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FREEZE-THAW INJURY: EVIDENCE FOR DOUBLE STRAND BREAKS IN ESCHERICHIA COLI DNA

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SUMMARY - Neutral sucrose gradient sedimentation studies indicate that freeze-thawing of Escherichia coli B/r results in double strand breakage of its DNA. Breaks were detected after one hour of frozen storage at -16°C, resulting in a distinct displacement of the major peak of [^{3}H]DNA toward the meniscus. In addition at this time a minor shallow peak of radioactivity toward the meniscus appeared indicating accumulation of some small molecular weight DNA fragments of heterogeneous size. These small DNA fragments seemed to become the major DNA breakage product after 1½ and 4 months of frozen storage. Double strand DNA breakage continued through 8 and 12 months of frozen storage. However, the sedimentation profile became progressively more broad and shallow suggesting that the initial DNA fragments may have reassociated into molecular aggregates of heterogeneous molecular composition.

INTRODUCTION

At least four mechanisms have been considered to explain cell death due to freeze-thawing (1): i. mechanical damage to the cell by ice crystals or by pressures generated by ice, ii. harmful effect of concentrated solutes in the cell after the water is "frozen out", iii. metabolic injury, an ill-defined "catch all" group of harmful effects, and iv. single strand breaks in the DNA (2). Using the alkaline sucrose gradient method (3), an apparent correlation was observed between the number of single strand breaks in Escherichia coli B/r DNA and the extent of cell death as the result of initial freeze-thaw treatment. When the cells were stored in the frozen state for longer periods of time, a similar correlation was noted between continued additional single strand breakage of DNA and progressive delayed cell death (2); such delayed death of frozen cells has also been reported previously by other investigators (4).

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Cryoprotective substances such as dimethylsulfoxide and glycerol protect cells against both loss of viability and induction of single strand breakage during freezing and thawing (5). Generally, however, it is thought that single strand breakage can be efficiently repaired especially in such repair competent organisms as Escherichia coli B/r, and that double strand breaks are more likely to be the lesions responsible for cell death (6). Induction of double strand breaks in Escherichia coli cells subjected to freezing and thawing had not been studied so far. However, under conditions of injury from mild heat in the case of Escherichia coli (7), or by radiation in the case of Micrococcus radiodurans (8), the existence of double strand breakage has been demonstrated by the neutral sucrose gradient centrifugation method (6).

In view of the evidence of the involvement of double strand breaks in heat and radiation injury and the possible relation of double strand breaks to loss of cell viability, it was of interest in the present study to investigate the possiblility of induction of double strand breaks in cells subjected to freeze-thaw injury.

MATERIALS AND METHODS - Escherichia coli B/r, a freeze-sensitive organism (9), was grown in L-broth at 37°C overnight on a shaker. Then 1.0 ml of the actively growing culture was transfered to another flask containing 25.0 ml L-broth and incubated on a shaker at 37°C. When an O.D. = 0.3 was attained, 2.0 ml of the cells were transferred to a flask containing 20.0 ml L-broth supplemented with 2.5 μCi/ml [^{3}H]thymidine to label the DNA. The culture was grown on a shaker at 37°C for a period of 4 hours at which time log phase growth was reached. At this point the cells were harvested, washed with 0.01 M Tris buffer (pH 8.0) and adjusted to a final concentration of 2-4 x 10^9 cells/ml. One ml aliquots were then taken and frozen at -16°C for periods varying from 1 hour to 1 year. After the desired storage time, the cells were thawed at 25°C and immediately assayed for DNA double strand breaks by the neutral sucrose gradient centrifugation method (pH 8.0) as described by Clewell and Helinski (10). No precautions were taken to protect the cells during freeze-thawing, since our aim was to study cell injury.

RESULTS AND DISCUSSION

As shown in Figure 1A(a), the [^{3}H]DNA from the control (unfrozen) cells consistently formed a sharp narrow peak at a sedimentation distance of 0.77 from the meniscus. In comparison, the sedimentation patterns of DNA from frozen cells (Figure 1A(b) to Figure 1E) shifted toward the meniscus

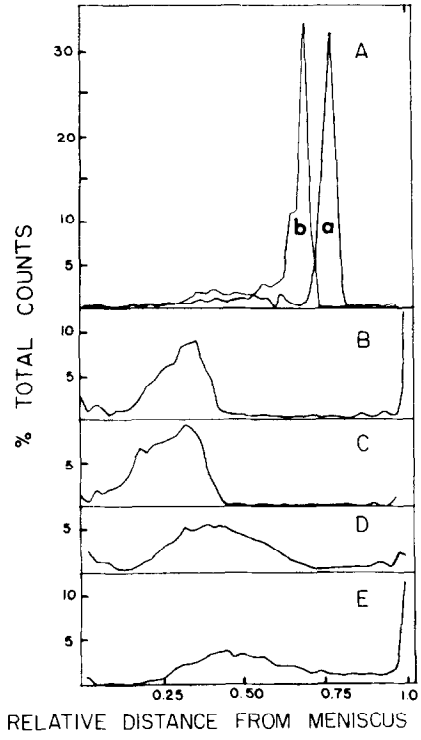

Figure 1. Neutral sucrose gradient sedimentation profiles of [^{3}H]DNA from Escherichia coli B/r subjected to freezing at -16°C, followed by frozen storage at -16°C for various periods of time and subsequently thawing at room temperature and analysis by the neutral sucrose gradient sedimentation method (10). Cold storage at -16°C was for the following periods of time: A(a). 0 hour (non-frozen control); A(b). 1 hour; B. 1½ months; C. 4 months; D. 8 months; and E. 12 months.

indicating that the DNA fragments from frozen cells were smaller than those from unfrozen controls and sedimented at a slower rate. As shown in Figure 1A(b), the DNA from cells frozen for one hour exhibited a sharp and narrow sedimentation profile similar to that of the DNA from unfrozen cells except that: i. the sharp peak shifted toward the meniscus (fractions 0.63 to 0.73), and ii. a second (flat) peak of [^{3}H]DNA seemed to emerge between fractions 0.30 to 0.53 suggesting that the DNA from frozen cells contained a sub-population of relatively small molecular weight fragments of heterogeneous sizes. The flat peak at this sedimentation distance became increasingly pronounced on longer storage. After 1½ and 4 months at -16°C (Figures 1B and 1C), the DNA profiles showed additional shifts toward the meniscus indicating that the DNA fragments isolated from these stored cells became progressively

smaller. On frozen storage for 8 to 12 months (Figures 1D and 1E), the DNA sedimentation profiles became increasingly flat and wide with no distinct peaks such as were observed in unfrozen cells (Figure 1A-a) or in cells frozen and thawed after one hour of frozen storage (Figure 1A-b).

From the observed shifts of the [^{3}H]DNA sedimentation profiles toward the meniscus in Figure 1, it seems clear that DNA breakage had occurred as the result of cold injury. Furthermore, the DNA breaks must have been double strand breaks since single strand breaks are not detected by the neutral sucrose gradient method used in this study. The double strand breakage of the DNA was especially pronounced during the initial freeze-thawing as well as during the first 4 months of frozen storage. However, on frozen storage for longer than 4 months it appeared that in addition to continued DNA breakage there might have been some random reassociation and aggregation of the initial DNA fragments resulting in increased heterogeneity of the molecular population as suggested by the exceedingly shallow and broad shape of the DNA sedimentation profiles obtained after these prolonged periods of cold storage.

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REFERENCES

1. Broom, B. (1967) Thermobiology (edited by A. H. Rose), pp. 511-534, Academic Press, New York.
2. Alur, M. D. and Grecz, N. (1975) Biochem. Biophys. Res. Comm. 62, pp. 308-312.
3. McGrath, R. A. and Williams, R. W. (1966) Nature 212, pp. 534-535.
4. Weiser, R. S. and Osterud, C. M. (1945) J. Bact. 50, pp. 413-439.
5. Alur, M. D., Lewis, N. F., and Grecz, N. (1977) FEMS Letters 1, pp. 367-369.
6. Kaplan, H. S. (1966) Proc. U.S. Nat. Acad. Sci. 55, pp. 1442-1446.
7. Woodcock, E. and Grigg, G. W. (1972) Nature New Biol. 237, pp. 76-79.
8. Kitayama, S. and Matsuyama, A. (1971) Agr. Biol. Chem. 5, pp. 644-652.
9. Christophersen, J. (1973) Temperature and Life (edited by H. Precht, J. Christophersen, H. Hensel, and W. Larcher), p. 2-59, Springer Verlag, New York.
10. Clewell, D. B. and Helinski, D. R. (1969) Proc. U.S. Nat. Acad. Sci. 62, pp. 1159-1166.