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Cold Shock Lethality and Injury in *Clostridium perfringens*

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Several observations have been made in regard to cold shock lethality of *Clostridium perfringens*: (i) loss of viability was not consequence of exposure of the cells to air; (ii) stationary-phase cells were much more resistant to cold shock at 4 C than exponential-phase cells; (iii) at 4 C 96% of an initial population of exponential-phase cells was killed upon cold shock and 95% of the remaining population was killed within 90 min of continued exposure at 4 C; (iv) the minimal temperature differential for detectable cold shock lethality was between 17 and 23 C, and the maximum beyond which lethality was not appreciably increased was between 28 and 33 C. Up to 75% of viable cold-shocked cells were injured, as demonstrated by cold shocking late exponential-phase cells at 10 C and using differential plating procedure for recovery. Repair of injury was temperature dependent, and occurred in a complex medium and 0.1% peptone but not water. Nalidixic acid, chloramphenicol, and rifampin did not inhibit repair of injury.

Generally, cold shock can be defined as either injury or death or both of organisms caused by sudden chilling in a suitable menstruum. Susceptibility is usually limited to exponential-phase cells. Cold shock may be dependent on rate of temperature drop, the length of time of exposure to low temperature, and the cold shock menstruum.

Gram-positive organisms generally have been considered insensitive to this stress, and therefore much of the literature cites results of work done with *Escherichia coli* (G. Houghtby and J. Liston, Bacteriol. Proc., p. 19, 1965; 7, 19) and other gram-negative organisms (1, 2, 21). However, a similar phenomenon has been described in *Streptomyces hydrogenans* (16) and *Bacillus subtilis* (20).

The present study establishes that both injury and death resulting from cold shock also can occur in the anaerobe *Clostridium perfringens*.

MATERIAL AND METHODS

Cultural procedures and harvesting of cells. A 10% (by volume) inoculum of *C. perfringens* type A, strain NCTC 8798, was made from a cooked-meat medium (Difco) working stock culture into 4.5 ml of Trypticase (1.5%; BBL), yeast extract (1.0%; Difco), and filter-sterilized glucose (0.5%) broth (TYG broth) at pH 6.9. The inoculated tube was incubated at 37 C for 18 to 24 h. A 10% (by volume) inoculum from this 18- to 24-h culture was made into 18 ml of TYG broth. The latter culture was incubated at 37 C until the culture reached approximately midexponential phase. This was equivalent to an optical density (OD) at 660 nm of about 0.5 reached in approximately 45 min. The culture was placed in a 20 C water bath and held at this temperature for 15 min. The culture was then incubated at 37 C for 5 min before inoculating 20 ml into 180 ml of prereduced (flushed with N_2 gas for 1 h) TYG broth at 37 C. Growth was monitored by measuring OD_{660} with a Bausch and Lomb Spectronic 20 Colorimeter.

Cells at a desired phase of growth were harvested by centrifugation for 10 min at 3,400 rpm. After pelleting, a cell preparation with an OD_{660} of about 1.0 was made by the addition of an appropriate amount of 0.1% peptone (3) at 37 C. A temperature of 37 C was maintained during centrifugation and subsequent manipulations. When desired, growth and harvesting conditions were maintained at 43 rather than 37 C.

Lethality techniques. Routinely, a 10% (by volume) sample from a cell preparation, adjusted to an OD_{660} of 1.0 and a temperature of 37 or 43 C, was squirted into prechilled 0.1% peptone and held at the cold shock temperature for 10 min. Cold shock temperatures were maintained in a temperature-controlled bath containing aqueous propylene glycol. The sensitivity of the bath was ±0.02 C. Unshocked control cells were held at 20 C for 10 min.

Injury techniques. Late exponential-phase cells were obtained as outlined above. A 10% (by volume) inoculum was made from a cell preparation at an OD_{660} of 1.0 and a temperature of 37 C into a cold shock menstruum at 10 C followed by holding at that temperature for 10 min.

Repair of injury. A 10% (by volume) inoculum of the injured, cold-shocked cell suspension was placed in a prereduced suspension medium and incubated at a specified experimental temperature in a water bath. A control flask containing an equal volume of unshocked cells from the same cell preparation was also

tested. Single samples were withdrawn at intervals from these systems, which were constantly flushed with N_2. Experiments were replicated at least twice to verify results.

Plating media, incubation, and determination of cell death and injury. Cells were surface plated in triplicate on TYG containing 1.35% agar (TYGA) or both TYGA and Trypticase sulfite neomycin (TSN) agar (BBL), modified by adding 0.6% NaCl and 0.5% filter-sterilized glucose (m-TSN).

All plating media were used within 2 to 3 h after sterilization by autoclaving. Plates were incubated at 37 C anaerobically, using GasPak anaerobic systems (BBL). The colonies were enumerated after 24 h of incubation. Further incubation did not demonstrate a significant increase in counts. The anaerobic system was rendered free of O_2 (as determined by the reduction of methylene blue indicators in the chamber as well as in an agar control) within 1 to 1.5 h.

Petri plates fitted with metal tops and absorbent disk inserts (BBL) were used to eliminate spreading of colonies due to moisture condensation. The moisture absorbing ability of the disk inserts also made it possible to use prepared plates approximately 5 to 10 min after pouring.

The amount of cell death after cold shock was determined from the difference in colony-forming units on TYGA before and after cold shock. The level of cell injury was calculated from the difference in colony-forming units per milliliter on TYGA and m-TSN at any particular sampling time. In repair studies, initial m-TSN count represented the number of uninjured cells present. Any increase in the m-TSN count during the test period was regarded as due to repair. Conversely, simultaneous reductions in the count on TYGA and m-TSN were considered as death or an increase in injury, respectively. Simultaneous increase in counts on TYGA and m-TSN were assumed to be due to cell multiplication.

Metabolic inhibitor studies. Three cellular functions that might be related to repair of injury were studied, namely deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein synthesis. The inhibitors used to investigate these metabolic functions were aqueous nalidixic acid (Sigma Chemical Co.), rifampin (Calbiochem) dissolved in methanol, and chloramphenicol (Parke, Davis and Co.) dissolved in ethanol. Each solution was filter sterilized, except the rifampin in methanol.

RESULTS

Effect of the growth phase on the sensitivity of cells to cold shock. Figure 1 shows the relationship between the growth phase and cell susceptibility to cold shock in 0.1% peptone (pH 6.9). Exponential-phase organisms, obtained after 60 to 120 min of growth, were most sensitive to sudden chilling as evidenced by a range of 80 to 95% drop in viability during this sampling time. As the cells approached and entered stationary phase, they became less sensitive.

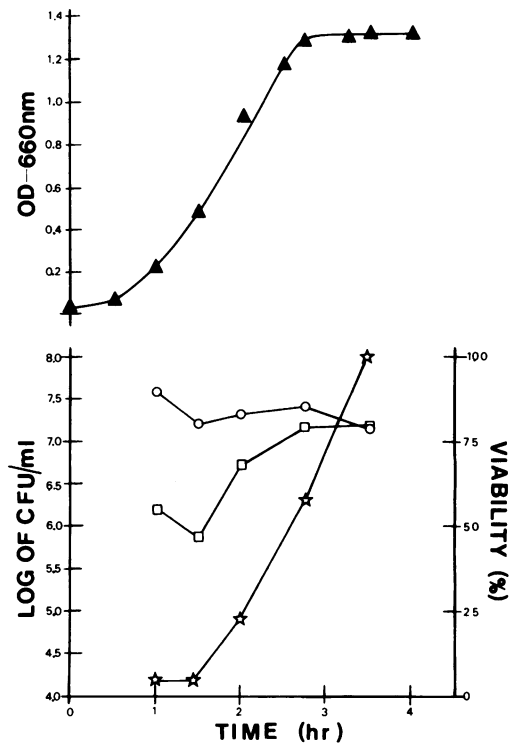

FIG. 1. *Effect of cold shock under aerobic conditions on the viability of cells of C. perfringens obtained from various phases of growth. Symbols: ▲, growth of strain H-9 in TYG broth under anaerobic conditions at 37 C; ○, controls held at 20 C for 10 min; □, cold-shocked cells held at 4 C for 10 min; ☆, percentage of viability after cold shock. Each value represents an average of two cell preparations that were sampled once and plated in triplicate. CFU, Colony-forming units.*

To confirm the lack of influence of anaerobic conditions on cold shock, similar experiments were conducted under strict anaerobic conditions obtained by using N_2-flushed, stoppered serum vials in conjunction with syringes for cold shocking and diluting. Results were similar to those obtained from experiments run under aerobic conditions.

Effect of the length of time of exposure of exponential-phase cells at the cold shock temperature on cell viability. Progressive loss of viability occurred with cold-shocked exponential-phase cells held at 4 C. Some 96% of the population was killed upon initial cold shock, and exposing the cold shock preparation for up to 90 min at 4 C resulted in an additional 3.8% loss in viability of the initial population (Fig. 2). This was equivalent to a decrease in viability of approximately 95% of the population that was

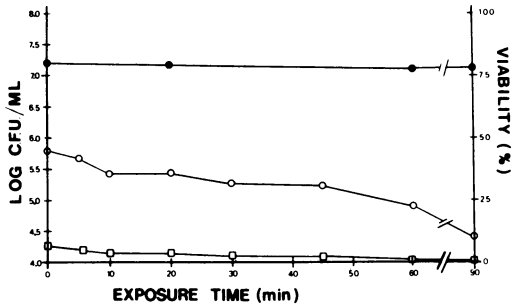

FIG. 2. *Effect of the length of time of exposure to low temperature after the initial cold shock on the viability of exponential cells of C. perfringens. Symbols: ●, control samples taken from a cell preparation that was held at 20 C for the indicated lengths of time; ○, samples taken from a cell preparation that was cold shocked from 37 to 4 C and held at 4 C for the indicated lengths of time; □, percentage of viability after cold shock and exposure. Each value represents an average of two cell preparations that were sampled once and plated in triplicate. At zero time, cells were squirted into the cold shock menstruum and immediately removed for plating. CFU, Colony-forming units.*

not killed upon initial cold shock. Thus, although the greatest loss of viability (96%) occurred with the initial cold shock, a progressive loss of viability of the survivors occurred with increasing time of exposure at the cold shock temperature.

Effect of the growth-cold shock temperature differential on viability. To ascertain whether or not lethality was dependent on the growth-cold shock temperature differential, cells were grown at 37 or 43 C and cold shocked at 4, 15, and 20 C, an approximately 20% greater loss of viability occurred after cold shock of cells grown at 43 C versus those grown at 37 C. Cells grown at 43 C and cold shocked at 30 C showed no loss of viability. The percentage of survival was essentially identical after shocking at 4 C, regardless of the growth temperature.

Effect of the growth versus incubation temperature on viability after cold shock. The data above raised the question of whether or not the cold shock effect was dependent on the growth temperature or simply on the temperature at which the cells were incubated prior to cold shock. The data in Fig. 3 indicates that cold shock lethality is dependent on the temperature differential but not necessarily the growth temperature, since cells grown at 37 C and cold shocked at 15 C or 20 C were less sensitive than cells from the same batch that were incubated at 43 C for 3 min prior to cold shock at 15 or 20 C. These data are similar to those obtained with cells grown at 43 C rather than merely being incubated at 43 C. Since cells incubated at both 37 and 43 C exhibited similar decreases in viability upon cold shock at 4 C, it would appear that although the differential between the temperature of incubation prior to cold shock and that of cold shock effects lethality, there is a maximal differential beyond which the extent of lethality is not increased. Under the present experimental conditions, the maximal differential would appear to be between 28 C (the difference between 43 and 15 C) and 33 C (the difference between 37 and 4 C). The minimal temperature differential for detectable lethality was between 17 C (a drop from 37 to 20 C) and 23 C (a drop from 43 to 20 C).

Establishment of a repair of injury system. It was experimentally determined that cold shocking late, exponential-phase cells at 10 C for 10 min resulted in an adequately injured population that would grow on TYGA but not m-TSN.

Cold shocking under a N_2 atmosphere or air made no difference in the number of cells of *C. perfringens* injured. Thus, no special precau-

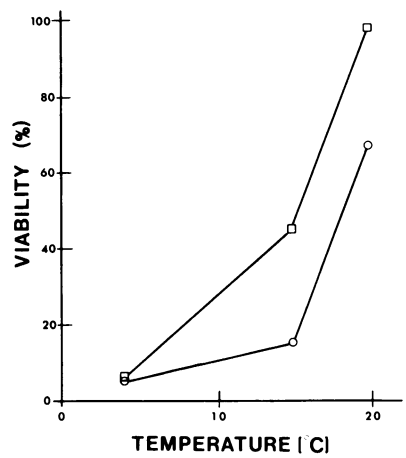

FIG. 3. *Comparison of the viability of exponential cells of C. perfringens grown and harvested at 37 C and then cold shocked versus cells grown and harvested at 37 C followed by incubation for 3 min at 43 C prior to cold shock. Symbols: □, exponential cells grown and shocked from 37 C; ○, exponential cells grown at 37 C and cold shocked from 43 C. Initial count for cells grown at 37 C was 2.0 × 10⁷. Initial count for cells grown at 37 C and incubated at 43 C prior to cold shock was 2.0 × 10⁷. Each value represents an average of two cell preparations sampled once and plated in triplicate. The cold shock temperature is indicated on the abscissa.*

tions were made to provide anaerobic conditions during routine shocking experiments.

The effect of temperature on the repair of injured cells in Trypticase (1.5%) yeast extract (1.0%) broth (TY broth) is shown in Fig. 4. A 10% (by volume) inoculum of injured, late exponential-phase cells was made into 180 ml of TY broth and held at 37, 30, 25, 20, and 15 C. The recovery system was held under anaerobic conditions to optimize chances for repair of the injured cells. At 37 C repair took place within 10 to 30 min, as indicated by an increase in counts on m-TSN. However, these results were clouded by the fact that multiplication also occurred

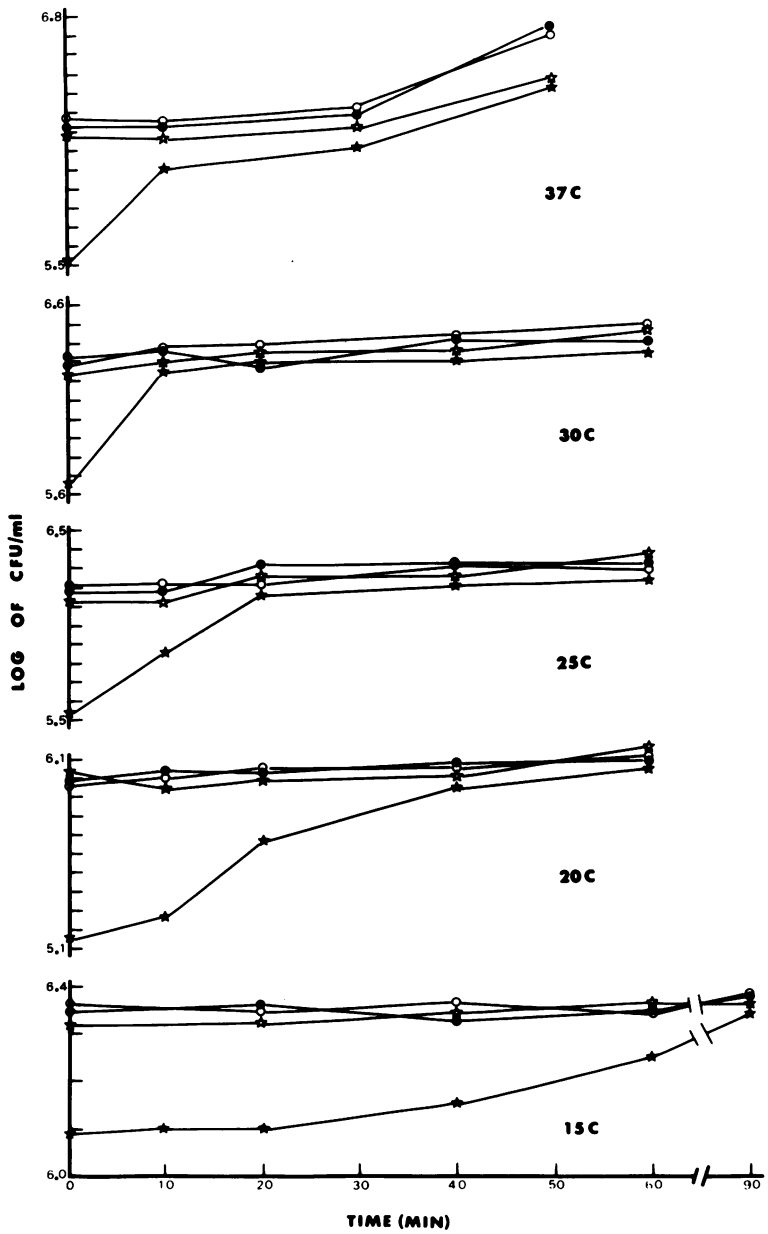

FIG. 4. *Repair of injury of cold-shocked C. perfringens cells in TY broth at 15, 20, 25, 30, and 37 C. Symbols: O, unshocked cells spread plated on TYGA; ●, unshocked cells spread plated on m-TSN; ☆, shocked cells spread plated on TYGA; ★, shocked cells spread plated on m-TSN. CFU, Colony-forming units.*

during this time period. Repair at 30 and 25 C occurred within 10 to 20 min without cellular multiplication. At 20 C repair was complete within 40 to 60 min; at 15 C, which is at or near the minimal temperature for growth of *C. perfringens*, repair was completed within 60 to 90 min.

Effect of the nutrient level on the repair of injury of cold-shocked C. perfringens cells. Figures 5 and 6 show results on repair of injury in 0.01% peptone and water, respectively. In the former experiment, late exponential cells were injured in 0.1% peptone at 10 C for 10 min, and a 10% inoculum was made from this injured cell preparation into a recovery medium of prereduced water. This resulted in a recovery medium with final concentration 0.01% peptone. Recovery was considerably less than that which occurred in TY broth at 20 C. Only 24% of the injured cells recovered after 60 min. Cold-shocked cells were pelleted at 10 C and suspended in water at 10 C before being used to inoculate a recovery system containing prereduced water. Here, repair of the injury did not occur (Fig. 6).

Effect of nalidixic acid, chloramphenicol and rifampin on repair of injury of cold-shocked cells. Nalidixic acid, an inhibitor of DNA synthesis, at 40 μg/ml did not impede recovery of cold-shocked cells (Fig. 7). Since this concentration was sufficient to inhibit vegetative multiplication of *C. perfringens*, it may imply that DNA synthesis is not involved in repair of injury.

Neither chloramphenicol at 4 μg/ml (the minimal inhibitory concentration for vegetative multiplication) nor rifampin at 0.01 μg/ml inhibited repair (Fig. 7). Thus, neither protein synthesis nor DNA-dependent RNA polymerase seem to be involved in repair of cold shock injury.

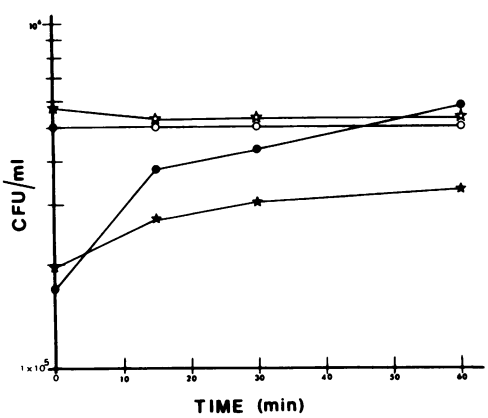

FIG. 5. *Repair of injury of cold-shocked C. perfringens cells in 0.01% peptone at 20 C. Symbols: O, shocked cells plated on TYGA from TY broth; ●, shocked cells plated on m-TSN from TY broth; ☆, shocked cells plated on TYGA from 0.01% peptone; ★, shocked cells plated on m-TSN from 0.01% peptone. CFU, Colony-forming units.*

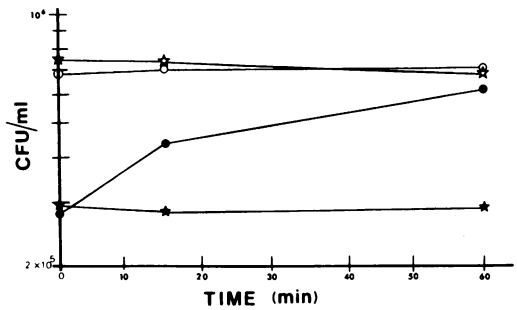

FIG. 6. *Repair of injury of cold-shocked C. perfringens cells in water at 20 C. Symbols: O, shocked cells plated on TYGA from TY broth; ●, shocked cells plated on m-TSN from TY broth; ☆, shocked cells plated on TYGA from water; ★, shocked cells plated on m-TSN from water. CFU, Colony-forming units.*

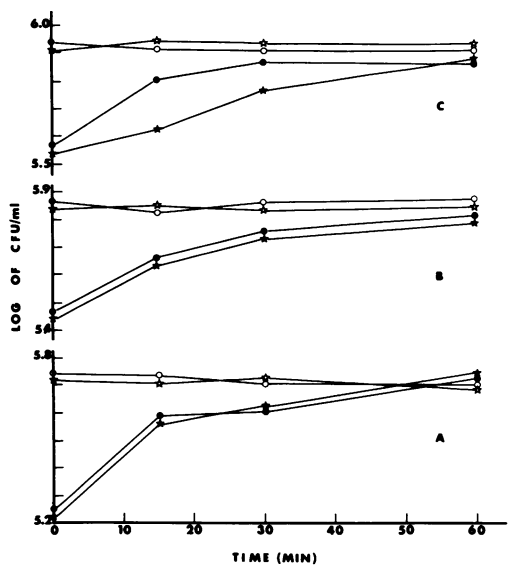

FIG. 7. *Repair of injury of cold-shocked C. perfringens cells in TY broth containing: (A) nalidixic acid, 40 μg/ml; (B) chloramphenicol, 4 μg/ml; (C) rifampin, 0.01 μg/ml. Symbols: O, shocked cells plated on TYGA from TY broth plus methanol, ethanol, or water solvent as a control; ☆, shocked cells plated on TYGA from TY broth plus inhibitor; ●, shocked cells plated on m-TSN from TY broth plus methanol, ethanol, or water solvent as a control; ★, shocked cells plated on m-TSN from TY broth plus inhibitor. CFU, Colony-forming units.*

DISCUSSION

Several observations have been made concerning cold shock lethality in *C. perfringens*. (i) The level of survival depended on the phase of growth of the cells at the time of cold shock. Exponential-phase cells were more sensitive to cold shock, whereas stationary-phase cells were resistant. Similar results have been reported for *E. coli* (7) and *Pseudomonas pyocyanea* (2). (ii) Cold shocking cells under aerobic versus anaerobic conditions did not effect the loss of cell viability, regardless of the phase of growth of the cells. (iii) Although the greatest percentage of lethality of exponential-phase cells was manifested upon initial cold shock at 4 C, a progressive loss of viability of the survivors occurred with increasing time of exposure to the cold shock temperature. (iv) The incubation-cold shock temperature differential influenced the degree of cold shock lethality. Sato and Takahashi (18, 19) reported finding two differential temperature zones through which exponential cells had to be suddenly chilled to obtain a maximal lethal effect. These findings were in contrast to the report of Smeaton and Elliott of one critical temperature zone in the case of *B. subtilis* (20). Our results are somewhat in agreement with those of the latter authors in that only one differential temperature zone was found for maximal cold shock lethality. However, the zone for lethality was broader than those reported by either Smeaton and Elliott or by Sato and Takahashi. With *C. perfringens*, the minimal temperature differential for detectable lethality was between 17 C (a drop from 37 to 20 C) and 23 C (a drop from 43 to 20 C). The maximal temperature differential beyond which lethality was not appreciably increased was between 28 C (a drop from 43 to 15 C) and 33 C (a drop from 37 to 4 C).

The minimal temperature differential for detectable lethality of *C. perfringens* (17 to 23 C) suggests that it would be easy to inadvertantly cold shock cells maintained at 37 C by exposing them to temperatures in the range of 15 to 20 C. Obviously, the use of cold buffer in washing cell suspensions should be avoided if viability is to be maintained.

Based on the information acquired from a partial characterization of cold shock lethality, a cold shock procedure for obtaining a large population of injured cells was devised. With such a system, repair of injury can be studied, and possibly the site(s) of injury can be defined. Naturally, such information would be quite helpful in recovering debilatated *C. perfingens* from food products. This type of system has been used in investigations of thermal injury (4, 8, 22), as well as in studies on freeze injury (12, 14). The results presented here establish that repair of injury does occur at 30, 25, 20, and 15 C in a complex recovery medium. Repair of injury at 37 C probably also occurs, but the results are clouded by the cell multiplication that occurred during the repair of injury period. Also established is that the lower the temperature of the recovery system, the longer it takes for injury to be repaired. This would imply that certain enzymatic reaction(s) are involved in repair.

By employing the "repair of injury" model, it has also been shown that there seems to be a minimal nutritional requirement for repair, since at 20 C repair is complete in 40 to 60 min in TY broth, is only partially complete in 0.01% peptone, and does not occur in water. In the experiments reported here, only 24% of the population repaired injury in 0.01% peptone. This is in agreement with similar reports of a complex nutrient requirement for repair of injury caused by freezing (9, 11) or heating (17).

Investigations into the site of injury were conducted by the addition of nalidixic acid, chloramphenicol, and rifampin to cells undergoing repair in TY broth. It was found that none of the aforementioned metabolic inhibitors, at levels capable of inhibition of vegetative growth, inhibited repair. Based on these results, this would seem to rule out DNA, protein, and RNA synthesis as sites of injury and repair.

Similar results were reported on repair of freeze-drying injury in *Salmonella anatum* (13), and on repair of freeze injury in *S. anatum* (12) and *E. coli* (15). In these studies the cell wall, cell membrane, or adenosine 5'-triphosphate-synthesizing machinery was cited as being possibly involved in cellular injury.

It is interesting to note that Ring (16), while studying the active transport of amino acids in *S. hydrogenans*, found that permeability drastically increased upon sudden cooling of the cell suspension. He postulated from uptake and leakage studies that there must be an alteration of the membrane structure, probably due to a phase transition of some of the membrane lipids. Work done by Ring (16) and Ingrahm (5) supports this view; they observed that the critical temperature for increasing permeability is shifted to lower values when cells are grown at lower temperatures, and that bacteria adapt to lower temperatures by shifting the fatty acid content of their membranes in favor of unsaturated acids which would lower the melting point of the lipids. Farrell and Rose, while working with a mesophilic and psychrophilic pseudomo-

nad (1), and Leder, while working with *E. coli* (6), also noted a permeability change upon sudden chilling. They too attributed this change to crystallization of the liquid lipids within the cell. It is plausible that if the cell membrane of *C. perfringens* is damaged in this way by cold shock, repair may depend on certain biosynthetic reactions as well as energy metabolism, as has been found in freeze and freeze-drying injury (12, 13).

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