664 nm with 580 nm excitation (Rao and Wang, unpublished data). We interpret this as an increase in allophycocyanin fluorescence resulting from blocking of energy transfer from phycobilisomes to membrane Chl.

Comparison with other Blue-Green Algae. We have looked for the cold shock syndrome in other blue-green algae grown at 39 C. Four cultures from C. Van Baalen showed no loss of viability or subsequent differential bleaching following 10 min at 0 C: *Agmenellum quadruplicatum*, strains PR-6 and BG-1, and *Coccochloris elabens*, strains 17A and DI. Two cultures of *Synechococcus* obtained from R. Stanier showed characteristic cold shock effects. Strain 6311 was about two times more sensitive than Tx20 as estimated by viability *versus* time at 0 C. Strain 6908 did not give reliable quantitative colony formation, but showed growth delay and differential pigment bleaching following cold exposure; we judge that it is substantially less sensitive than Tx20. Strains 6311 and 6908 were chosen because they have been grouped together with 6301 (historically identical to Tx20) in strain cluster 4 of *Synechococcus* species (18).

DISCUSSION

Intent of the present work has been to explore and further describe the cold shock syndrome rather than to reach explanation in terms of molecular events. Our discussion is framed around the following hypothesis. Cold shock is a membrane-related phenomenon. In cells exposed to a low temperature, some critical event of membrane disorganization occurs at random in the population (Fig. 1). The probability of occurrence of the critical event (sensitivity) is dependent upon growth temperature (Fig. 2A).

In certain heterotrophic bacteria, a cold shock phenomenon is also demonstrable and has some of the characteristics seen in *A. nidulans:* (*a*) sensitivity requires growth at elevated temperatures (>30 C) and is not observed in cells grown at lower temperatures (2); (*b*) cells grown at higher temperatures have a lower content of unsaturated fatty acids (2); (*c*) sensitivity is greater when chilling is rapid (5); (*d*) sensitivity is greater during rapid growth (5, 20); (*e*) cold shock results in loss of various cell constitutents: UV absorbing compounds (5, 20, 21), ninhydrin-reacting substances (20, 21), and permease-accumulated substances (12).

Because of loss of cell constituents in heterotrophic bacteria, the site of damage induced by cold shock has been attributed to the outer limiting membrane. In *A. nidulans*, there is a similar loss of cell material observed in terms of pteridines (4, 9) and glutamate (4). Janz and Maclean (9) considered loss in selective permeability as the probable cause of loss in viability. They also recognized a concomitant damage to photosynthetic membranes seen in their work as a decay in photophosphorylation in cell-free extracts during incubation at 0 C. Our observations of photochemical pigment bleaching are taken as evidence that disorganization within the lamellar membranes, as well as in the outer limiting membrane, contributes to the total cold shock syndrome.

The irreversible changes seen in the cold shock syndrome may be related to the reversible phase changes occurring in lamellar membranes as observed by Murata *et al.* (13). For *A. nidulans* grown at 28 C, they observed a transition temperature at 10 to 13 C while for cells grown at 38 C the transition temperature was at 18 to 24 C. Evidently there is a still lower transition temperature at which the probability of some critical event of membrane disorganization becomes significant. There are now three correlated characteristics of *A. nidulans* with respect to the growth temperature at which its membranes are constructed: the reversible phase change (13), the content of unsaturated fatty acid (6), and sensitivity to cold shock. The reversible phase change has been studied only at two growth temperatures (38 and 28 C) and it is not clear whether it will show the same discontinuity at 34 to 35 C which is reported for decrease in unsaturated fatty acids (6) and development of sensitivity to cold shock (Fig. 2A).

The cold shock phenomenon provides a means of following what appear to be changes in cellular membranes *in situ* (Fig. 3). Development of the sensitive condition at 39 C occurs slowly at a rate comparable to the growth rate. It is tempting to suppose that development of sensitivity depends upon synthesis of new (39 C) membrane with low unsaturated fatty acid. The opposite

case of loss of sensitivity at 25 C is dramatically rapid and clearly not growth-dependent. Presumably it represents *in situ* changes which conceivably can be accomplished by desaturation of membrane lipids (15).

Our exploration of immediate and subsequent metabolic effects of cold shock obviously is incomplete. Measurements of photosynthesis were made in terms of net O_2 evolution. They are clouded by the possibility of an attendant O_2 uptake in a Mehler reaction, conceivably increasing in cold shocked cells via endogenous autooxidizable acceptors (7). An essential question is whether the primary lesion can be assigned to photophosphorylation or to electron transport. Janz and Maclean (8), using cell-free extracts in 0.3 M sucrose, found cold shock inhibition of phosphorylation but no inhibition of electron flow measured in terms of ferricyanide reduction or Cyt *c* oxidation. Our consistent observation of inhibition of O_2 evolution with quinone, using whole cells, leads us to be skeptical of a simple conclusion that electron transport is not inhibited by cold shock.

Our comparison of different species suffices only to show that cold shock is not a general phenomenon in blue-green algae. Our comparative material included two strains, PR-6 and 17A, known to be high in polyunsaturated fatty acids (10). *A. nidulans* (Tx20) and related strains 6311 and 6908, which show cold shock, contain negligible amounts of polyunsaturated acids (6, 10, 15, 18). It is tempting to use data on fatty acid contents to suggest other blue-green algal strains as prime candidates in further exploration for cold-sensitive strains. There are numerous discrepancies in analytical data reported (10), perhaps because few workers have given more than casual consideration to growth conditions (temperature and growth rate). Tx20 and related strain 6311 appear to be the most cold-sensitive microorganisms so far examined. We note from other experiments of our laboratories (unpublished) that, in terms of loss of viability of Tx20, 10 min at 0 C can be more damaging than 100 krads of x-rays.

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LITERATURE CITED

1. Chu N-H 1972 The methyl viologen-catalyzed Mehler reaction and catalase activity in blue-green algae and *Chlamydomonas reinhardi*. Biochim Biophys Acta 245: 277-287
2. Farrell J, AH Rose 1968 Cold shock in a mesophilic and psychrophilic *Pseudomonad*. J Gen Microbiol 50: 429-439
3. Fogg GE 1953 The Metabolism of Algae. Methuen and Co, London
4. Forrest HS, C Van Baalen, J Myers 1957 Occurrence of pteridines in a blue-green alga. Science 125: 699-700
5. Gorrill RH, EM McNeil 1960 The effect of cold diluent on viable count of *Pseudomonas pyocyanea*. J Gen Microbiol 22: 437-442
6. Holton RW, HH Blecker, M Onore 1964 Effect of growth temperature on the fatty acid composition of a blue-green alga. Phytochemistry 3: 595-602
7. Honeycutt RC, DW Krogman 1970 A light-dependent oxygen-reducing system from *Anabaena variabilis*. Biochim Biophys Acta 197: 267-275
8. Janz ER, FI Maclean 1972 Photosynthetic properties of extracts of *Anacystis nidulans* prepared by lysozyme digestion. Can J Microbiol 18: 1727-1731
9. Janz ER, FI Maclean 1973 The effect of cold shock on the blue-green alga *Anacystis nidulans*. Can J Microbiol 19: 381-387
10. Kenyon CN 1972 Fatty acid composition of unicellular strains of blue-green algae. J Bacteriol 109: 827-834
11. Kratz WA, J Myers 1955 Nutrition and growth of several blue-green algae. Am J Bot 42: 282-287
12. Leder IG 1972 Interrelated effects of cold shock and osmotic pressure on permeability of the *Escherichia coli* membrane to permease accumulated substrates. J Bacteriol 111: 211-219
13. Murata N, JH Troughton, DC Fork 1975 Relationships between transition of physical phase of membrane lipids and photosynthetic parameters in *Anacystis nidulans* and lettuce and spinach chloroplasts. Plant Physiol 56: 508-517
14. Myers J, JR Graham 1971 The photosynthetic unit in *Chlorella* measured by repetitive short flashes. Plant Physiol 48: 282-286
15. Nichols BW, RV Harris, AT James 1965 The lipid metabolism of algae. Biochem Biophys Res Commun 20: 256-262
16. Patterson CO, J Myers 1972 Photosynthetic production of hydrogen peroxide by *Anacystis nidulans*. Plant Physiol 51: 104-109
17. Sell JL 1960 Study of effects of low temperature on *Anacystis nidulans*. MA thesis. Univ of Texas, Austin
18. Stanier, RY, R Kunisawa, M Mandel, G Cohen-Bazire 1971 Purification and properties of blue-green algae. Bacteriol Rev 35: 171-205
19. Stevens CLR, J Myers 1976 Characterization of pigment mutants in a bluegreen alga, *Anacystis nidulans*. J Phycol 12: 99-105
20. Strange RE, FA Dark 1962 Effect of chilling on *Aerobacter aerogenes* in aqueous suspension. J Gen Microbiol 29: 719-730
21. Strange RE, AG Ness 1963 Effect of chilling on bacteria in aqueous suspension. Nature 197: 819
22. Van Baalen C 1967 Further observations on growth of single cells of coccoid blue-green algae. J Phycol 3: 154-157