

# Characterization of the Repair of Injury Induced by Freezing *Salmonella anatum*<sup>1</sup>

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Fast freezing and slow thawing of *Salmonella anatum* cells suspended in water resulted in injury of more than 90% of the cells that survived the treatment. The injured cells failed to form colonies on the selective medium (xylose-lysine-peptone-agar with 0.2% sodium deoxycholate) but did form colonies on a nonselective (xylose-lysine-peptone-agar) plating medium. In Tryptic soy plus 0.3% yeast extract broth or minimal broth, most of the injured cells repaired within 1 to 2 hr at 25 C. Tryptic soy plus yeast extract broth supported repair to a greater extent than minimal broth. Phosphate or citrate at concentrations found in minimal broth supported repair of some cells. MgSO<sub>4</sub>, when present with inorganic phosphate or citrate or both, increased the extent of repair. The repair process in the presence of phosphate was not prevented by actinomycin D, chloramphenicol, and D-cycloserine, but was prevented by cyanide and 2,4-dinitrophenol (only at pH 6). This suggested that the repair process might involve energy metabolism in the form of adenosine triphosphate. The freeze-injured cells were highly sensitive to lysozyme, whereas unfrozen fresh cells were not. In the presence of phosphate or minimal broth this sensitivity was greatly reduced. This suggested that, at least in some of the cells, the injury involved the lipopolysaccharide of the cell wall and adenosine triphosphate synthesis was required for repair.

Microbial cell injury has been induced by various sublethal stresses (10, 19, 20, 22-25, 27). When the stress was removed and the cells were exposed to a suitable environment, the repair of damage in these cells was observed (10, 12, 15, 16, 21, 25). The nature of the injury produced by exposing the microbial cells to different environmental stresses and cell repair mechanisms have been studied. In general, the repair process(es) has been characterized by restoration of altered permeability, rebuilding of the lost intracellular nutrient pool, regeneration of ribonucleic acid (RNA; especially ribosomal RNA), and synthesis of proteins, etc. (12, 14, 15, 18, 23, 25). Although the repair mechanisms appeared to differ depending upon the nature of stress or injury, recent reports have indicated one characteristic common to all injury repair. The injured cells required energy synthesis, at least during the early phase of repair. Cells injured by freeze-drying (21), heat (25), ethylenediamine-

tetraacetic acid treatment (10), N-hydroxyurethane treatment (16), X-ray radiation (14), and, possibly, air drying (28) required energy metabolism during repair. In many cases the energy synthesis possibly occurred in the form of adenosine triphosphate (ATP) synthesis by oxidative phosphorylation through the electron transport system (10, 13, 16, 21, 25).

In this paper we characterize the repair process of freeze-injured *Salmonella anatum* cells. A preliminary report of these findings has been presented previously (B. Ray, D. W. Janssen, and F. F. Busta, *Bacteriol. Proc.*, p. 6, 1971).

## MATERIALS AND METHODS

**Freezing and thawing of cell suspensions.** *S. anatum* NF3 was propagated and maintained in reconstituted nonfat dry milk (10% solids, not fat) as described previously (20). A 1-ml portion of this culture was inoculated in 100 ml of Tryptic soy broth containing 0.3% yeast extract (TSY) and grown for 16 to 20 hr at 35 C. The cells were centrifuged at 2,520 × g for 10 min in a Sorvall RC2-B automatic, refrigerated centrifuge (Ivan Sovall, Inc., Newton, Conn.) and washed three times, each time with 100 ml of sterile water. The cells then were suspended uni-

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formly in water with a Vortex mixer (Scientific Products, Evanston, Ill.) to a concentration of  $10^8$  to  $2 \times 10^8$  cells/ml. Portions of 10 ml each were placed in test tubes (150 by 25 mm) and frozen rapidly by static placement in a dry ice-acetone bath for 10 min. The contents were thawed in a water bath at 4 C for about 75 min and tested for cell injury and repair immediately.

**Determination of cell injury and repair.** A method described previously was used with the following slight modification (21). A 1-ml portion of the thawed sample was diluted in 9 ml of water. Then 1-ml portions were added to 9 ml of each of the several solutions used in a particular test. The test solutions containing the cells were incubated in a forced-air incubator at 25 C. At indicated intervals, cells from the test solutions were counted on xylose-lysine-peptone-agar (XLP) and XLP with 0.2% sodium deoxycholate added (XLDP). A 0.1-ml quantity was surface-plated in each of three plates of each medium. Death, injury, and repair were determined as described previously (20). The results were reported with respect to the cell concentration used for freezing and not to the concentration used in the test solutions, which was a 1:100 dilution.

**Composition and preparation of minimal broth.** Minimal broth contained  $K_2HPO_4$ , 0.25%;  $MgSO_4$ , 0.01%;  $(NH_4)_2SO_4$ , 0.1%; sodium citrate, 0.01%; NaCl, 0.01%; and glucose, 0.25%. To prepare a 100-ml equivalent of the broth, all the ingredients, except glucose, were dissolved in 50 ml of water; glucose was dissolved separately in 30 ml of water. The two solutions were separately sterilized and mixed together, and the pH was adjusted with sterile 0.1 N sodium hydroxide and hydrochloric acid solutions. The final volume was adjusted to 90 ml. A 9-ml portion of minimal broth was used at each test solution to which 1 ml of cell suspension was added.

In tests in which components of minimal broth were studied, either individually or in combinations, solutions of each component were made separately in  $10\times$  concentrations and were used in 1-ml quantities in the test solutions. When required, the volumes of the test solutions were adjusted to 9 ml with sterile water. All chemicals were of analytical grade.

**Antimicrobial agents and biochemicals.** Agents used in test solutions were adenosine 5'-triphosphate disodium; actinomycin D; D-cycloserine; 2,4-dinitrophenol (DNP); lysozyme, egg white (all from Mann Research Lab., N.Y.); chloramphenicol (Parke, Davis & Co., Detroit, Mich.); and sodium cyanide (J. T. Baker Chemical Co., Phillipsburg, N.J.).

Solutions of each agent in  $10\times$  concentration were prepared with sterile water and used while fresh. Unless stated, the pH was adjusted to 7.0 with 0.1 N sterile sodium hydroxide or hydrochloric acid. Solutions were diluted to the desired concentration where appropriate (final volume 10 ml).

## RESULTS

**Rate of repair of freeze injury.** Repair of injury caused by freezing and thawing was studied by suspending the freeze-injured *S.*

*anatum* cells in test solutions containing water, TSY broth, and minimal broth. The test suspensions were evaluated after 0, 10, 20, 30, 40, 50, 60, and 120 min of incubation at 25 C. The results shown in Fig. 1 indicated about a 97% difference in numbers on the nonselective (XLP) and selective (XLDP) plating media in all three test solutions. Unfrozen cells exhibited only 10 to 15% differences in number between the two plating media; these large differences were due to cell injury caused by freezing and thawing. Numbers of colonies formed on the XLP medium from both TSY and minimal broths remained almost the same during the 2-hr test period. However, numbers of colonies formed on the XLDP medium increased rapidly up to 1 hr and then increased at a reduced rate. After 2 hr, the differences in counts between the two types of plating media were about 37% in TSY broth and 49% in minimal broth. This increase in counts on XLDP medium was due to repair of the injured cells. The rate of repair was extremely rapid in both broths, but TSY broth provided a better environment than minimal broth for repair. The injured cells failed to repair when suspended in water, and some reduction in number occurred in both plating media.

**Effect of ingredients of minimal broth on repair.** The freeze-injured cells were suspended in minimal broth and in solutions containing individual components of this broth (at the same concentration used in the minimal broth). Water was used for a control. The test solutions were incubated at 25 C and plated at 0 and 1 hr. Since the repair was measured as an increase in counts of colony-forming units on XLDP medium, only counts on this medium after 0 and 1 hr from different test solu-

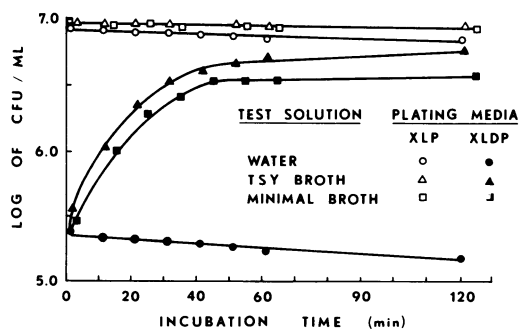


FIG. 1. Repair of injury of frozen *Salmonella anatum* cells in water, Tryptic soy broth with 0.3% yeast extract (TSY broth), and minimal broth. The test solutions were incubated at 25 C and plated on xylose-lysine-peptone-agar (XLP) and XLP plus 0.20% sodium deoxycholate (XLDP). CFU indicates colony-forming units.

tions were presented (Fig. 2). During this period, the cell counts increased in varying amounts in the presence of minimal broth, phosphate, and citrate (i.e., repair occurred). No repair was observed in the presence of  $MgSO_4$ ,  $(NH_4)_2SO_4$ , or glucose, or in water. The greatest amount of repair occurred in minimal broth and the least in citrate. The extent of repair that occurred in the presence of 0.25% phosphate ( $K_2HPO_4$ ) was greater than that which occurred in citrate (sodium salt, 0.01%) but less than in minimal broth. The number of survivors after freeze treatment (generally less than 10% of the untreated cells) was determined by colony count on the XLP medium and was essentially the same at 0 hr in all test solutions; therefore, only data from the water system are presented. Numbers observed with XLP remained almost constant after 1 hr in the presence of minimal broth, phosphate, and citrate but were reduced considerably in other test solutions (data not presented).

The ingredients of minimal broth were tested in different combinations and data indicating additive effects on repair were observed (Fig. 3). A combination of phosphate, citrate, and  $MgSO_4$  provided repair comparable to minimal broth. Phosphate alone produced less

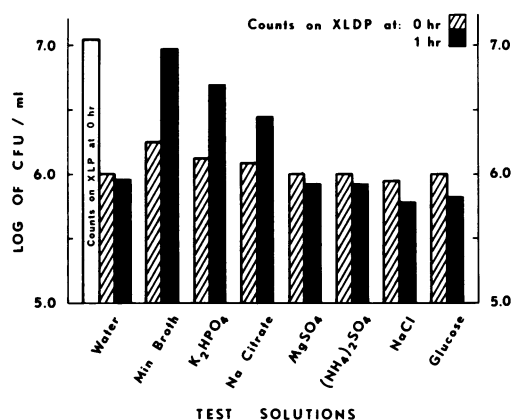


FIG. 2. Repair of freeze-injured *Salmonella anatum* cells at 25 C in water, minimal broth (MM), and components of MM. The concentrations of the individual components were the same as in MM. To indicate repair, counts on xylose-lysine-peptone-agar with 0.2% sodium deoxycholate (XLDP) at 0 and 1 hr are presented. CFU indicates colony-forming units. The number of survivors after freezing in water [numbers on xylose-lysine-peptone-agar (XLP) at 0 hr] is presented separately. Numbers on XLP at 0 and at 1 hr remained essentially the same in MM,  $K_2HPO_4$ , and sodium citrate, but were reduced in  $(NH_4)_2SO_4$ ,  $MgSO_4$  (about 30% in each), water, NaCl, and glucose (about 65% in each) after 1 hr.

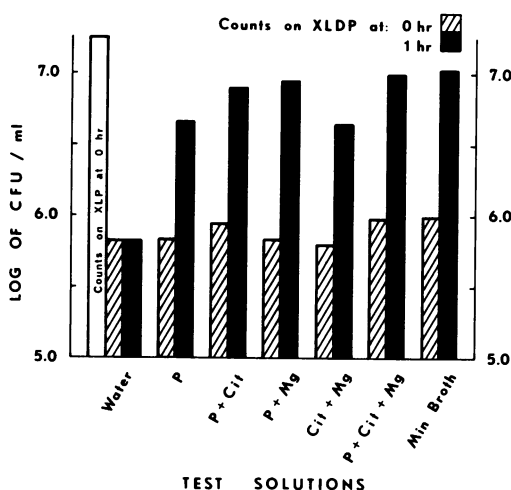


FIG. 3. Repair of freeze-injured *Salmonella anatum* cells at 25 C in water, minimal broth, 0.25%  $K_2HPO_4$  (P), 0.01% sodium citrate (Cit), 0.01%  $MgSO_4$  (Mg), and combinations. To indicate repair, the numbers on xylose-lysine-peptone-agar with 0.2% sodium deoxycholate (XLDP) at 0 and 1 hr are presented. CFU indicates colony-forming units. The number of survivors after freezing in water [numbers on xylose-lysine-peptone-agar (XLP) at 0 hr] is shown separately. Numbers on XLP at 0 and 1 hr remained essentially the same in all solutions except water. In water, about 60% reduction occurred within 1 hr.

repair than when in combination with  $MgSO_4$  or citrate. The citrate and  $MgSO_4$  combination resulted in repair almost equal to that observed with phosphate, whereas citrate alone produced less repair than phosphate (Fig. 2).

**Effect of pH on repair in phosphate.** Some of the injured cells were able to repair in the presence of phosphate alone. To determine whether this repair in phosphate was dependent on pH, injured cells were suspended in test solutions containing 0.25% phosphate at pH levels between 5 and 9, incubated at 25 C, and plated at 0 hr and 1 hr. To show the amount of repair, the counts on XLDP medium are presented (Fig. 4). Although essentially the same number of injured cells were introduced in each test solution at 0 hr (a 1-ml portion from the same sample was added to each test solution), relatively higher counts were obtained as the pH increased from 5 to 9. After 1 hr of incubation, increased counts indicating repair were observed at tested pH levels of 6, 7, 8, and 9. Essentially no repair was observed at pH 5.0. Maximum repair was observed at pH 8.0 where after 1 hr only 37% of the treated cells exhibited damage. At the pH used in other tests (pH 7), a considerable number of cells were able to repair.

**Effect of phosphate concentration on repair.** Injured cells were suspended in test solutions containing 0 to 1.0%  $K_2HPO_4$  at pH 7.0. Water was used for a control. The data from XLDP medium after 0 and 1 hr of incubation at 25 C are presented (Fig. 5). At 0 hr, counts at all concentrations were about the same (i.e., the number of uninjured or injured cells were equivalent at all concentrations at 0 hr). After 1 hr, counts increased in all concentrations tested. Here, 0.5% phosphate supported the greatest repair; however, a 0.25% concentration was used in other tests.

**Effect of antimicrobial agents on repair in phosphate.** Injury repair in the presence of phosphate (0.25%  $K_2HPO_4$ ) was studied by suspending the freeze-injured cells in test solutions containing phosphate and one of the several antimicrobial agents at pH 6.0 or 7.0. The results obtained at pH 6.0 are presented (Fig. 6). The counts on the XLDP medium increased after 1 hr in the presence of actinomycin D (10  $\mu\text{g}/\text{ml}$ ), chloramphenicol (100  $\mu\text{g}/\text{ml}$ ), and D-cycloserine (20  $\mu\text{g}/\text{ml}$ ). Thus, the repair process in phosphate was not affected by these three agents. In the presence of sodium cyanide (50  $\mu\text{g}/\text{ml}$ ) or DNP (75  $\mu\text{g}/\text{ml}$ ),

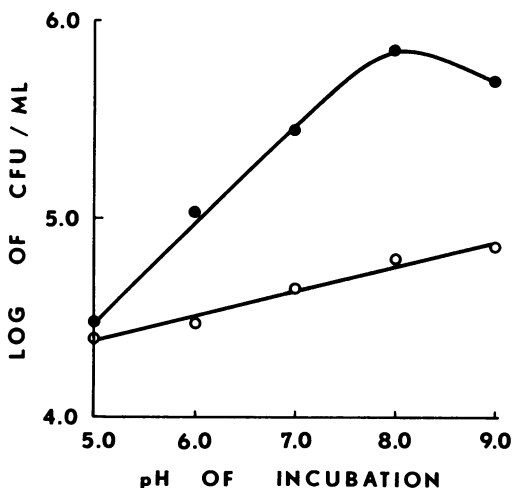


FIG. 4. Effect of pH on the repair of freeze-injured *Salmonella anatum* cells in the presence of  $K_2HPO_4$  (0.25%) at 25 C. To indicate repair, counts on xylose-lysine-peptone-agar with 0.2% sodium deoxycholate (XLDP) are presented. CFU indicates colony-forming units. Data obtained at 0 hr are indicated by open circles and at 1 hr are indicated by closed circles. The number of survivors on xylose-lysine-peptone-agar (XLP) at 0 hr was about  $10^4/\text{ml}$ . Numbers at 1 hr on XLP remained essentially the same at all pH values except at pH 5.0 and 9.0, where about 40% reduction occurred.

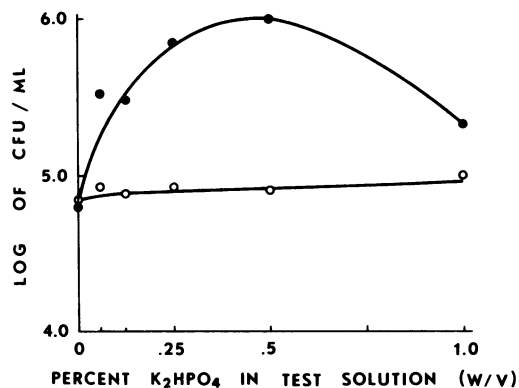


FIG. 5. Effect of concentration of  $K_2HPO_4$  (pH 7.0) on repair of freeze-injured *Salmonella anatum* cells at 25 C. To indicate repair, numbers on xylose-lysine-peptone-agar with 0.2% sodium deoxycholate (XLDP) are presented. CFU indicates colony-forming units. Data obtained at 0 hr are indicated by open circles and at 1 hr are indicated by closed circles. Numbers on xylose-lysine-peptone-agar (XLP) at 0 and 1 hr remained essentially the same in all concentrations except in water.

the repair process was greatly inhibited. DNP prevented repair at pH 6.0 but not at pH 7.0. Results obtained with the other antimicrobial agents at pH 7.0 were similar to the corresponding results obtained at pH 6.0 (data not presented).

**Effect of lysozyme on the survival of freeze-injured cells.** Freeze-injured *S. anatum* cells were suspended in the presence and absence of lysozyme (100  $\mu\text{g}/\text{ml}$ ) in test solutions containing water, phosphate (0.25%), and minimal broth. For comparison, unfrozen cells were also suspended in water with and without lysozyme. The samples were incubated at 25 C and plated on XLP medium at 0 and 1 hr. For a particular combination (e.g., in the presence of lysozyme in water), data for 0 hr are presented for only the sample without lysozyme, whereas data for 1 hr evaluations are presented for systems with and without lysozyme (Fig. 7). The freeze-injured cells showed susceptibility to lysozyme. In water the counts reduced in the absence of lysozyme, but the reduction was much greater in the presence of lysozyme. With phosphate or minimal broth, the sensitivity of the freeze-injured cells to lysozyme was reduced greatly. The fresh cells, however, were unaffected by lysozyme.

## DISCUSSION

Fast freezing followed by slow thawing of *S. anatum* cells suspended in water inactivated more than 90% of the cells. This death was

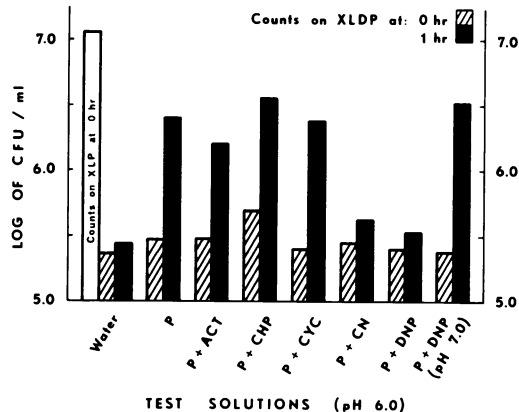


FIG. 6. Repair of freeze-injured *Salmonella anatum* cells at 25 C in water, 0.25%  $K_2HPO_4$  (P), and solutions of P plus different antimicrobial agents. The agents used were actinomycin D (ACT, 10  $\mu$ g/ml), chloramphenicol (CHP, 100  $\mu$ g/ml), D-cycloserine (CYC, 20  $\mu$ g/ml), sodium cyanide (CN, 50  $\mu$ g/ml), and 2,4-dinitrophenol (DNP, 75  $\mu$ g/ml). To indicate repair, the numbers on xylose-lysine-peptone-agar with 0.2% sodium deoxycholate (XLP) at 0 and 1 hr are presented. CFU indicates colony-forming units. The number of survivors after freezing in water [numbers on xylose-lysine-peptone-agar (XLP) 0 hr] is presented separately. Numbers on XLP at 0 and 1 hr remained essentially the same in all test solutions except in water (60% reduction), ACT (70% reduction), CN (40% reduction), and DNP at pH 6.0 (40% reduction).

determined from the differences in numbers of colony-forming units observed on XLP medium before and after freezing (data not presented). About 90% of the viable survivors were injured as they failed to form colonies on a plating medium containing deoxycholate as the selective agent (XLP) but not on a non-selective medium (XLP). Similar high percentages of cell injury due to freeze-drying (20) and sublethal heat treatment (25) have been reported previously. The susceptibility of the injured cells to the selective agent disappeared when these cells were exposed to nutritive environments, such as TSY broth, minimal broth, etc. This resulted in an increase in counts on only the selective plating medium, indicating repair of injury of the cells. The rate of repair was rapid initially and approached completion after 60 min at 25 C. The extent of repair at 25 C depended upon the nature of the medium in which the injured cells were suspended. TSY broth provided an environment that supported more repair than minimal broth, indicating possible requirements of some complex nutrients for injury repair. This would be consistent with reports that meta-

bolic damage by freezing (14, 19), freeze-drying (21), other sublethal stresses (18, 28), and consequent loss of cellular components required complex nutrients for the repair.

All freeze-injured cells could not repair their injury in minimal broth in 2 hr at 25 C. The capacity of minimal broth to support repair could be duplicated by a combination of inorganic phosphate, citrate, and  $MgSO_4$ , all of which were present in the broth. However, a certain fraction of the cells also were able to repair in the presence of either phosphate or citrate alone. Other ingredients of minimal broth when present alone were unable to produce any repair. The addition of  $MgSO_4$  increased the amount of repair produced by phosphate (phosphate repair) or citrate (citrate repair).

The ability of some of the freeze-injured cells to repair in the presence of phosphate or citrate indicates that the repair process, at least in these cells, was related to energy metabolism. Energy metabolism during repair of injury by cells that survived sublethal stresses has been reported (10, 13, 16, 21, 25). Citrate transport in many citrate-utilizing species in the *Enterobacteriaceae* depends upon a specific inducible permease system (9, 26). *S. anatum* NF3 used in this study was able to grow with citrate as sole carbon source. If we assume that citrate utilization in this strain is

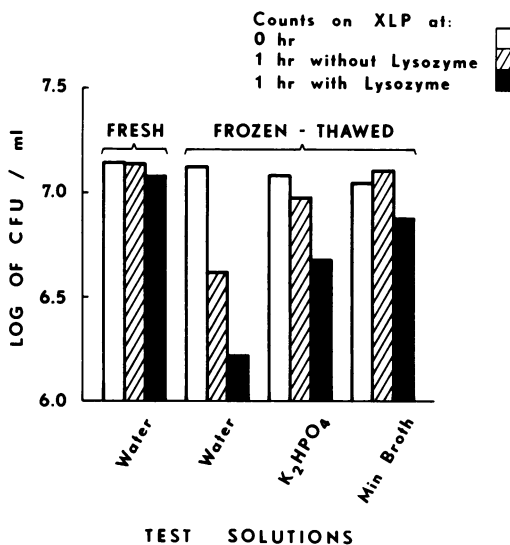


FIG. 7. Survival of fresh and frozen *Salmonella anatum* cells in different test solutions at 25 C in the presence and absence of lysozyme (100  $\mu$ g/ml). To show reduction in survivors only on xylose-lysine-peptone-agar (XLP) numbers at 0 and at 1 hr are presented. CFU indicates colony-forming units.

also dependent upon such an inducible permease system, cells grown in TSY broth, and subsequently injured, will not have this enzyme. However, citrate permease could be induced within a few minutes after the cells were exposed to citrate (26), and thus citrate could be utilized for energy metabolism and repair during a 1-hr incubation period. Freeze-injured *S. anatum* cells also repaired in the presence of pyruvate, oxaloacetate, acetate, succinate, fumarate, or malate (all sodium salts used at 100  $\mu\text{g/ml}$  at pH 7.0; data not presented).

The ability of some freeze-injured cells to repair in the presence of inorganic phosphate was dependent upon pH and phosphate concentration. This phosphate repair was prevented in the presence of sodium cyanide or DNP (at pH 6.0). Both cyanide and DNP interfere with ATP synthesis during oxidative phosphorylation in the electron transport system (1, 11). Therefore, the data suggested the probable requirement of energy metabolism during repair. Repair in the presence of pyruvate also was inhibited by cyanide and DNP (data not presented). DNP inhibition of repair of injury has been reported by several workers (10, 13, 16, 21, 26). Increases in the amount of phosphate or citrate repair in the presence of  $\text{MgSO}_4$  could be due to  $\text{Mg}^{2+}$ . This divalent cation is necessary for the activity of many enzymes that take part in the energy metabolism.

Actinomycin D, chloramphenicol, and D-cycloserine are inhibitors of the synthesis of ribonucleic acid, proteins, and cell wall mucopolysaccharide, respectively (2, 3, 17). The repair process in phosphate was not inhibited by any of these three agents. This suggested the probable lack of involvement of RNA, protein, and mucopolysaccharide synthesis during the repair process of the freeze-injured cells. The synthesis of RNA and protein during repair of cells injured by freeze-drying and heat treatment has been reported (15, 23, 25). Gram-negative organisms are impermeable to actinomycin D; however, after some sublethal treatments this barrier is impaired. The injured cells become permeable to actinomycin D, at least until the injury is repaired (10, 23). In the present study, the number of cells repaired in the presence of phosphate plus actinomycin D was less than those repaired in the presence of phosphate alone. Also, a large number of freeze-injured cells showed susceptibility to actinomycin D (data not presented).

During this study, we observed that a large reduction in numbers of colony-forming units

of *S. anatum* cells occurred when the freeze-injured cells were suspended in water or in test solutions that did not support repair. This reduction was observed only with XLP medium. In water, the reduction varied between 40 to 70% during 1 hr of incubation at 25 C (data not presented). We suspected that this cell death (inability to form colonies on XLP medium) might be due to inability to maintain cell integrity (6, 9). The cell wall which helps to maintain cell integrity might be altered in some way in the freeze-injured cells. These cells also were susceptible to lysozyme. Lysozyme causes degradation of the mucopolysaccharide layer, but in gram-negative bacteria this layer lies beneath the lipopolysaccharide (LPS) layer of the cell wall and usually remains inaccessible to lysozyme action (4, 5). However, once this LPS layer is removed or undergoes some steric or chemical change, lysozyme is able to hydrolyze mucopolysaccharide (6, 7, 10). The lysozyme susceptibility of the freeze-injured *S. anatum* cells may have been due to some alteration in the LPS layer. However, when the cells were given a chance to repair (in minimal broth or in phosphate), their susceptibility to lysozyme was reduced. The untreated cells were not sensitive to lysozyme. The beneficial effect of  $\text{MgSO}_4$  on phosphate repair could be due to the  $\text{Mg}^{2+}$ . Divalent cations ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ) are known to stabilize the cross-linkage of the LPS layer in gram-negative bacteria (4, 5). Freezing might have interfered with the cross-linkage and brought about some steric or other reversible change, at least in some of the injured cells. The repair of this injury required energy metabolism, possibly in the form of ATP. Although inorganic phosphate (with or without  $\text{Mg}^{2+}$ ) was able to support repair in some of the freeze-injured cells, a greater number of cells repaired in presence of complex organic nutrients.

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