MECHANISM OF INJURY OF ESCHERICHIA COLI BY FREEZING AND THAWING

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SUMMARY: Freezing and thawing of Escherichia coli B/r has two types of lethal effect, (i) immediate, i.e. that occurring during freezing at -20°C or -196°C and immediate thawing, and (ii) delayed, i.e. that occurring during frozen (-20°C) storage. Alkaline sucrose gradient (5-20%) centrifugation revealed 1 to 6 single strand breaks (SSB) in the [^{3}H] DNA from frozen E. coli cells. The number of SSB showed a quantitative correlation with the extent of cell death, immediate as well as delayed. On this basis we conclude that cell death of E. coli by freezing, cold storage, and thawing is due to DNA degradation and loss of its vital integrity.

At least four theories have been proposed to explain cell death due to freezing and thawing (1): i. mechanical crushing resulting in "holes" and "cracks" in the cell; ii. harmful effect of concentrated solutes remaining after water is selectively "frozen out"; iii. intra-cellular ice crystals; and iv. metabolic injury, an ill-defined "catch all" group of harmful effects. None of these can be categorically supported or denied by current experimental data. The observations reported here raise yet another possibility, viz. v. DNA fragmentation as the possible cause of cell death from freezing and thawing. Knowledge of underlying molecular events is essential in low temperature preservation of biomedical materials: bacteria, blood, sperm, bone marrow, and various tissues used to replace damaged or diseased parts in patients, e.g., cornea, tendon, skin, bone, blood vessel and heart valve grafts, etc. (1)

MATERIALS AND METHODS. E. coli B/r, a highly freeze-sensitive organism (2) was grown in M-9 broth for 3-4 hrs at 37°C, then a 10% inoculum was transfered to fresh M-9 broth containing 10μCi/ml ^{3}H - thymidine for labeling of the cells DNA. After 3-4 hrs of growth the cells were harvested by centrifugation, washed thrice with cold M-9 broth and resuspended in the same broth to a final concentration of 2 x 10^8. These cells were immediately subjected to experimental treatments viz, either lysed on top of alkaline sucrose gradients (5-20%) (3) or immediately frozen as described in Table 1. The cells were frozen slowly in a -20°C freezer or rapidly in liquid nitrogen at -196°C. Cells were thawed at 25°C. No attempt was made to protect the cells, since it was

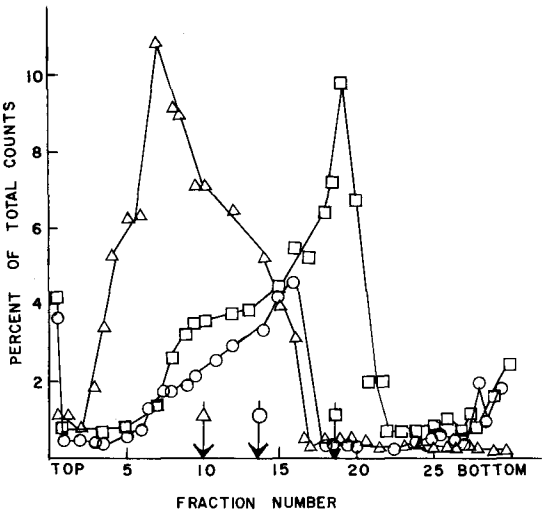

Fig. 1. Alkaline sucrose gradient (5-20%) sedimentation patterns of [^{3}H] DNA from frozen and thawed cells of E. coli B/r. Cells frozen and immediately thawed at 25°C, -[]-, unfrozen control; -0- frozen at -20°C; -Δ-, frozen at -196°C. The marked arrows indicate the position of the appropriate sedimentations distances (D) as explained in Table 1 (footnote*).

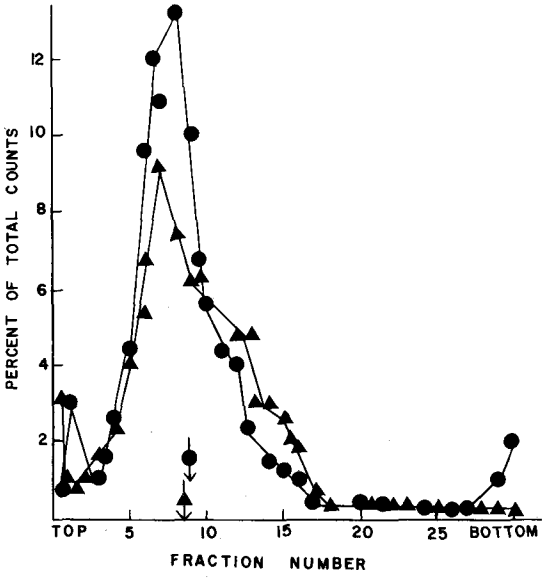

Fig. 2. Alkaline sucrose gradient (5-20%) sedimentation patterns of [^{3}H] DNA from frozen and thawed cells of E. coli B/r. Cell frozen, cold stored at -20°C for 24 hrs and then thawed at 25°C. -●-, frozen at -20°C; -▲- frozen at -196°C. The marked arrows indicate the position of the appropriate sedimentation distances (D) as explained in Table 1 (footnote*).

Table 1. Effect of freezing and thawing on E. coli B/r: Viability of cells and fragmentation of DNA as determined by alkaline sucrose gradient centrifugation.(3)

Sample and method of freezing (cells frozen in broth).	Time of thawing	Viable counts	% cell death	Alkaline sucrose gradient centrifugation of DNA		
				(D)*	MW, daltons**	#SSB***
a. unfrozen control	- - - - -	2.2×10^8	00.0	18.5	1.51×10^8	- - -
b. slowly frozen, -20°C	immediate	1.69×10^8	22.7	13.8	6.50×10^7	1.16
c. slowly frozen, -20°C	after 24 hrs at -20°C	1.28×10^7	94.1	8.8	1.80×10^7	6.05
d. rapidly frozen, -196°C	immediate	7.12×10^7	67.6	10.0	2.60×10^7	4.04
e. rapidly frozen, -196°C	after 24 hrs at -20°C	3.29×10^7	85.0	9.1	2.00×10^7	5.46

* Sedimentation distance $D = \Sigma XiYi/\Sigma Yi$ where Yi is the fraction of radioactivity in the i-th fraction at a distance Xi from the meniscus.(4)

** Calculated using the formula $\frac{M_1}{M_2} = \log^{-1} 2.86 \log \frac{D_1}{D_2}$, where D_1 and D_2 represent the sedimentation distances of unfrozen control and frozen cells' DNA, respectively.(6)

*** Number of DNA single-strand breaks (SSB) calculated from the formula $SSB = \left(\frac{D_1}{D_2}\right)^{2.63} - 1$, where D_1 and D_2 are the average sedimentation distances of unfrozen and frozen cells' DNA, respectively.(6)

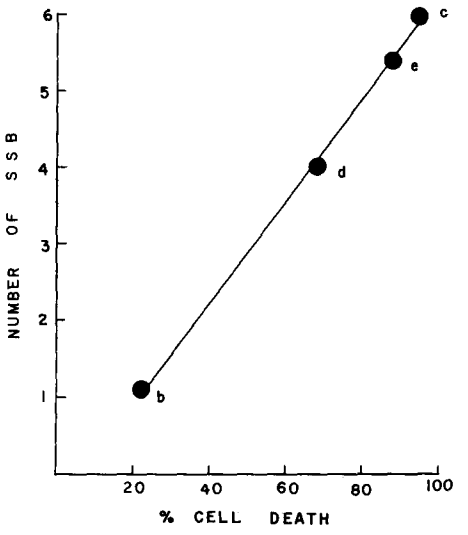

Fig. 3. Correlation between the number of DNA single strand breaks and the extent of cell death of E. coli B/r due to freezing, cold storage and thawing. For method of SSB calculation see Table 1. The samples are marked by letters next to the points, the letters are the same as those used for corresponding samples in Table 1.

our aim to study the nature of cell injury. Viability was assessed on the surface of L-agar plates.

RESULTS AND DISCUSSION. Sedimentation patterns of [^{3}H] DNA clearly show that in all cases the DNA from frozen cells was extensively fragmented. Rapid freezing (-196°C) caused more extensive immediate DNA fragmentation than slow freezing (-20°C) (Fig. 1). However, on storage at -20°C for 24 hrs additional (delayed) fragmentation of DNA occured in the slowly frozen cells so that in the end all samples showed approximately the same extend of DNA fragmentation (Fig. 2).

From Table 1, freezing and thawing had two types of lethal effects, an immediate and a delayed. The immediate death i.e. that after freezing and immediate thawing, was considerably higher when cells were rapidly frozen at -196°C (ca. 68% death) vs. slowly frozen cells (-20°C) and immediately thawed (ca. 23% death). The delayed or storage death, i.e. that which occurred during storage of cells in the freezer at -20°C for 24 hrs showed just the reverse pattern; it was 71% for slowly frozen cells, but only 17% for rapidly frozen

cells. In the end, both freezing schedules yielded practically identical cell death after storage, i.e. 85-94%.

A remarkable correlation emerged from Table 1 between percent cell death, immediate as well as delayed, and the number of single strand breaks (SSB) in the DNA (Fig. 3). Thus, freezing at -196°C which caused a high rate of immediate cell death caused also a high degree of DNA fragmentation (SSB=4.04). Slow freezing at -20°C which caused a relatively smaller degree of immediate cell death caused also a smaller amount of DNA breakage (SSB=1.16). For unfrozen control SSB=0, by definition. The same trend appeared to hold for the delayed cell death during storage at -20°C for 24 hrs. This was particularly striking with the slowly frozen cells where SSB increased from 1.16 to 6.05 while an additional 71% of the cells suffered loss of viability. With rapidly frozen cells, both loss of viability and change in SSB were considerably smaller during storage at -20°C for 24 hrs.

The picture which emerges from the studies indicates a molecular basis for cell death by freezing, cold storage and thawing as being due to DNA degradation and loss of its vital integrity. A quantitative correlation seems to exist between the number of SSB and percent cell death. Since DNA is attached to plasma membrane (5), our idea can conceivably be reconciled with the older ideas attributing freeze death to plasma membrane injury.

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