APPLIED MICROBIOLOGY, Jan. 1969, p. 169-175
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Vol. 17, No. 1
Printed in U.S.A.

Effect of Cold Temperatures on the Viability of *Chromobacterium violaceum*

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Received for publication 18 October 1968

The effect of low, nonfreezing temperatures on the viability of five strains of *Chromobacterium violaceum* was studied. The viability of cultures grown at 30 C was determined after exposure to various diluents held at 0 to 2 C. A culture diluted at its growth temperature served as the control. Cells of strain N were most sensitive in the early part of the exponential phase of growth. Cells of strains 252 and 341 were most sensitive in the late exponential, early stationary phase of growth. Cells of strain 9 showed greatest loss of viability during the maximal stationary phase. Strain 69 was completely resistant throughout its growth cycle to cold injury. Cell viability was much greater in buffered salts solution than in distilled water, broth, or physiological saline, whether cultures were diluted at room temperature or in the cold. The proportion of cells surviving after exposure to cold, however, was the same regardless of the composition of the diluent. Loss of viability was progressive at 0 to 2 C and reached a maximum after 2 hr. There was no loss of viability of cells exposed to 20 C, but there was some loss at 12 C. Strain 341 cultivated at 15 C was much less sensitive to 0 to 2 C than when it was cultivated at 30 C. The composition of the growth medium seemed to have no effect on the survival of cells exposed to cold. The polyamines, spermine and trimethylenediamine, as well as erythritol and sucrose, exerted some protective action against the effects of cold but not uniformly for all strains studied.

In his monograph on the genus *Chromobacterium*, Sneath (15) described the difficulties involved in keeping the organisms alive. He pointed out that "some cultures survive for weeks at 37 C but die in a week or two at 4 C." We had similar experiences in our laboratory, especially in trying to maintain newly isolated cultures of *C. violaceum*. The practice of refrigeration of stock cultures until their next transfer had to be abandoned since even brief exposure to cold resulted in an essentially "sterile" culture.

It has been recognized for some time that gram-negative bacteria can be killed by exposure of young cells to nonfreezing, cold temperatures. Hegarty and Weeks (7) and Meynell (11), working with strains of *Escherichia coli*, confirmed earlier reports (13, 14) that sudden chilling was necessary to elicit this type of temperature-dependent cell death. The term "cold shock" has come into common use in describing this phenomenon (5, 8), mainly because several workers (6, 17) found that *sudden* exposure to low temperatures was necessary but also generally agreed that the phenomenon was most easily demonstrated when using young, exponential-phase cells.

Preliminary experiments carried out on several strains of *C. violaceum* showed that neither sudden exposure of cells to cold nor exponential-phase cells were necessarily required for demonstration of injury due to cold.

MATERIALS AND METHODS

Organisms. The organisms selected for this study were maintained as laboratory stock cultures on a medium containing Difco Peptone, 0.5% (w/v); glucose, 1% (w/v); and agar, 1.5% (w/v). Five strains of *C. violaceum* were studied. Strains N, 9, and 69 were isolated in this laboratory; strains 252 and 341 were kindly supplied by P. H. A. Sneath; *E. coli* KH and *Pseudomonas fluorescens* were laboratory stock cultures.

Experimental cultures. Peptone-glucose broth was used in most experiments. The medium contained Peptone (Difco), 5 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; and 1,000 ml of distilled water. The *p*H was adjusted to 7.0 before autoclaving. Sterile glucose solution was added aseptically to give a final concentration of 1% (w/v). When a solid medium was required, agar (Difco) was added to give a final concentration of 1.5% (w/v).

Glutamate-mineral salts medium was used in a few

experiments. It contained L-glutamic acid, 4.413 g; KH_2PO_4, 1.36 g; $Na_2HPO_4 \cdot 7H_2O$, 2.13 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; and $FeCl_3 \cdot 6H_2O$, 0.005 g. The *p*H was adjusted to 7.0.

Erlenmeyer flasks (125 ml) containing 25 ml of broth were inoculated from agar slant cultures and incubated overnight at 30 C on a rotary shaker. A 0.3-ml amount of the culture was inoculated into a flask of fresh medium and incubated for specified time periods. After various experimental treatments, cultures were diluted and plated on the surface of agar with a sterile glass spreader. Each dilution was plated at least in triplicate. Plate counts were subjected to statistical analysis using Student's "*t*" test (16).

Cold treatment of bacterial cultures. Volumes (1 ml) of an experimental culture were removed periodically and pipetted into dilution fluids maintained in water baths adjusted to one of the following temperatures: 0 to 2 C, 15 C, 20 C, or 30 C. The suspensions were held for various periods of time, diluted further, and surface-plated on peptone-glucose-agar. A culture diluted at 30 C and plated immediately served as an untreated control.

Diluents studied. Dilution fluids evaluated in relation to sensitivity of cells to cold were: distilled water peptone-glucose broth medium, physiological saline (0.85%, w/v, NaCl in distilled water), and a modification of a complex salts buffer of Monod (12). The salts buffer contained the following (per liter of distilled water): KH_2PO_4, 13.6 g; Na_2SO_4, 2.0 g; $MgSO_4$, 0.2 g; $Ca(NO_3)_2$, 0.01 g; and $FeSO_4$, 0.0005 g. The *p*H was adjusted to 7.0 with 9.0 ml of a 10 N solution of KOH.

In one series of experiments, the following substances were added individually to distilled water in the final concentrations indicated and were used as the dilution fluid in which cold treatment was carried out. (Room temperature controls were also done in connection with these experiments.) Glucose, sucrose, and erythritol were used in concentrations ranging from 0.05 to 0.3 M; trimethylenediamine · 2HCl and spermine (Mann Research Labs, N.Y.) were used in concentrations ranging from 10^{-3} to 10^{-5} M.

RESULTS

The culture exposed to cold had a consistently lower plate count than did the room temperature control. Cultures 4 to 8 hr of age were particularly sensitive to cold (Fig. 1). In the 1st hr after inoculation, the plate count of the room temperature control decreased by 7% of the initial population.

Since young, 4-hr cultures of strain N had exhibited a rather dramatic sensitivity to cold (Fig. 1), the young cultures of the other *C. violaceum* strains and cultures of *E. coli* and *P. fluorescens* were exposed to similar conditions. The loss of cell viability was most pronounced in young cultures of *C. violaceum* strains N, 252, and 341, whereas cells of strains 9 and 69 were resistant to the cold diluent, inasmuch as plate counts of cold-treated and room temperature controls were almost identical (Table 1).

E. coli and *P. fluorescens* showed survivals of only 44 and 69%, respectively.

With increasing age, the sensitivity of cells of strain 9 to cold temperature increased considerably. Cells taken from a culture in the early part of the maximal stationary phase displayed a 13% loss in viability but, after 48 hr, when the culture was well into the maximal stationary phase of growth, there was an 87% loss in cell viability (Fig. 2).

Strain 252 had the greatest number of cold-sensitive cells during the maximal stationary phase (Table 2). Strain 341 showed greatest sensitivity during the late exponential or early maximal stationary phase, whereas the cells of strain 69 were completely resistant to the cold diluent at all culture ages examined.

Rate of cell death in cold diluent. Young cells of strain N held at 0 C in distilled water lost viability gradually, with no significant change in the viable count until after 30 min (Fig. 3). After 60 min,

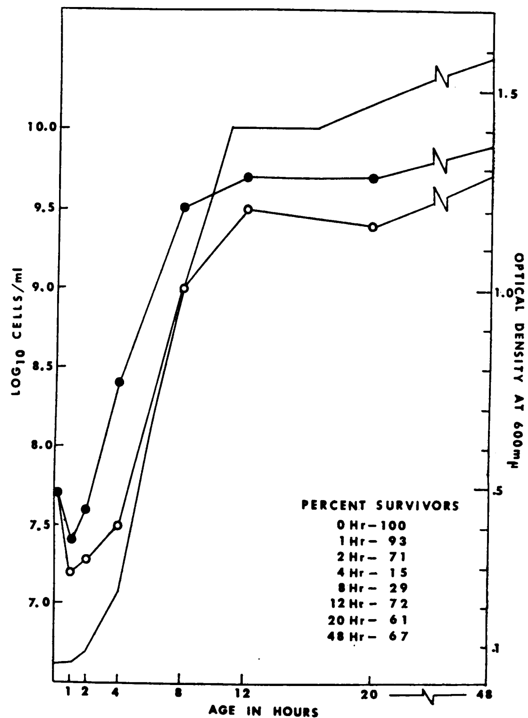

FIG. 1. *Relationship of cell age to viability in cells of C. violaceum strain N exposed to 0 to 2 C for 2 hr. The uninterrupted line represents optical density measurements taken during normal growth of the parent culture at 30 C. Symbols: (●) viable count of the parent culture at the specified hours; (○) viable count of the culture after exposure for 2 hr in the cold.*

TABLE 1. *Sensitivity of young cultures of bacterial aerobes to cold*[a]

Organism	OD[b]	DF[c]	No. of cells/ml[d]		"t" value[e]	Per cent killed
			0 hr (room temp ± SD)	2 hr (OC ± SD)		
C. violaceum N	0.255	3	320 ± 3	108 ± 6	6.0	67
C. violaceum 341	0.143	3	138 ± 7	86 ± 3	13	38
C. violaceum 69	0.181	3	198 ± 6	215 ± 8	3.3	0
C. violaceum 252	0.230	3	305 ± 5	166 ± 3	46	46
C. violaceum 9	0.224	3	299 ± 2	334 ± 4	16	0
E. coli	0.096	3	22 ± 3	10 ± 3	5.0	56
P. fluorescens	0.190	4	209 ± 6	130 ± 3	23	38

[a] All cultures were 3 to 5 hr old.
[b] Optical density at 600 nm.
[c] Degrees of freedom, the plate number of each set less one.
[d] Each result and its standard deviation (SD) must be multiplied by the dilution factor, 10^6.
[e] Critical value for t at the 5% level, 3 DF is 3.182; 4 DF is 2.776; critical values for t at the 1% level, 3 DF is 5.841, and 4 DF is 4.604.

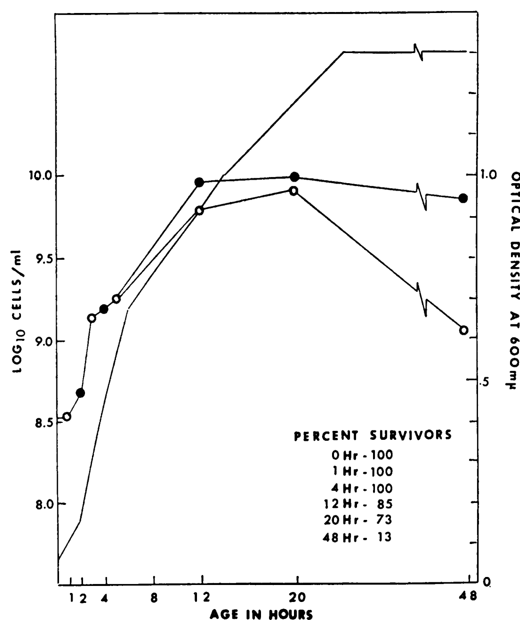

FIG. 2. *Relationship of cell age to viability in cells of C. violaceum strain 9 exposed to 0 to 2 C for 2 hr. The uninterrupted line represents optical density measurements taken during normal growth of the parent culture at 30 C. Symbols: (●) viable count of the parent culture at the specified hours; (○) viable count after samples taken from the parent culture were held 2 hr in the cold.*

the survival rate was 72%; after 120 min, 52% of the cells had been killed. The last plating, done after 24 hr of holding at 0 to 2 C, showed 36% survivors, which was only 14% fewer than the percentage of survivors at 2 hr. The upper curve in Fig. 3 shows that holding cells in distilled water at room temperature permitted cell growth to continue, although certainly at a different rate than in undiluted cultures.

Influence of magnitude of temperature shift on survival. Cultures of strain N, growing at 30 C, were diluted into distilled water maintained at 0, 12, and 30 C, held for 2 hr, and diluted further; plate counts were then performed. A comparison of the number of survivors (Fig. 4) showed that the greatest loss of viability occurred when the temperature "shift" was from 30 to 0 C. Cells from 4-hr cultures were most sensitive, but even cells of cultures 24 hr old suffered some loss of viability when "shifted" to the 0 C temperature. This confirmed an earlier finding that "shifts" from 30 to 20 C or from 30 to 12 C, while causing some cell death especially in cultures of 2 and 4 hr, were clearly less damaging than "shifts" to 0 C.

Effect of composition of dilution fluid on cell death at 0 to 2 C. Broth cultures of strain N were pipetted into several diluents held at 0 to 2 C and at room temperature. The diluents used were distilled water, physiological saline (0.85%), Monod's buffer, and peptone-glucose growth medium. Essentially the same percentage of surviving organisms was obtained with each diluent used. However, dilution in Monod's buffer at room temperature gave a viable count twice that found when distilled water or peptone broth were used as diluting fluid at room temperature (Table 3). These results suggest that diluents are not without effect on cell viability, even though they were ineffective in protecting cells from the effects of cold temperature, since the percentage of cells killed at 0 to 2 C was the same in all dilution fluids used. Sucrose, in various concentrations,

TABLE 2. *Effect of cold treatment on three strains of C. violaceum*[a]

Strain	Temp of growth	OD at 600 mm	Age	Initial no. of viable bacteria/ml[b]	Percentage of survival[c]	Estimated growth phase
	C		hr			
341	30	0.143	4	14.20	60	Lag
		0.720	16	100.00	12	Late exponential
		1.000	24	92.00	14	MSP[d]
		2.000	48	0.77	43	Death
252	30	0.260	4	13.4	46	Lag
		0.824	16	66.0	25	Late exponential
		1.000	24	104.0	14	MSP
		2.000	48	0.33	33	Death
69	30	0.071	4	1.9	100	Lag
		0.237	16	7.7	100	Exponential-MSP
		0.301	24	0.05	100	MSP
		1.000	48	0.006	100	Death

[a] Broth cultures of each strain were cold-treated at the specified times and portions from serial dilutions of the resulting suspensions were surface-spread on a total of 10 peptone-glucose-agar plates.

[b] Number of bacteria per ml as obtained from colony counts of the five plates spread from the 0-hr room temperature control suspensions. Each result must be multiplied by 10^8.

[c] Percentage of organisms surviving the 2-hr exposure to cold.

[d] Maximal stationary phase.

does not protect strain N against cold, but it was quite effective in the protection of strain 252 and 341 (Table 4). Erythritol and the polyamines, trimethylenediamine-hydrochloride and spermine, gave partial protection.

When the cultures were diluted and held at room temperature rather than at 0 to 2 C, the viable count was invariably the same or higher after 2 hr than at zero time, except when sucrose solution was the diluent. Sucrose, in concentrations of 0.05, 0.1, 0.2, and 0.3 M, gave survival values of 100, 57, 11, and less than 1%, respectively.

Effect of growth medium on survival of cells diluted in the cold. Strain N was grown on glutamate-salts medium which was also used as a diluent and, after the addition of agar, as a plating medium. The percentage of glutamate-grown cells surviving the cold teatment was compared with results using PG medium. In both cases, there were losses in cell viability due to exposure to cold. The glutamate-grown cells seemed to show a greater loss in cell viability than did the peptone-glucose-grown cells, but, when the Student's "*t*" test was applied to the numerical results, it was found that the difference between the two survival values was significant only to the 50% level. In other words, the difference in count could have occurred by chance, so that statistically significant differences were not discernible.

Influence of gradual elevation and lowering of temperature on cell survival. Strain N cultures grown at 30 C for 4 hr were diluted in distilled water at 0 to 2 C; then, over a period of 2 hr, the temperature was raised to 30 C. The survival rate was 93%. A control held for 2 hr at 2 C showed only 40% survivors. As a parallel experiment, the temperature of the culture diluted at room temperature was lowered stepwise to 0 to 2 C; 47% of the cells survived.

These results were not surprising in view of the data in Fig. 3 and 4, which indicate that the killing effect of cold is not immediate and that temperatures between 30 and 0 C are intermediate in their injurious properties.

Relationship of growth temperature on sensitivity of cells to dilution in the cold. Cultures of *C. violaceum* strain 341 grown at 15 C were less sensitive to cold treatment than were the same cultures grown at 30 C (Table 5). Both exponential-phase cultures had similar optical densities and therefore probably were of similar physiological age, although the 30 C culture had been incubated for 16 hr and the 15 C culture for 72 hr.

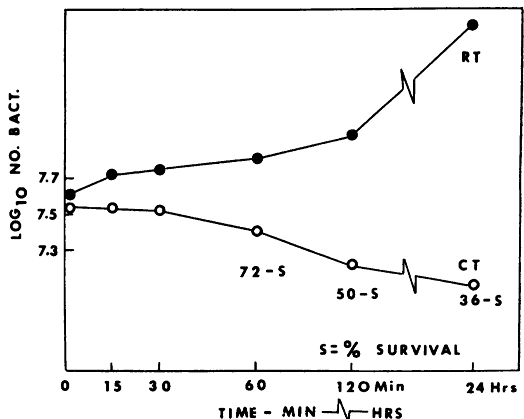

FIG. 3. *Influence of the duration of the holding time on the viability of C. violaceum strain N cells exposed to cold. Symbols: (●) viable population in a sample of cells held at room temperature for 24 hr; (○) surviving population in a similar sample of cells held at 0 to 2 C for 24 hr. Both cell samples were taken from a parent culture incubated at 30 C for 4 hr and were maintained, without shaking, in sterile distilled water dilution blanks throughout the test period.*

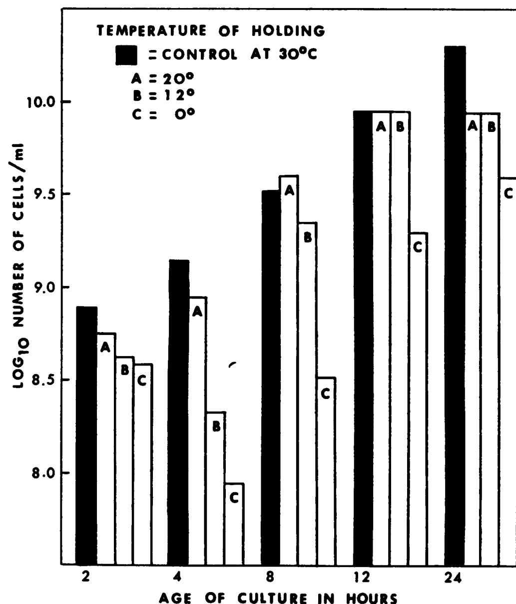

FIG. 4. *Determination of the downward temperature shift most effective in causing death to cells of C. violaceum strain N. Solid bars: test culture grown at 30 C. Samples of the test culture were shifted to 20 C (A), 12 C (B), and 0 C (C) and held (2 hr) at specified times in their growth cycle.*

TABLE 3. *Effect of the composition of the diluent on the survival of C. violaceum strain 341 exposed to cold*

Diluent	No. of cells/ml after[a]		Percentage of survival
	0 hr (room temp)	2 hr (0 C)	
			%
Distilled water.....	1.24×10^8	0.68×10^8	54
Peptone-glucose broth, 1.5%......	1.48×10^8	0.76×10^8	51
Buffer[b].............	2.95×10^8	1.55×10^8	52
Saline (0.85%)......	2.86×10^8	1.44×10^8	50

[a] Microscopic examination showed cells were not clumped before or after cold treatment in any diluent.

[b] Modification of Monod buffer (phosphates and salts, *p*H 7.0).

TABLE 4. *Protective action of various substances against death[a] of cells in the cold*

Strain	Diluent	Percentage of survival
N	Distilled water	50
	plus	
	Sucrose, 0.05 M	20
	Sucrose, 0.10 M	2
	Sucrose, 0.20 M	2
	Sucrose, 0.30 M	2
	Erythritol, 0.04 M	50
	Erythritol, 0.08 M	65
	Erythritol, 0.10 M	82
	Erythritol, 0.20 M	80
	Erythritol, 0.30 M	82
	Trimethylenediamine-hydrochloride, 10^{-3}	100
	Trimethylenediamine-hydrochloride, 10^{-5}	81
	Spermine, 10^{-3}	87
	Spermine, 10^{-5}	70
252	Distilled water	50
	plus	
	Sucrose, 0.3 M	100
341	Distilled water	59
	plus	
	Sucrose, 0.3 M	100

[a] Substances were dissolved in distilled water in the concentrations shown. The result of a dilution and plating of the culture at room temperature was taken at 100%.

TABLE 5. *Effect of the temperature of growth on the survival of C. violaceum strain 341 cells exposed to cold*

Expt	Temp of growth	Age	OD at 600 mm	No. of cells/ml after		Percentage of survival
				0 hr (room temp)	2 hr (0 C)	
	C	hr				%
I	30	16	0.80	64×10^8	15×10^8	23
II	15	72	0.70	24×10^8	21×10^8	89
III	15	72	0.65	23×10^8	21×10^8	91
IV	15	96	0.82	34×10^8	40×10^8	100

DISCUSSION

Experiments relating to the adverse effects of suboptimal, nonfreezing temperature on the viability of bacteria have been reported for many years in the bacteriological literature. Sherman and Cameron (14) pointed out, in their experiments with *E. coli*, that abrupt temperature shifts and other environmental changes proved lethal to "physiologically young" cells from the late lag and early exponential phase of growth. Similar studies with *P. pyocyanea* by Gorrill and McNeil (6) and *Aerobacter aerogenes* by Strange and Dark (17) also described the unusual sensitivity of exponential-phase cells to cold. Abrupt temperature changes were employed in all these experiments and the term "cold shock" came into common use.

Results reported here show that cells from

young cultures of some strains of *C. violaceum* were resistant to cold, whereas cells from older cultures of these strains were quite sensitive. In still other cases, cells from cultures of any age were completely insensitive to cold. The inconsistencies observed regarding cold sensitivity in relation to age among strains of chromobacteria suggested the desirability of reevaluating the phenomenon to more clearly define the mechanism of cell death.

The exact meaning of "physiological youth" is not clear, but the expenditure of cellular energies for young, exponential-phase bacterial cells is in the direction of cell division; therefore, the construction and maintenance of membranes and walls, for example, might be temporarily abandoned, leaving young cells vulnerable to the influences of sudden environmental changes. Umbreit (18) pointed out that the processes involved in cell division are sensitive to harmful environmental influences and, since cell division is a predominant part of the early stages of growth, young exponential-phase cells are presumably more sensitive than older ones. Cyclical fluctuations in the susceptibility of exponential-phase cells of *E. coli* to cold were attributed by Hegarty and Weeks (7) to a partial synchronization of cell division, since each synchronous division was accompanied by a greater cell loss than was observed between divisions.

The late lag- and early exponential-phase cells of strain N were most sensitive to cold (Fig. 1), but strain 341 displayed maximal cold sensitivity during the late exponential phase of growth (Table 2). Maximal stationary-phase cells of strain 9 (Fig. 2) and 252 (Table 2) were most affected by cold. *C. violaceum* strain 69 showed no sensitivity to cold at any part of its growth cycle. Houghtby and Liston (Bacteriol. Proc., p. 146, 1964) found extreme variability in cold sensitivity for various cultures of *E. coli* and *Salmonella*, but such variability was a matter of extent of cell death induced in exponential-phase cells. Cells from the lag and stationary phases of growth were virtually insensitive.

It is possible that variability in the susceptibility of cells of *C. violaceum* to cold may reflect differences in their optimal temperature of growth. Strain N has an optimum in a range of 25 to 30 C and displays typical "early log-phase" sensitivity to cold, whereas strains 9, 341, and 252 have growth optima in the 30 to 37 C temperature range and cold-sensitive cells apppear in these strains in the late exponential and maximal stationary phases of the growth cycle. *C. violaceum* strain 69, although capable of growth at 30 C, grows optimally at 20 to 25 C.

The rate of cooling *Chromobacterium* cells was not as significant in causing cell death as was the length of time the cells were held in the cold (Fig. 1). Gradual loss in cell viability suggests the disappearance of some essential component required for cell division, rather than a massive cell injury, as the cause of death. Strange and Dark (17) made similar observations on the exponential-phase cell of *A. aerogenes*. Cells plated immediately after chilling were completely viable, but death was progressive over a period of 60 min at 0 C. These observations contrast sharply, however, with the earlier results of Hegarty and Weeks (7) who found that 90% of the viable exponential-phase cells of *E. coli* were instantly destroyed by dilution in the cold.

Figure 3 shows that susceptible cells of chromobacteria must be held at 0 to 2 C for at least 30 to 40 min before injurious effects of cold can be realized. When the temperature was lowered to 0 to 2 C gradually, taking less than 2 hr, a larger number of cells survived. When the procedure was reversed, that is, the culture grown at 30 C, diluted at 2 C, then the temperature raised in steps to room temperature, 93% of the population survived. At least two explanations seem reasonable: (i) cells held in the cold suffer membrane damage and lose some cell materials necessary for survival, and (ii) the damage and losses cannot be repaired and replaced as long as the temperature is below that which would permit rapid respiration and synthesis.

When cells of *C. violaceum* strain 341 were grown at a temperature of 30 C and then exposed to 0 to 2 C for 2 hr, only 23% survived. In contrast, cells grown at 15 C to about the same cell density and similarly tested showed 90 to 100% survivors. An implication of this experiment is that cell enzymes concerned with respiration and synthesis of cells grown at 15 C may be functional at 0 to 2 C, whereas those of cells grown at 30 C are not. Another possible explanation is that membranes of cells grown at 30 C may experience a disorganization of greater magnitude than those of cells grown at 15 C when both are exposed to 0 C. Considering the complexity of plasma membranes, it is difficult to visualize the sort of significant change that could be responsible.

Studies with *E. coli* by Marr and Ingraham (10), with *S. marcescens* by Bishop and Still (2), and with *Pseudomonas* sp. by Farrell and Rose (5) have shown that an increase in unsaturated fatty acids and a decrease in saturated fatty acids accompanies growth at lower temperatures. Possibly membrane lipids with unsaturated fatty acids are stable and "most functional" at low

temperatures, whereas membrane lipids with saturated fatty acids undergo phase changes, even crystallization, when cooled. It is known that at zero temperature the fatty acid side chains in membrane phospholipids solidify (3).

Washing cells of *C. violaceum* in aqueous solvents results in the removal of an appreciable quantity of surface material with a composition of protein, lipid, and carbohydrate in the approximate proportions found in isolated cell envelopes (4). Loss of some envelope material, however, does not necessarily effect cell death and, in any case, seems to be unrelated to the injurious effect of cold.

Cells of *C. violaceum* strain 341 lost some of their viable population after they were diluted into distilled water, broth, and physiological saline at room temperature (Table 3). Diluting cells into the buffer at room temperature (Table 3), on the other hand, resulted in maximal viability of the cells. Although the buffer or the ions it contained (Mg^{2+}, PO_4^{3-}, Ca^{2+}) probably protected sensitive cells at room temperature, they did not seem to protect cells against cold, since cells diluted in the buffer held at 2 to 0 C showed 50% loss in viability, identical to that observed in distilled water. This suggests that damage inflicted on the cells by the cold is distinct from that caused by an "osmotic effect" of a diluent transfer.

No loss of viability resulted, either at room temperature or in the cold, when 10^{-3} M solutions of the polyamine, trimethylenediamine-hydrochloride, were incorporated in the dilution fluid in tests with *C. violaceum* strain N (Table 4). Bachrach and Cohen (1) reported on the protective action by several polyamines to the cytoplasmic membranes of bacterial cells. Mager (9) found that polyamines enhance bacterial growth by preventing leakage of nutrients from the cell, which may be the protective action they exert against death in the cold.

ACKNOWLEDGMENT

The senior author (M.H.E.) acknowledges with thanks a fellowship from the American Association of University Women.

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