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Characterization of Injury Incurred by *Escherichia coli* upon Freeze-Drying[1]

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When cells of *Escherichia coli* ML30 were suspended in 2% gelatin and frozen at −40 C, no appreciable metabolic damage or death occurred. After freeze-drying for 8 hr at a platen temperature of 49 C and rehydration with a mineral salts medium, survival of the cells was 0.6%. Metabolic damage of the survivors was found to be 23%. Permeability alterations were detected by several criteria. Freeze-dried cells were susceptible to antibiotics normally ineffective against *E. coli* and leakage of ribonucleic acid (RNA) occurred. Analysis of ribosomal extracts of rehydrated freeze-dried cells demonstrated the presence of appreciable degradation products. Permeability alterations were shown to be reversible by the observation that antibiotic susceptibility was a time-dependent process and that the gratuitous inducer of β-galactosidase was not concentrated by freeze-dried cells until the injured cells had been incubated in a nutrient medium for 300 min or more. At approximately the same time, metabolic damage was repaired. RNA synthesis preceded protein synthesis by about 150 min, and deoxyribonucleic acid synthesis occurred with the resumption of normal growth. This was interpreted to be the result of repair of RNA taking place before protein synthesis and growth could resume. A pronounced increase in the lag time of freeze-dried cells was also observed. Peptides and Casamino Acids shortened the lag time for freeze-dried cells but not for the controls. Glycerol and glucose were found to be better carbon sources for growth of freeze-dried cells than sodium lactate or sodium succinate.

The relationship between cellular injury and death is a perplexing problem in nature. Exposure of an organism to unfavorable environmental conditions or sublethal stresses, by either physical or chemical means, may result in physiological injury which is not immediately lethal. Subsequent treatment of such a cell, by supplying the proper nutrients or a more suitable environment, may afford the cell an opportunity of overcoming the injury and thus continuing as a viable organism.

Injury has been demonstrated in cells exposed to heat (12–14), chilling or freezing (3, 17, 18, 20, 27, 28), ionizing radiation (1, 5), various chemicals (11), and to freeze-drying (10, 23, 24). Characteristics of injured organisms are an extended lag time, leakage of cellular materials (primarily nucleic acids), an increased sensitivity to selective media, and an increased nutritional requirement (12, 13, 17, 18, 20, 23, 26–28).

In the present study, the injury incurred by *Escherichia coli* during freeze-drying was characterized in an attempt to determine the similarities or differences between injury caused by freeze-drying and by other types of stress. In addition, the sequence of metabolic events that occur during recovery of the injured cells was investigated.

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MATERIALS AND METHODS

Media employed for the growth and enumeration of E. coli. The test organism used in these studies was *E. coli* ML30 obtained from J. Ingraham, University of California, Davis. The complete medium used for determining the number of viable cells was Trypticase Soy Agar (BBL) supplemented with a 0.5% yeast extract (TSY). The minimal salts-agar (MM) employed had the following composition (grams/liter): sodium citrate, 2.5; ammonium sulfate, 5.0; magnesium sulfate, 1.0; potassium phosphate (monobasic), 2.0; sodium chloride, 1.0; glycerol, 2.0; and agar, 15.0.

In preparing MM, the basal salts and agar were prepared separately at double strength and were combined aseptically after sterilization. Both TSY and MM agars were sterilized with steam at 15 psi for 15 min. The minimal medium without agar (MB) served

as the primary growth and rehydration medium for freeze-dried *E. coli* cells.

Desoxycholate Agar (Difco) was used to evaluate the ability of freeze-dried *E. coli* cells to grow on a selective medium.

Enumeration of the organisms on all agar media was performed by the pour plate method. For each serial dilution assayed, four replicate plates were prepared. The diluent employed for all assays was chilled: (3 C) 0.1% Trypticase (BBL) in 0.0003 M KH_2PO_4 (*p*H 7.0).

The TSY plates were incubated at 37 C for 24 to 48 hr, whereas the MM and Desoxycholate plates were incubated for 48 to 72 hr at 37 C.

Variability in the determination of the plate counts was measured by a standard error analysis. The Student *t* test (4) was employed to determine the statistical significance of the differences in recovery from the various media.

Preparation and freeze-drying of E. coli. An inoculum of *E. coli* cells from a 24-hr stock culture was grown in minimal broth on a rotary shaker at 37 C. After incubation for 24 hr, 0.1 ml of broth was transferred to fresh growth medium. After two additional transfers in fresh broth, the cells were harvested during the stationary phase of growth by centrifugation for 10 min at 27,000 × *g* in a Sorvall-RC-2B centrifuge maintained at 2 C. The supernatant fluid was discarded, and the cells were washed twice in chilled growth medium.

A quantity of washed cells sufficient to give a final concentration of 10^8 to 3×10^8 viable cells per ml was added to sterilized 2% gelatin (Difco) solution at room temperature. After thorough mixing, 10-ml portions of the suspensions were transferred to washed and sterilized 5-cm diameter aluminum dishes and frozen at −40 C for 12 hr. The frozen samples were removed from the aluminum dishes and placed on sterile, prechilled perforated aluminum trays.

Samples were dried in a VirTis freeze-dryer for 8 hr at a constant platen temperature of 49 C and at a gas pressure of 10 μm of Hg. The final moisture content was usually less than 1.5% as determined by the official method for analysis of gelatin of the Association of Official Agricultural Chemists. The freeze-drying chamber was filled with prepurified nitrogen gas (Air Reduction Co., Natick, Mass.), and the samples were removed from the freeze-drier and sealed in cans. The cans were punctured, replaced in the vacuum chamber, and flushed twice with nitrogen. The hole was then sealed with solder.

Rehydration procedure employed for determining viability and various types of injury caused by freeze-drying of E. coli. Preliminary investigations established the effects of rehydration temperature, glycerol concentration, and incubation temperature on the viability of freeze-dried *E. coli* cells. It was found that high viability of the freeze-dried cells could be obtained by adding the freeze-dried gelatin samples containing *E. coli* cells to 100 ml of MB in 300- or 500-ml culture flasks (Bellco Glass Inc., Vineland, N. J.) at a temperature of 37 ± 1 C in a model 6-77 New Brunswick rotary shaker set at 150 rev/min. The initial concentration of viable cells ranged from 10^5 to 3×10^5 cells per ml.

As a control in each experiment, *E. coli* cells in the stationary phase of growth were inoculated into MB at the same initial viable cell concentration range used in the freeze-dried samples. The growth of the control cells and freeze-dried cells was followed turbidimetrically by use of a Klett-Summerson colorimeter with a 600-nm filter. At various times, samples were removed for chemical and biochemical tests.

Effect of nitrogen sources, carbon sources, antibiotics and sodium deoxycholate on freeze-dried E. coli cells. The effect of various nitrogen sources on the growth of freeze-dried *E. coli* cells was determined by supplementation of MB. Casamino Acids (Difco) were added to the MB at a final concentration of 0.1%. Glucose, sodium lactate, and sodium succinate (all reagent grade) were added at a final concentration of 0.2%.

Actinomycin D (Merck, Sharp, and Dohme, Rahway, N.J.) was used at a final concentration of 5 μg/ml of MB. The effects of streptomycin sulfate (Nutritional Biochemicals Corp., Cleveland, Ohio) and chloramphenicol (Parke, Davis & Co., Detroit, Mich.), both at a final concentration of 10 μg/ml, were determined by adding the antibiotics to the minimal broth for 30 min, removing a sample, washing the cells, and determining the number of viable cells.

Lysis in the presence of the sodium deoxycholate (Ealing Corp., Cambridge, Mass.) was determined by adding the agent to the minimal broth at a final concentration of 1% and following the change in optical density of a dense suspension of control and freeze-dried cells.

Chemical analysis and biochemical procedure employed. To measure incorporation of L-leucine-*1*-^{14}C into protein, a mixture of L-leucine-*1*-^{14}C (New England Nuclear Corp., Boston, Mass.; specific activity, 25.4 mc/mmole), at a final concentration of 0.05 μc/ml, plus 0.076 mM nonradioactive L-leucine was added to the minimal broth. At various intervals, 2-ml samples were transferred to acid-cleaned tubes (18 × 150 mm) containing an equal volume of cold (0 C) 10% trichloroacetic acid. After cooling, the insoluble residue was collected on membrane filters (25 mm in diameter with a pore size of 0.45 μm; Millipore Corp., Bedford, Mass.), and the precipitate was washed three times with a total of 45 ml of cold 5% trichloroacetic acid. The filters were then glued onto stainless-steel planchets [1.5 × 0.063 × 0.05 inches (3.8 × 0.16 × 0.13 cm), Planchets Co., Chelsea, Mich.] and were dried at room temperature. Radioactivity was measured in a low-background gas-flow counter (model 181A, Nuclear-Chicago Corp., Des Plaines, Ill.). The results were expressed as counts per minute per viable cell.

Uracil-*6*-^3H or thymidine-methyl-^3H (New England Nuclear Corp.; specific activity, 9.6 c/mmole) was added to the minimal broth at a final concentration of 0.25 μc/ml. Nonradioactive uracil or thymidine was also added at a final concentration of 0.00004 or 0.0009 mM, respectively. After 2-ml volume of culture in minimal broth were mixed with an equal volume of cold (0 C) 10% trichloroacetic acid, the acid-insoluble fraction was collected on membrane filters in the same manner as in the experiments with L-leucine-*1*-^{14}C.

After drying at room temperature, the filters were dissolved in 15 ml of scintillation fluid (7), and radioactivity was measured in a model 2002 Packard liquid scintillation spectrometer.

As a measurement of the ribonucleic acid (RNA) leakage by freeze-dried cells, the pentose content of the supernatant fluid obtained by removal of the cells by centrifugation was determined by the orcinol test as described by Merchant et al. (16). Yeast RNA (Worthington Biochemical Corp., Freehold, N.J.) was used as a standard.

Induction and assay of β-methylthiogalactopyranoside (grade A; Calbiochem, Los Angeles, Calif.) were done at a final concentration of 10^{-5} M.

After the addition of the inducer, β-galactosidase was assayed in 1-ml samples of culture at various times. Samples were transferred to tubes containing 1 ml of distilled water, 0.2 ml of 0.013 M *O*-nitrophenyl-D-galactopyranoside (ONGP; grade A, Calbiochem), and one drop of toluene at 30 C. The reaction was stopped by the addition of 2 ml of 1 M Na_2CO_3, and the optical density of the resulting solution was measured at 420 nm.

To measure the effect of freeze-drying on the ability of cells to retain and to concentrate the inducer of β-galactosidase, β-methyl-^{14}C-D-thiogalactopyranoside (New England Nuclear Corp.; specific activity, 0.014 mc/mg) was added to cultures at a final concentration of 10^{-3} M. A 2-ml sample of the radioactive culture was collected on a membrane filter (25 mm in diameter, 0.45 μm pore size) and was washed with a total of 45 ml of MB at 37 C. The samples were dried at room temperature, and then the filters were dissolved in 15 ml of liquid scintillation fluid (7). Radioactivity was measured in a model 2002 Packard liquid scintillation spectrometer.

Sucrose density gradient analysis of crude ribosomal RNA extract. Cells were removed from the suspension in minimal broth 30 min after rehydration by centrifuging at 27,000 × *g* for 10 min in a Sorvall-RC-2B refrigerated centrifuge at 3 C. The resulting pellet of cells was resuspended in and washed twice with 10 ml of 10^{-2} M tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 7.2 (Calbiochem), containing either 10^{-2} or 10^{-4} M $MgCl_2$. The washed cells were disrupted at 0 C by two 1-min sonic treatments (Branson model 25, Rosette cooling cell). During the sonic treatment, 5 μg/ml (30,000 Dornase units of deoxyribonuclease; Nutritional Biochemicals Corp.) was added to the suspension of cells. Whole cells and cell debris were removed by a second centrifugation at 27,000 × *g* for 10 min at 3 C. The supernatant fraction was centrifuged at 110,923 × *g* for 3 hr at 3 C in a Spinco model L preparatory ultracentrifuge with a no. 40 rotor. The resulting pellet was then suspended in 2 ml of Tris buffer (10^{-2} M, *p*H 7.2) containing the desired magnesium ion concentration and 1.5% sucrose, and the amount of RNA in the sample was determined by measuring the optical density at 260 nm of a 0.1-ml sample.

Equal amounts of RNA from freeze-dried and control cells were layered on 5 to 30% sucrose gradient tubes (6). The sucrose solutions were prepared in 10^{-2} M Tris buffer with either 10^{-2} or 10^{-4} M $MgCl_2$. The samples were centrifuged at 90,137 × *g* for 4 or 8 hr in a Spinco model L preparatory ultracentrifuge with an SW-25 rotor. The bottoms of the tubes were then punctured, and 40-drop fractions were collected. Each fraction was then diluted with 3 ml of distilled water, and the absorbancy was measured at 260 nm.

RESULTS

Effects of incubation temperature on the recovery of and extent of damage to freeze-dried E. coli. Freeze-dried cells were rehydrated at 37 C and then incubated at 37 and 20 C. No differences in the recovery of feeeze-dried cells of *E. coli* were observed on TSY incubated at 37 and 20 C (Table 1). In contrast, 54% more cells were recovered on MM at 37 C than at 20 C. Freeze-drying injury to the cells of *E. coli* at the 37 C incubation temperature was 23%, whereas that at 20 C was 58%. The differences in recovery on MM agar versus TSY were significant at the 1% confidence level, as were the differences in the recovery of cells incubated at 37 and 20 C on MM.

Effect of freezing and freeze-drying on survival and injury of E. coli. Experiments were performed to establish the efficiency of recovery of *E. coli* cells on TSY and MM before freezing, after freezing, and after freeze-drying. No significant differences in the recovery on TSY and MM were detected before freezing and after freezing (Table 2). With respect to the cells of *E. coli* frozen in 2% gelatin at −40 C for 12 hr, a slight reduction in viable cells occurred, but, more important, no significant amount of injury (at the 5% confidence level) resulted from the freezing procedure. Freeze-drying at 49 C resulted in a marked decrease in viability of the cells (0.6% survival) and a significant increase in damage (25%) at the 1% level, as determined by the percentage differences in recovery on TSY and MM.

After rehydration of freeze-dried cells, two to three times more RNA could be detected in the

TABLE 1. *Effect of incubation temperature on recovery of and injury to freeze-dried E. coli cells*[a]

Incubation temp	Millions of organisms/g[b]		Damage[c]
	TSY	MM	
C			
37	112 ± 4.0	87 ± 2.0	23
20	112 ± 3.0	47 ± 2.0	58

[a] Cells were rehydrated at 37 C.

[b] Average values of four replicate plates ± SE.

[c] The percentage of damage = (1 − MM/TSY) × 100.

TABLE 2. *Determination of viable cells before freezing, after freezing, and after freeze-drying on TSY and MM*

Conditions	Millions of organisms/g[a]		Damage[b] (%)
	TSY	MM	
Initial numbers in 2% gelatin before freezing	200 ± 2	196 ± 2	2
After freezing in 2% gelatin at −40 C for 12 hr	195 ± 8	189 ± 4	3
After freeze-drying in 2% gelatin at 49 C for 8 hr	1.16 ± 0.02	0.87 ± .01	25

[a] Average of four replicate plates ± SE of plating.
[b] The percentage of damage = (1 − MM/TSY) × 100.

supernatant fluid of the freeze-dried cells than in that of the control cells (Table 3).

By using washed-cell suspensions and layering equivalent amounts of RNA from the control and freeze-dried cells on the separate sucrose gradients, it was found that freeze-dried cells had a lower concentration of 70*S* ribosomes (Fig. 1) than did the control cells. An analysis of the pellet showed that some of the RNA from freeze-dried cells had aggregated and was on the bottom of the tube, thus accounting for differences in the areas under the respective curves. The 50*S* and 30*S* components were examined by conducting the centrifugation in 10^{-4} M Mg^{++} (Fig. 2). Both the 50*S* and 30*S* fractions were identified in the control cells. For the freeze-dried cells, the 50*S* and 30*S* fractions were displaced and were present in reduced amounts.

Sensitivity of freeze-dried cells to various inhibitors. The effects of chloramphenicol, streptomycin, and actinomycin D on the recovery process were determined. None of the antibiotics demonstrated any significant effect on the control cells when present in MB for 30 min at 37 C. However, freeze-dried cells showed a marked susceptibility to each of the antibiotics. Exposure to chloramphenicol resulted in a recovery of 25%; streptomycin, 0.0%; and actinomycin D, 10%.

Freeze-dried *E. coli* cells were also found to be

TABLE 3. *Loss of RNA from freeze-dried E. coli cells during rehydration*

Time after rehydration	RNA (μg/ml of supernatant fluid)	
	Control cells[a]	Freeze-dried cells
min		
1	4.0 ± 2[b]	11 ± 2
90	3.0 ± 2	14 ± 1

[a] For comparative purposes, the leakage from 10^8 control cells was compared with that from the same quantity of freeze-dried cells.
[b] Values represent average of duplicate samples ± SE.

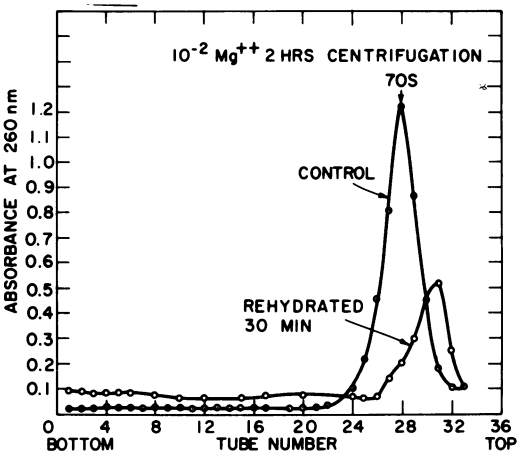

FIG. 1. *Distribution of RNA after 2 hr of centrifugation in a sucrose gradient containing 0.01 M Mg.*

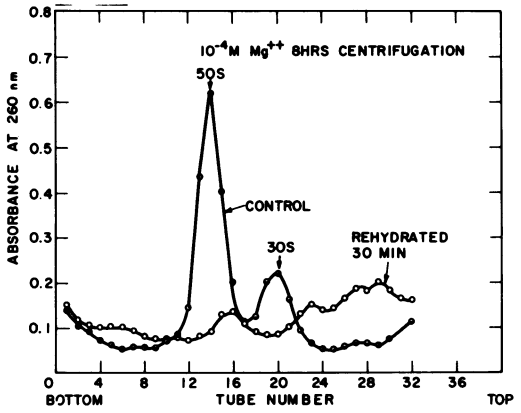

FIG. 2. *Distribution of RNA after 8 hr of centrifugation in a sucrose gradient containing 10^{-4} M Mg.*

more sensitive to the surface-active agent sodium deoxycholate than were control cells. Furthermore, freeze-drying caused a marked and significant decrease in the ability of *E. coli* to grow on Desoxycholate Agar.

Effect of freeze-drying on the induction of β-galactosidase. Equal numbers of control cells and viable freeze-dried cells were induced with 10^{-5} M methylthiogalactoside (TMG). The ability of the freeze-dried cells to synthesize new enzyme was severely depressed (Table 4). After 30 min of incubation, control cells formed four times as much enzyme, and after 60 min of incubation about seven times as much enzyme, as the freeze-dried cells. It was only at 500 min that the amount of enzyme present in the freeze-dried cells was equal to that of the control cells. The ability of freeze-dried cells to concentrate the labeled inducer was also determined because the cells may have been able to concentrate the inducer but unable to synthesize the enzyme (Fig. 3). Although freeze-dried cells showed a slight initial uptake of inducer, it was only after 300 min of incubation that appreciable uptake of the inducer occurred, whereas control cells were able to concentrate the inducer immediately. It is thus seen that the ability to concentrate the inducer precedes enzymatic synthesis by about 200 min.

Determination of the time necessary for the complete repair of freeze-drying injury to E. coli. Samples of freeze-dried cells of *E. coli* were rehydrated in MB, and the extent of injury, as measured by differences in recovery on TSY and MM during incubation, was determined (Fig. 4). The percentage of injury appeared to remain constant for almost 5 hr and then rapidly decreased to zero during the 6th hr of incubation.

TABLE 4. *Synthesis of β-galactosidase in minimal broth*

Time of incubation	β-Galactosidase activity[a]	
	Control cells	Freeze-dried cells
min		
0	0.05 ± 0.01[b]	0.01 ± 0.01
30	3.3 ± 0.4	0.8 ± 1.0
60	7.1 ± 0.3	1.1 ± 0.2
300	7.2 ± 0.4	1.5 ± 1
400	7.3 ± 0.3	1.8 ± 1
500	8.0 ± 0.5	8.3 ± 0.5

[a] β-Galactosidase activity is expressed as 10^{-3} units/viable cell. One unit of β-galactosidase activity is defined as the amount of enzyme that causes 1 μmole of a product (ONGP) to appear per min under the assay conditions.

[b] Values represent average of duplicate samples ± SE.

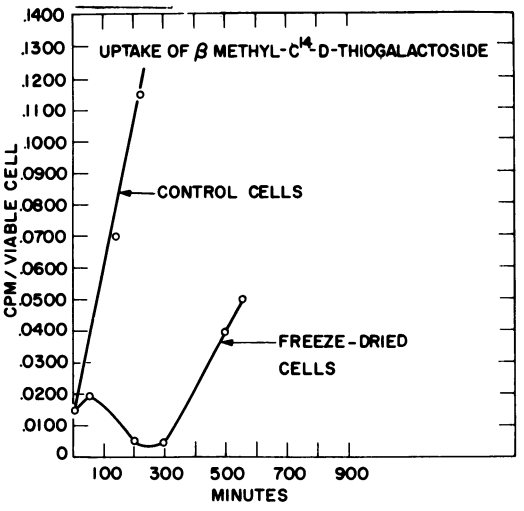

FIG. 3. *Radioactivity of cells exposed to β-methyl-^{14}C-D-thiogalactoside.*

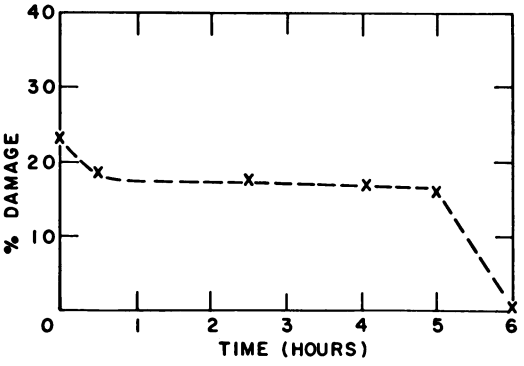

FIG. 4. *Time required for freeze-dried cells to recover from metabolic damage. Per cent damage = (1 − MM/TSY) × 100.*

Other experiments demonstrated that other repair processes were also completed within 5 hr after incubation. One such indication was obtained by studying the effect of actinomycin D on the growth of rehydrated, freeze-dried cells. Actinomycin D is a powerful inhibitor of deoxyribonucleic acid (DNA)-dependent RNA synthesis and is normally ineffective against *E. coli* (14). The lag time for freeze-dried cells in the presence of actinomycin D added at the start of incubation is extended to about 800 min. Figure 5 shows that the addition of actinomycin D after 300 min caused only a slight inhibition of growth of freeze-dried cells, indicating that, in this period, both permeability and metabolic damage were repaired. Growth in the presence of actinomycin D is probably due to the growth of undamaged cells.

Effect of freeze-drying on the synthesis of protein, DNA, and RNA by E. coli. In the case of freeze-dried cells, it was found (Fig. 6) that an increase in uracil-6-^3H uptake begins at 300 min. This was then followed by an increase in protein synthesis after an additional 150 min of incubation (Fig. 7). DNA synthesis, as measured by thymidine uptake (Fig. 8), occurred approximately 100 min after protein synthesis was initiated. In the control cells, the same sequence of events occurred but the intervals of time between synthesis of RNA, protein, and DNA were shorter.

Nutritional aspects of the recovery of freeze-dried E. coli. Figure 9 shows that glucose and glycerol were equally effective as carbon sources for both the control and freeze-dried cells. The other two carbon sources were much less effective

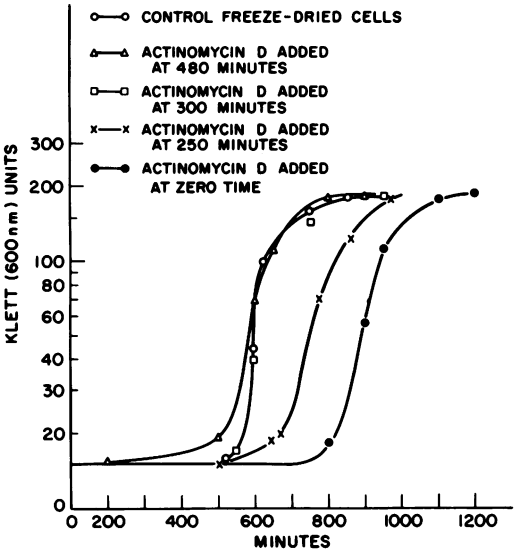

FIG. 5. *Effect of actinomycin D on the growth of freeze-dried cells.*

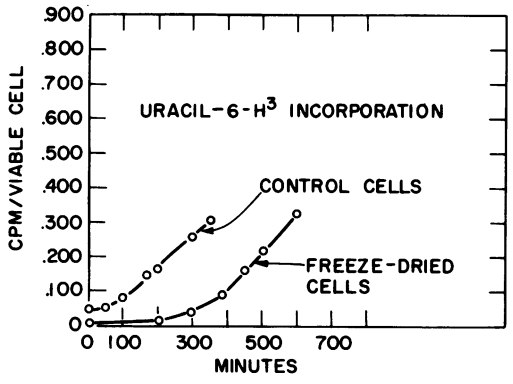

FIG. 6. *Radioactivity of cells exposed to uracil-6-^3H.*

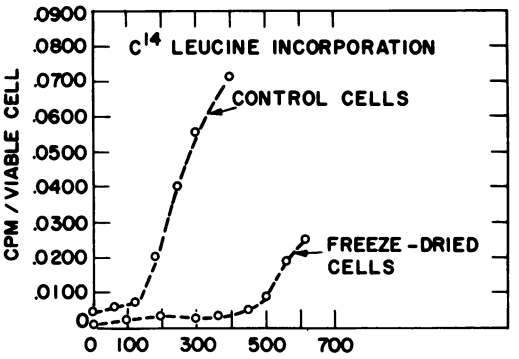

FIG. 7. *Radioactivity of cells exposed to ^{14}C-leucine.*

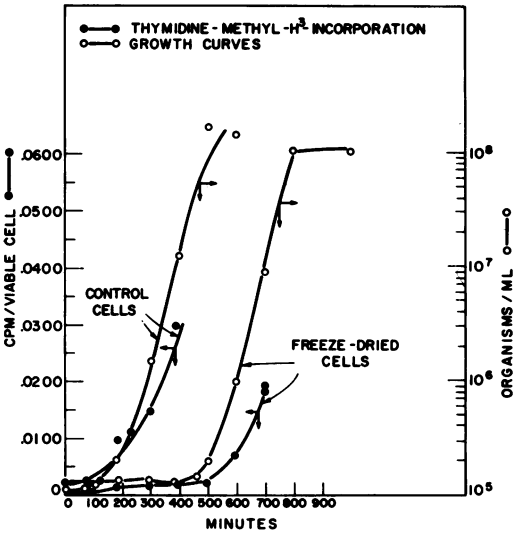

FIG. 8. *Radioactivity of cells exposed to thymidine-methyl-^3H.*

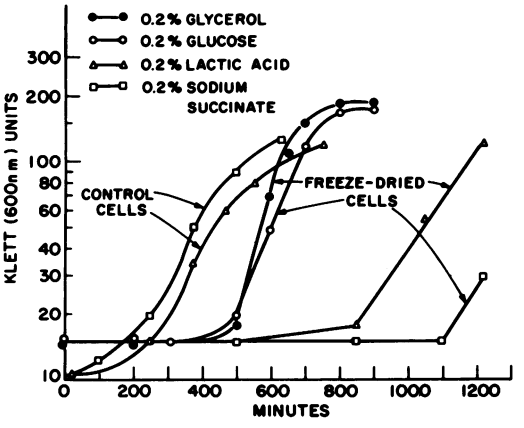

FIG. 9. *Effect of carbon sources on the growth of freeze-dried cells.*

for the growth of freeze-dried cells. Even though the growth rate of control cells with sodium succinate or sodium lactate was only slightly less than with glycerol, the lag time of freeze-dried cells was extended to about 850 min with sodium lactate and to about 1,150 min with sodium succinate (Fig. 9).

In contrast to the effects of the carbon sources examined, the nitrogen sources, such as Casamino Acids, N-Z case, and Trypticase, appreciably decreased the lag time of freeze-dried cells (Fig. 10). Although an initial injury of about 25% was detected for freeze-dried cells in the presence of each of the nitrogen sources investigated, no injury could be detected after 150 min of incubation compared with 300 min in MB. Casamino Acids gave the shortest lag time, followed by Trypticase and N-Z case. However, even in the presence of Casamino Acids, freeze-dried cells still exhibited an extended lag time as compared with control cells.

DISCUSSION

In this study, freezing injury was eliminated by controlling the freezing rate; therefore, the majority of the injury could be attributed to the freeze-drying operation per se. The average number of cells of *E. coli* which survived freeze-drying in 2% gelatin was 0.6% of the frozen cells, and the amount of damage incurred by freeze-drying under these conditions was 28% for the surviving cells. It was previously reported that the survival of *Salmonella typhimurium* freeze-dried in a similar manner was 2%, and the damage ranged from 14 to 34% (23, 24).

The evidence obtained for injury due to freeze-drying and the events occurring before normal growth resumes are schematically illustrated in Fig. 11.

A pronounced initial effect of freeze-drying and rehydration of *E. coli* is an altered permeability of the cell wall and cytoplasmic membrane. This was demonstrated by the observation that freeze-dried cells were lysed by the surface-active agent sodium deoxycholate. Wagman (29) has presented evidence of cytoplasmic injury in dried bacteria by demonstrating the loss of RNA.

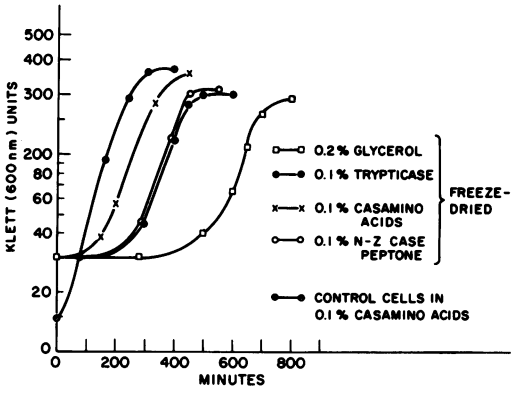

FIG. 10. *Effect of nitrogen sources on the growth of freeze-dried cells.*

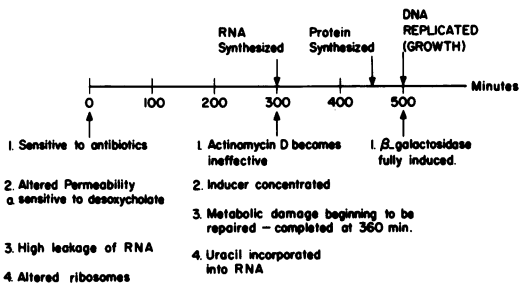

FIG. 11. *Summary of the known events occurring in the recovery and growth of freeze-dried cells.*

Further evidence for cytoplasmic membrane and cell wall damage is indicated by the fact that chloramphenicol, actinomycin D, and streptomycin were all lethal to freeze-dried cells but not to control cells.

The treatment of bacterial cells with chloramphenicol does not result in membrane damage (8), but, rather, in a block in protein synthesis. Normally, actinomycin D is effective in *E. coli* only if the organism is previously treated with the chelating agent ethylenediaminetetraacetate (15). Streptomycin (8) apparently affects both the cell membrane and the ribosomes of exposed organisms. The fact that streptomycin affects the cell in a dual manner may help to explain why freeze-dried cells are much more sensitive to it than to either actinomycin D or chloramphenicol.

Although an increased permeability of freeze-dried cells is evident, the permeability alteration is a reversible phenomenon. This was demonstrated by the fact that actinomycin D became ineffective after 300 min of incubation (Fig. 5).

Concurrent with the regained resistance to actinomycin D, the freeze-dried cells were able to begin concentrating the radioactive inducer TMG. Shortly thereafter, metabolic damage was not detectable. An additional 140 min were required for the initiation of growth, and after this interval normal protein and DNA synthesis began. However, at 300 min, when permeability is repaired, the cells start synthesizing RNA. The interval between synthesis of RNA and synthesis of protein is extended in the freeze-dried cells as compared with control cells. Synthesis of RNA and replenishment of the amino acid pools must take place before protein synthesis can occur; only then can there be repair with resumption of

growth. The lost RNA must be replaced and the damaged ribosomes must be repaired. This is indicated by the fact that uracil uptake precedes leucine uptake by 150 min.

Each of the three amino nitrogen sources examined reduced the lag time necessary for repair, with Casamino Acids being superior to either N-Z case or Trypticase. Casamino Acids notably shortened the lag period, but not to the extent observed for the control cells. It is conceivable that altered permeability and the loss of cellular constituents upon rehydration result in cells that are depleted of amino acids and RNA. This is the same type of result that one obtains in "shift-down" experiments, in which it is thought that the cells are starved for amino acids (19).

Furthermore, Dresden and Hoagland (9) have shown that if *E. coli* is starved of glucose a rapid and marked breakdown of the polyribosomes occurs. The effect of Casamino Acids and Trypticase might be twofold. The first effect is in the replenishment of the amino acid pools, and the second effect is in the stabilization of the ribosomes associated with the bacterial membrane. Membrane-bound polysomes have been described in bacterial systems (21), but it has not been established that the association exists in vivo. However, electron microscopy of membrane sections has revealed clusters of ribosomes near or associated with the membrane (22). A recent report by Aronson (2) demonstrated that, for the binding of ribosomes to bacterial membranes, the presence of nascent polypeptides is the critical factor in establishing association with the membrane. In the presence of polypeptides, polysomes adsorbed on the membrane were more resistant to the action of ribonuclease than were free polysomes. However, this work was done primarily with *Bacillus subtilis*, and it is not at present certain that it is also applicable to *E. coli*.

Freeze-drying combines a number of stresses that may cause injury to microorganisms. A number of characteristics of freeze-dried injured cells have been reported for other organisms injured in a variety of ways. For instance, an extended lag time has been demonstrated as a culture response to thermal injury (12, 13). Furthermore, an increased nutritional requirement, as well as leakage of nucleic acids, is also a common occurrence in freeze-injured cells (23, 24, 29).

The evidence reported here indicates that RNA and permeability alterations may be the most sensitive sites detectable in sublethally injured microorganisms. Recently, Sogin and Ordal (25) demonstrated that the site of thermally induced injury to *Staphylococcus aureus* may be ribosomal RNA and that the ribosomal RNA is resynthesized during the extended lag time.

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