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Latent injury in frozen–thawed bacteriophage T4Bo

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SUMMARY

Two interesting new phenomena have been observed in suspensions of T4Bo bacteriophage which were frozen to temperatures below the eutectic temperature of the salt (sodium chloride) in the suspending medium. Approximately 10% of the phage appeared to survive such a phase change as determined by plaque titre. However, exposure of these survivors to ultrasonic vibration or repeated freezing showed them to be hypersensitive and thus latently injured. The hypersensitivity was lost on incubating the phage at 37° C. for 3 hr. Furthermore, following a eutectic phase change, the surviving phage could be inactivated by rapid cooling to −90° C. followed by slow rewarming. Such inactivation cannot be accounted for by accepted theories of freezing injury.

INTRODUCTION

The T4 and T4Bo (osmotic shock-resistant) bacteriophage of *Escherichia coli* have proved a useful experimental model for investigating the effects of freezing and thawing on biological systems, and have allowed osmotic shock, salt denaturation and eutectic phase change injury (eutectic injury) to be identified as distinct mechanisms of freeze–thawing inactivation (Leibo & Mazur, 1966; Steele, Davies & Greaves, 1969*a*, *b*; Steele, 1972*a*). The nature of the eutectic injury is the least understood, although it has been suggested that the change of the external phase from liquid to solid may result in loss of water essential for the stability or interactions of structural proteins. The prevention of eutectic injury by low concentrations ($\sim 2 \times 10^{-3}$ M) of small basic peptides and the inhibition of such protection by amino acids (Steele, 1972*b*), supports the argument for such a physico-chemical basis of inactivation, rather than a direct mechanical effect of salt and ice crystals. In a re-examination of the inactivation of T4Bo phage in saline suspension at and below the eutectic point of sodium chloride (−21·8° C.) two interesting new phenomena have been observed, namely a reversible latent eutectic injury and inactivation below the eutectic temperature following rapid cooling and slow warming of samples.

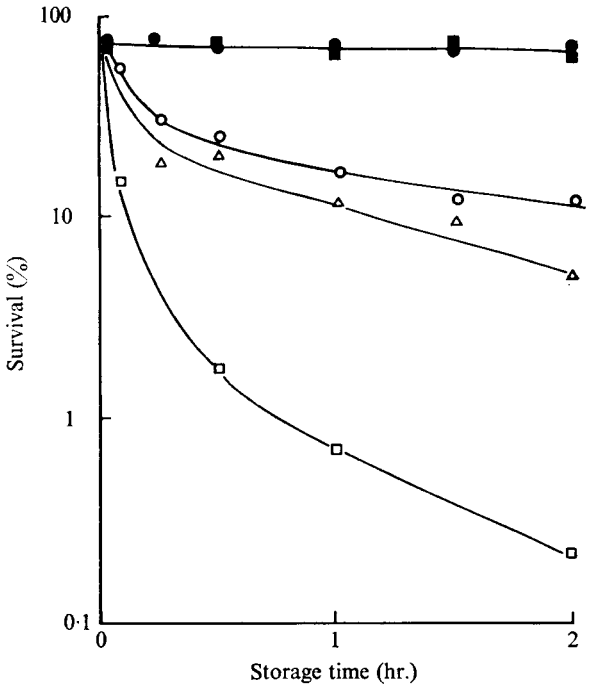

Fig. 1. Effect of the eutectic phase change on the survival of T4Bo phage suspended initially in 0·1 molal NaCl. In the single storage cycle samples were stored at −23° C, with the eutectic unseeded (●) or seeded (○) and thawed rapidly (in a 37° C, water bath) at the indicated times. Survivals for all points in this Figure and in Figs. 2–4 were determined by the standard plaque assay procedure. In double storage cycles samples were seeded and stored at −23° C, for 2 hr., thawed rapidly and then stored again at −23° C. with the eutectic unseeded (■) or seeded (□) and thawed rapidly at the indicated times. One set of samples (△) was incubated at 37° C. for 3 hr. between the first and second storage periods. For these double storage cycles the survivals shown in the Figure are expressed as a percentage of plaque forming units remaining after the first storage cycle.

MATERIALS AND METHODS

The methods of phage preparation, purification and titre determination, and the methods employed for freezing and thawing experimental samples were the same as those described previously (Steele *et al.* 1969*a*).

EXPERIMENTS AND RESULTS

Samples (0·1 ml.) of T4Bo phage ($2{\cdot}5 \times 10^6$ p.f.u./ml.) suspended in 0·1 molal sodium chloride were frozen at −3° C. for 15 min. and then cooled at 1° C./min. to −23° C. (which is just below the eutectic temperature). Seeding of the eutectic, by touching the frozen surfaces of the samples with solid eutectic, resulted in a sixfold fall in survival over a 90 min. period when compared with unseeded samples (Fig. 1). When samples were seeded and stored at −23° C. for 2 hr., thawed, refrozen and reseeded at −23° C. there was a 50-fold fall in survival over a 2 hr. period when compared with unseeded samples (Fig. 1). However, when the latter

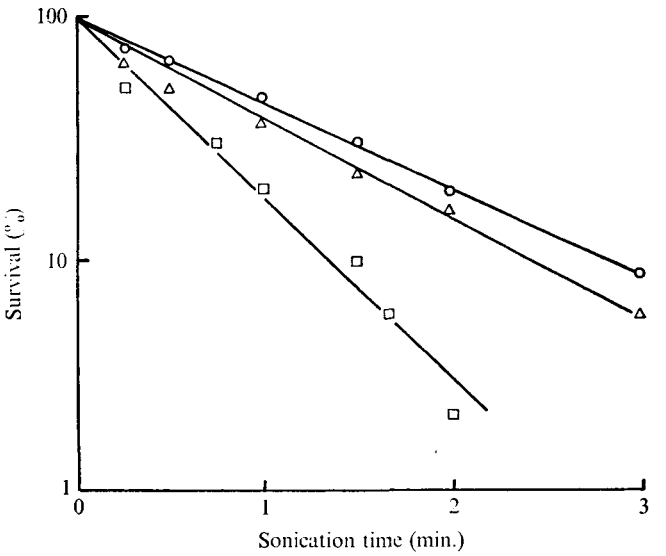

Fig. 2. Effect of the eutectic phase change on the sensitivity of T4Bo phage to ultrasonic vibration (20 kHz, 66 μ). Samples of T4Bo phage suspended in 0·1 molal NaCl were ultrasonicated for the indicated times subsequent to the following pre-treatment: (i) ○, control, no pre-treatment. (ii) □, stored at −23° C. for 2 hr. with the eutectic seeded and thawed rapidly. (iii) △, stored at −23° C. for 2 hr. with the eutectic seeded, thawed rapidly and then incubated at 37° C for 3 hr.. In (ii) and (iii) survivals are expressed as a percentage of the plaque forming units remaining after the pre-treatment procedures.

samples were incubated for 3 hr. at 37° C. after thawing from a first storage period of 2 hr at −23° C., their sensitivity to a second eutectic phase change returned almost to normal (Fig. 1). The results summarized in Fig. 2 show that T4Bo phage which survive the sodium chloride eutectic phase change are also hypersensitive to ultrasonic vibration and again this hypersensitivity is almost lost on incubation at 37° C. for 3 hr.

These results indicate that T4Bo phage which survive the eutectic phase change are in some way injured despite their production of plaques, and that this latent injury resolves upon incubation at 37° C.

When the eutectics were seeded at −23° C. as in the preceding experiments and the samples immediately cooled to −90° C. at slow (3° C./min.) or rapid (180° C./min.) rates, survivals were the same as that observed following the eutectic phase change at −23° C. so long as rewarming from −90° C. was rapid. When rewarming was slow (3° C./min. and 1° C./min.) samples which had been rapidly cooled to −90° C. underwent considerable additional inactivation during the rewarming which began below −40° C. as demonstrated by interrupting the slow rewarming with rapid thawing (Fig. 3). Slowly cooled samples were stable to slow rewarming as were samples which had been seeded and stored at −23° C. for 3 hr. before rapid cooling to −90° C. (Fig. 3). A similar effect is shown in Fig. 4 in which samples were seeded at −23° C. and stored at −42·5° C. (cooling rate 60° C./min.) or cooled to −90° C. at slow (3° C./min.) or rapid (180° C./min.)

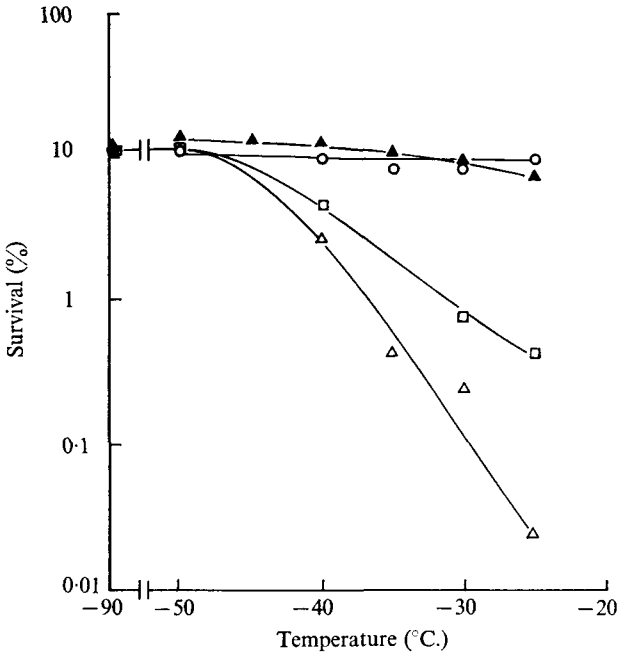

Fig. 3. Effect of cooling and rewarming rates below the eutectic temperature (−21·8° C.) on the survival of T4Bo phage initially suspended in 0·1 molal NaCl. All samples were seeded with solid eutectic at −23° C. and then stored at −90° C. for 15 min. prior to rewarming. At the indicated temperatures rewarming was interrupted by rapid thawing at 37° C. The following cooling rates (CR) and rewarming rates (WR) were used: ▲, CR 3° C./min, WR 3° C./min.; □, CR 180° C./min., WR 3° C./min.; △, CR 180° C./min., WR 1° C/min.; ○, stored at −23° C. for 3 hr. with the eutectic seeded, then CR 180° C./min., WR 3° C./min.

rates and then rewarmed at 4° C./min. to the storage temperature of −42·5° C. The samples cooled rapidly to −90° C. before storage at −42·5° C. were unstable and rapidly inactivated.

DISCUSSION

It has long been believed that rapid freezing to low temperature followed by slow rewarming is lethal to plant and animal cells because of intracellular ice formation, and that this is avoided by slow freezing because the cells are dehydrated at high sub-zero temperatures by concentrated non-penetrant extracellular solutes (Mazur, 1965). T4Bo phage heads are, however, permeable to sodium chloride and thus during freezing the internal aqueous phase of the phage head remains in equilibrium with the external aqueous phase. The explanations of the rapid cooling-slow rewarming inactivation of T4Bo phage below the eutectic temperature (of sodium chloride) cannot be that of the conventional intracellular ice injury hypothesis, since the internal aqueous phase is the same as regards volume and salt concentration at the eutectic temperature for all the samples, whether subsequent cooling to −90° C. is rapid or slow. We propose that following the eutectic phase change a dehydration of phage structural proteins occurs with

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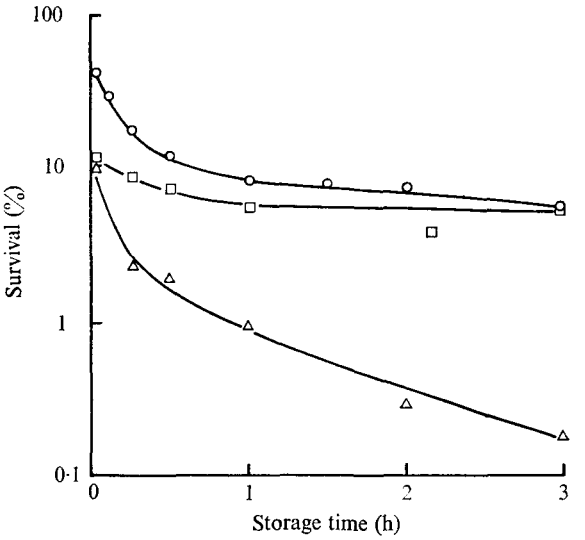

Fig. 4. Effect of prestorage treatment on the survival of T4Bo phage initially suspended in 0·1 molal NaCl stored at −42·5° C. Samples were seeded with solid eutectic at −23° C. and then stored at −42·5° C. in the following ways: ○, cooled directly to −42·5° C. (cooling rate 60° C./min.); □, cooled to −90° C. at 3° C./min. and after 15 min. equilibration at −90° C. rewarmed to −42·5° C. at 4° C./min. △, cooled to −90° C. at 180° C./min. and after 15 min. equilibration at −90° C. rewarmed to −42·5° C. at 4° C./min. After the indicated storage periods at −42·5° C. the samples were thawed rapidly at 37° C.

the external ice acting as a condenser. If this dehydration proceeds slowly at near eutectic temperatures the phage remain stable. If, however, this dehydration is avoided by rapid cooling to low temperatures labile subunit interactions are made potentially unstable perhaps through mechanical stress, and dissociate during slow rewarming. This may represent a lethal extension of the latent eutectic injury, but the results are equally explicable if different sites of injury are involved.

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