Metabolic Process During the Repair of Freeze-Injury in *Escherichia coli* 1

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After *Escherichia coli* was injured by freezing, the repair process was studied during incubation of the cells for 2 hr at 25 C in 0.5% K2HPO4, at pH 7.0 in the presence of specific metabolic inhibitors. The repair in K2HPO4 was not affected by inhibitors of the synthesis of protein, nucleic acids, and mucoprotein. These inhibitors prevented growth of the repaired cells in a minimal broth at 35 C for 24 hr (except actinomycin D and hydroxyurea). Several uncouplers of adenosine triphosphate (ATP) synthesis reduced the repair process in K2HPO4, but only cyanide and azide prevented growth in minimal medium. Data indicated that the cells synthesized energy in the form of ATP and probably utilized it for the repair process. Addition of ATP also facilitated the repair of injury. The freeze-injured cells showed extreme susceptibility to surface-active agents and lysozyme. The repaired cells, like the uninjured cells, became relatively resistant to these compounds.

Injury induced by freezing and other sublethal stresses in gram-negative bacteria can be repaired in the presence of many simple and complex compounds. The cellular events which occur during the repair process involve the synthesis of: ribonucleic acid (RNA) and proteins in freeze-dried *Escherichia coli* (17); adenosine triphosphate (ATP) in freeze-dried *Salmonella anatum* (13); lipids and proteins in frozen *E. coli* (D. A. Gabis, Ph.D. thesis, North Carolina State University, Raleigh, 1970); ATP in frozen *S. anatum* (12); RNA, proteins, and ATP in heat-shocked *S. typhimurium* (19); ATP in ethylenediaminetetraacetate (EDTA)-and N-hydroxyurethan-treated *E. coli* (6, 9).

The repair process, in most cases, has been studied in media which also facilitate growth. In a previous paper (14) we reported that frozen *E. coli* cells repaired their injury in the presence of compounds which by themselves are unable to support growth, such as in K2HPO4 and sodium pyruvate. Similar observations have also been reported for freeze-injured *S. anatum* (12). In this report we describe the possible nature of the metabolic process that occurs during the repair of freeze-injury in *E. coli* cells in the presence of K2HPO4.

**MATERIALS AND METHODS**

*E. coli* NCSM was used in this study. The general procedures for growing, harvesting, resuspending, freezing, and thawing have been described earlier (14). Cells in 10 ml of aqueous suspension (2 x 109/ml) were frozen in dry ice-acetone (-78 C) and thawed at 8 C. Cell suspension in a 1-ml portion was then transferred to 9 ml of media containing various compounds used to study repair, growth, and survival at pH 7.0 and 25 C.

**Injury repair.** The freeze-injured cells were suspended in 0.5% K2HPO4, at pH 7.0 in the presence of different inhibitors and incubated at 25 C for 2 hr in a water bath. Appropriate controls were used in all tests. At appropriate intervals of time samples were taken, serially diluted in water, and surface plated on Trypticase soy agar containing 0.3% yeast extract (TSYA) and TSYA containing 0.1% sodium deoxycholate (TSYDA). The plates were incubated for 24 hr at 35 C and colonies were counted. Any increase in TSYDA count during the test period was regarded as due to repair. Conversely, reductions on TSYA and TSYDA counts were considered as death and increase in injury, respectively. Methods of preparation of the two plating media, plating of samples, and calculations of percentages of injury and death have been described before (14).

**Growth.** The freeze-injured cells were suspended in a minimal medium (14) in the presence of antimicrobial agents. The cell concentrations and the concentration of the inhibitors were the same as that used in the repair study. After 24 hr of incubation at 35 C evidence of growth (i.e., turbidity) was observed visually.

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Survival. Unfrozen and freeze-thawed *E. coli* cells in aqueous suspension were incubated at 25 C for 1 hr in the presence of 0.1% sodium deoxocholate, 0.1% sodium lauryl sulfate and 100 µg of lysozyme per ml at pH 7.0. Cells suspended in water were used as a control. Serially diluted samples were surface plated on TSYA at 0 hr (only control) and 1 hr. Colonies were counted after incubating the plates at 35 C for 24 hr. Similar tests were conducted with the repaired cells. The injured cells were allowed to repair in 0.5% K$_2$HPO$_4$, with or without 0.01% MgSO$_4$ for 1 hr at pH 7.0 and 25 C and then tested by methods described above.

Chemicals. The following compounds were used in this study: penicillin G sodium (1,650 units/mg), rifampin, and 2,4-dinitrophenol (Mann Research Lab, New York, N.Y.); d-cycloserine, streptomycin sulfate, actinomycin D, lysozyme from egg white (1,800 units/mg), ATP disodium salt (Schwarz Mann, Orangeburg, N.Y.); chloramphenicol, kanamycin, 5-fluouracil, hydroxurea, gramicidin, oligomycin, sodium lauryl sulfate (Sigma Co., St. Louis, Mo.); tetracycline-hydrochloride and puromycin (Nutritional Biochemicals Corp., Cleveland, Ohio); sodium cyanide, sodium azide, sodium deoxocholate (Fisher Scientific Co., Fairlawn, N.J.).

Concentrated solutions of the chemicals were prepared in sterile water and adjusted to pH 7.0 when necessary with sterile NaOH or HCl. The solutions were stored at 4 C and used within 7 days. Only fresh cyanide was used.

RESULTS

Protein synthesis inhibitors. The injured cells were suspended in 0.5% K$_2$HPO$_4$ at pH 7.0 with or without inhibitors, and incubated at 25 C for 120 min. The progress of repair was studied by plating serially diluted samples on TSYA and TSYDA. To reduce carry-over of the inhibitors to the plates, the samples were diluted at least 1,000-fold. A proper control with inhibitor alone was also used. These data with chloramphenicol indicated that initially a large percentage of the surviving cells were injured, as shown by the difference in counts on TSYA and TSYDA at 0 hr (Fig. 1). In the presence of K$_2$HPO$_4$, TSYDA counts increased rapidly, indicating repair had occurred. Chloramphenicol did not show any appreciable inhibition either on the rate or total amount of repair in K$_2$HPO$_4$ during the 120-min incubation period. Similar results but with greater amounts of repair were obtained with puromycin (10 µg/ml) and tetracycline (30 µg/ml) (data not presented).

The effect of another protein synthesis inhibitor, kanamycin, on repair of injury in K$_2$HPO$_4$ was studied (Fig. 2). In the presence of kanamycin the cells were able to repair in K$_2$HPO$_4$ at a slower rate up to 20 min. After about 40 min the cells showed an increase in injury and death. When MgSO$_4$ (0.04%) was added, the inhibition of repair and death by kanamycin were prevented. Streptomycin (10 µg/ml) produced similar results. All of the five inhibitors prevented growth of the injured cells in a minimal broth during 24 hr of incubation at 35 C (data not presented).

RNA synthesis inhibitors. Repair of freeze-injury of *E. coli* in 0.5% K$_2$HPO$_4$ at pH 7.0 and 25 C occurred in the presence of rifampin (10 µg/ml) up to 60 min (Fig. 3 and Table 1); then the cells started showing a gradual increase in injury. During the 120-min test period cell death occurred in rifampin; the amount was much higher when rifampin and MgSO$_4$ were added.
K₂HPO₄ were present together. Thus, a combination of K₂HPO₄ and rifampin produced both repair of some cells and death of other cells at the same time. Similar results, but with more extensive death, were obtained with actinomycin D (Table 1). A higher percentage of repair with actinomycin D was apparently due to a simultaneous increase in TSYDA counts (decrease in injury) and decrease in TSYA counts (increase in death). 5-Fluorouracil (5-FU, 100 μg/ml) did not inhibit repair in K₂HPO₄. Cell growth in minimal broth after incubation at 35°C for 24 hr was inhibited by rifampin and 5-FU but not by actinomycin D.

**DNA synthesis inhibitor.** The rate and the total amount of repair during the 120-min test period were much higher when hydroxyurea (HU, 500 μg/ml) was present in combination with K₂HPO₄ than when HU was present alone. In HU alone, cells showed a gradual increase in injury. Cell growth in minimal broth occurred in the presence of HU (data not presented).

**ATP synthesis inhibitors.** Uncouplers of ATP synthesis, such as 2,4-dinitrophenol (DNP, 100 μg/ml), when present along with K₂HPO₄, reduced both the initial rate and the total amount of repair during the 120-min incubation period (Fig. 4A and Table 2). DNP when present alone increased both death and injury of the cells. Other uncouplers, such as cyanide (100 μg/ml), azide (500 μg/ml), gramicidin (10 μg/ml), and oligomycin (10 μg/ml), also showed different amounts of inhibition (Table 2). Gramicidin and to some extent oligomycin produced cell death, both in the absence and presence of K₂HPO₄. Cell growth in minimal broth after 24 hr at 35°C was inhibited by cyanide and azide but not by DNP, gramicidin, or oligomycin.

The inhibition of injury repair in K₂HPO₄ by the uncouplers suggested that the repair process might be dependent upon the synthesis of ATP. This was observed to be true, as the numbers of uninjured cells (i.e. TSYDA counts) increased from an original 6% (control) to about 46% after incubation in 5 mM ATP for 2 hr at 25°C (Fig. 4B). MgSO₄ (0.01%) potentiated the repair in K₂HPO₄ whereas no such effect of MgSO₄ was observed with ATP.

**Mucopeptide synthesis inhibitors.** K₂HPO₄ when present with penicillin (100 μg/ml) produced a greater degree of repair than when present alone (Fig. 5). When present alone penicillin produced about 15% cell death. Similar results were obtained also with d-cycloserine (20 μg/ml). Both penicillin and d-cycloserine inhibited cell growth in minimal broth for 24 hr at 35°C (data not presented).

**Effect of surface-active agents and lysozyme on the survival of freeze-injured E. coli cells.** Unfrozen and frozen-thawed E. coli cells in water were treated with sodium deoxycholate (0.1%), sodium lauryl sulfate (0.1%), and lysozyme (100 μg/ml) for 1 hr at 25°C and plated on TSYA to measure survivors (Fig. 6). Unfrozen cells showed almost no susceptibility, but frozen cells (with 90% injured cells) showed 50% or more reduction in TSYA count. However, when the injured cells were first allowed to repair in either K₂HPO₄ (0.5%) or in K₂HPO₄ and MgSO₄ (0.01%) for 1 hr at 25°C and then exposed to these agents for

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**Table 1. Effect of RNA synthesis inhibitors on repair and growth of freeze-injured E. coli at 25°C**

| Inhibitors in suspending medium (pH 7.0) | Percent repair in 0.5% K₂HPO₄ at 20 min | Percent death at 120 min in K₂HPO₄ | Growth in minimal broth |
|------------------------------------------|----------------------------------------|---------------------------------|------------------------|
| Control                                  | 35                                     | 52                              | +                      |
| Rifampin (10 μg/ml)                      | 29                                     | 43                              | 8                      |
| Actinomycin D (10 μg/ml)                 | 62                                     | 78                              | 42 ++                  |
| 5-Fluorouracil (100 μg/ml)               | 42                                     | 48                              | 5                      |

*Initial amount of uninjured cells was 3%. Any increase above this was considered due to repair."
Another 1 hr, the susceptibility of the cells was greatly reduced. This was indicated by relatively less reduction in TSYA count of the repaired cells. Lysozyme (10 µg/ml) actually produced lysis of the freeze-injured cells, as observed by reduction on optical density at 420 nm (data not presented).

**DISCUSSION**

Synthesis of various cellular components during repair of injury induced by sublethal stresses has been observed in many gram-negative bacteria (17, 19). To determine whether similar processes also occurred during the repair of freeze-injury of *E. coli* cells in the presence of K$_2$HPO$_4$, various inhibitors were used. We assumed that the leaked materials from the dead and injured cells (14), as well as

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**TABLE 2. Effect of ATP synthesis inhibitors on the repair and growth of freeze-injured *E. coli* at 25 C***

| Inhibitors in suspending medium (pH 7.0) | Percent repair in 0.5% K$_2$HPO$_4$ at 20 min | Percent death at 120 min in K$_2$HPO$_4$-inhibitor | Growth in minimal broth |
|---------------------------------------|---------------------------------------------|-----------------------------------------------|------------------------|
| Control                               | 24                                          | 5                                             | +                      |
| 2,4-Dinitrophenol (100 µg/ml)         | 3                                           | 5                                             | +                      |
| Sodium cyanide (100 µg/ml)            | 10                                          | 5                                             | +                      |
| Sodium azide (500 µg/ml)              | 6                                           | 6                                             | +                      |
| Gramicidin (10 µg/ml)                 | 4                                           | 30                                            | +                      |
| Oligomycin (10 µg/ml)                 | 10                                          | 12                                            | +                      |

*Initial amount of uninjured cells was 3%.

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**Fig. 4. A, Effect of 2,4-dinitrophenol (DNP, 100 µg/ml) on the repair of freeze-injured *E. coli* in 0.5% K$_2$HPO$_4$ (P) at pH 7.0 and 25 C. For other abbreviations see Fig. 1. B, Effect of 0.5% K$_2$HPO$_4$ (P) and 5 mM ATP with or without 0.01% MgSO$_4$ (Mg$^{2+}$) at pH 7.0 on the repair of freeze-injured *E. coli* after incubation for 2 hr at 25 C. Initially all the samples had about 6% uninjured cells. After 2 hr this amount remained unchanged in water, but increased in other media. The samples were plated on TSYA and TSYDA. Percent uninjured = (TSYDA counts/TSYA counts) x 100.

**Fig. 5. Effect of penicillin (PEN, 100 µg/ml) on the repair of freeze-injured *E. coli* in 0.5% K$_2$HPO$_4$ (P) at pH 7.0 and 25 C. For other abbreviations see Fig. 1.
the intracellular pool of the injured cells, might serve as an immediate source for possible synthesis of the components that might be necessary for repair of injury. However, they could not aid in repair when K$_2$HPO$_4$ was absent.

The metabolic process which occurs during the rapid repair of freeze-injured E. coli in K$_2$HPO$_4$ may not involve the synthesis of proteins, RNA, mucoperptides, or deoxyribonucleic acid (DNA); however, it may involve the synthesis of an energy-rich compound in the form of ATP. The ATP, whether synthesized from K$_2$HPO$_4$ or added exogenously, is utilized in the repair process. ATP synthesis from K$_2$HPO$_4$ might occur through oxidative phosphorylation (7) or through proton motive force (4). Mg$^{2+}$ accelerates this process. ATP synthesis during the repair of injury caused by other kinds of sublethal stresses in gram-negative bacteria has been observed (6, 9, 12, 13, 19). The repair process of "metabolic injury" involving the synthesis of protein and RNA (8, 17) appears to be different from the ATP-dependent repair process. Assuming that they enter into the injured cells, the inhibitors of protein, RNA, and mucoperptide synthesis used (10, 11, 20) were effective against E. coli since they prevented growth of the uninjured cell in a minimal broth (except actinomycin D), even when they failed to inhibit the repair process of the injured cell in K$_2$HPO$_4$. In contrast, inhibitors of ATP synthesis (4, 7, 10) affected the repair in K$_2$HPO$_4$, but did not prevent growth of E. coli in a minimal broth (except cyanide and azide). HU, an inhibitor of DNA synthesis (15), did not inhibit the repair on K$_2$HPO$_4$ and growth in a minimal broth. It was thus difficult to determine whether or not DNA synthesis occurred during the repair of freeze-injury in E. coli.

The cause of enhanced repair observed in this study in the presence of puromycin, tetracycline, HU, penicillin, and n-cycloserine is not clear. It might be caused by their direct stimulation of the repair process, such as increase in the utilization of K$_2$HPO$_4$, or might be due to the presence of essential cations (e.g., Mg$^{2+}$) in the inhibitors.

Inhibitors of protein synthesis produced apparently contradictory results. The repair process appeared to be inhibited by kanamycin and streptomycin, whereas chloramphenicol, tetracycline and puromycin did not show any inhibition. This may have been due to chelation of divalent cations from the cellular pool by kanamycin and streptomycin, since addition of Mg$^{2+}$ neutralized their inhibitory effect. While kanamycin and streptomycin specifically inhibit protein synthesis, they have other nonspecific effects, such as binding of cations and

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**Fig. 6.** Effect of sodium deoxycholate (DC), sodium lauryl sulfate (LS), and lysozyme (LZ) on the TSYA counts (i.e., survival) of unfrozen, frozen, and repaired E. coli at pH 7.0 and 25°C. Initial cell counts in all suspending media were the same as in water at 0 hr for any particular group. (For other explanations see Results.)
lysis of the cell membrane (10, 11). Similarly, the data showing simultaneous death and repair in the presence of rifampin and actinomycin D are conflicting. We suspect that the freeze-thawed E. coli population has cells with a varying degree of injury. The ones that are extensively injured are very sensitive to these inhibitors whereas the ones which are less injured are relatively less sensitive to them and are also able to repair in K$_2$HPO$_4$. The enhanced death of frozen cells in actinomycin D and rifampin in the presence of K$_2$HPO$_4$ could be due to a high intracellular accumulation of these inhibitors facilitated by K$_2$HPO$_4$. Unfrozen E. coli cells normally are impermeable to actinomycin D but become permeable after freezing due to the impairment of the permeability barrier (1). Gram-negative bacteria have been shown to be permeable to actinomycin D after EDTA treatment (6), freeze-drying (13, 17), and freezing (12). The growth of the frozen E. coli cells in minimal broth containing actinomycin D was probably due to the growth of the small percentages of uninjured cells in the population.

The frozen E. coli cells become sensitive to surface-active agents, such as deoxycholate and lauryl sulfate, and to lysozyme at concentrations which otherwise do not have any effect on unfrozen cells. Sensitivity to many surface-active agents and hydrolytic enzymes by the sublethally stressed gram-negative bacteria has been reported (3, 5, 12, 16, 18). This increased sensitivity of frozen E. coli cells to surface-active agents and lysozyme may result from the damage to the structure(s) which normally protect the cells from the action of these agents. In the gram-negative bacteria, the outer lipopolysaccharide layer of the cell wall (2) protects the mucopeptide layer of the wall and the lipoprotein layer of the membrane against the lytic action of lysozyme and surface-active agents, respectively. In the freeze-injured cells, the lipopolysaccharide layer probably undergoes some kind of change and allows lysozyme and the surface-active agents to come in contact with their respective substrates. This change in freeze-injured cells is reversible and can be repaired in K$_2$HPO$_4$, probably through the synthesis and utilization of ATP.

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