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# Some factors affecting the viability of freeze-thawed T4 bacteriophage

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## INTRODUCTION

It was reported in a previous communication (Greaves, Davies & Steele, 1967) that the T4 bacteriophage of *Escherichia coli* was highly sensitive to both freeze-thawing and freeze-drying. The structure of this virus is known in considerable detail and its viability can be easily and accurately determined. Therefore it seemed to be a suitable model for investigation into injury by freezing followed by thawing or drying, and the mechanism of action of protective additives.

This paper reports some effects of freeze-thawing on purified T4 phage, and a preliminary investigation into the nature of the protection afforded by peptone, a product which has been widely used as a protective against freeze-thawing and freeze-drying injury.

## MATERIALS AND METHODS

### *Host bacteria*

The host organism *Eschericha coli* B was grown in nutrient broth (Hartley's tryptic digest broth pH 7·4) at 37° C. in 6 ml. volumes for titre determination, or in 500 ml. volumes with aeration for phage preparation.

### *Bacteriophage*

The T4 phage were prepared from a 500 ml. lysed culture of *E. coli* B. Bacterial debris was removed by centrifugation at 7500*g* for 30 min. The supernatant was centrifuged at 30,000*g* for 60 min., and the pellet resuspended at 4 °C. overnight in 0·13M phosphate buffer ($KH_2PO_4$-$Na_2HPO_4$), with 2 $\mu$g./ml. of DNA-ase. The T4 phage were purified by two further cycles of low- and high-speed centrifugation. The final translucent pellet was resuspended in 0·13M phosphate buffer. Purified phage stocks were stored at 4° C. The phage titres were determined by the standard top-agar technique of Adams (1959): phage samples were mixed with bacteria in 2·5 ml. liquefied agar at 44° C., and the mixtures poured as a thin layer onto agar plates. The plates were incubated overnight at 37° C. and the next day the bacteriophage plaques in the bacterial lawn were counted. Duplicate samples, each plated on three separate plates, were used for every experimental determination of viability.

### *Freezing and thawing procedure*

Experimental samples, consisting of 0·1 ml. T4 phage suspended in 0·13M phosphate buffer or 0·13M phosphate buffer plus additive were measured into glass

freeze-drying tubes 10 cm. in length and 5 mm. internal diameter (Johnson and Jorgensen Ltd.). Unless otherwise stated, the concentration of T4 phage in the experimental samples was $1–2 \times 10^6$ p.f.u./ml. The samples were cooled at 1° C./min. on the freezing stage of the experimental freeze-drying unit previously described by Greaves & Davies (1965). At −5° C. freezing was induced by touching the surface of the sample with a fine wire cooled in liquid nitrogen. Rapid thawing was achieved by shaking the frozen sample in a 37° C. water bath. Samples were thawed slowly by placing them in a thick block of polystyrene maintained at 4° C.

*Peptone*

The peptone used in this investigation was a papaine digest of muscle ('Bacteriological Peptone', Evans Ltd.).

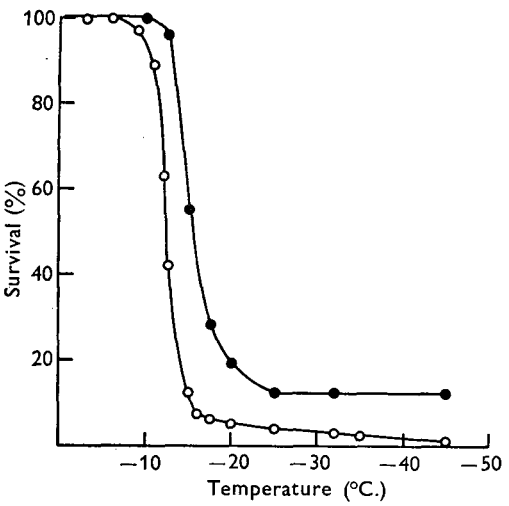


Fig. 1

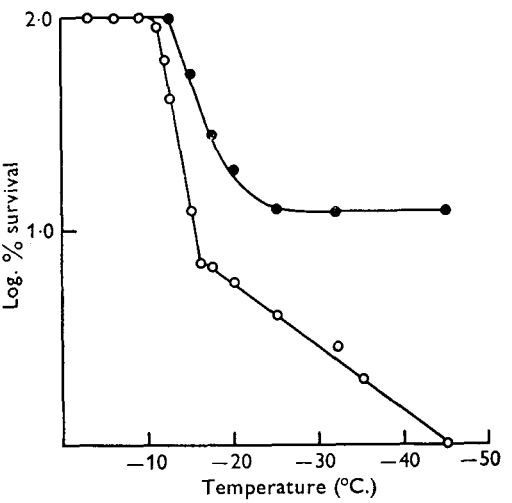


Fig. 2

Fig. 1. The percentage survival of frozen-thawed T 4 phage suspended in 0·13 M phosphate buffer. Samples were cooled at 1° C./min. to the indicated temperatures and thawed either rapidly (○) or slowly (●).

Fig. 2. The logarithmic plot of the results shown in Fig. 1. The rapidly thawed T 4 phage appear to be inactivated by two separate processes.

## RESULTS

### *Effect of the suspending medium*

*Phosphate buffer*

Samples of T4 phage suspended in 0·13 M phosphate buffer were cooled at 1° C./min. to temperatures from 0° to −45° C., and thawed either rapidly or slowly, and the percentage survival determined (Fig. 1). The viability of rapidly thawed phage fell sharply between the freezing temperatures of −11° and −15° C. to 10% survival. Thereafter the viability fell slowly to 1% survival following rapid thawing from −45° C. The logarithmic plot of the results (Fig. 2) suggests that the rapid-thaw inactivation results from two separate processes. The inactivation of slowly thawed T4 phage occurred between −10° and −25° C.

*Addition of sodium chloride*

Sodium chloride was added to experimental samples in concentrations of 0·15 M and 0·5 M. Despite the large difference in initial molarity both concentrations of NaCl had a similar effect on the viability of freeze-thawed phage (Fig. 3). Most of the inactivation of rapidly thawed samples occurred in a temperature range 3° C. higher

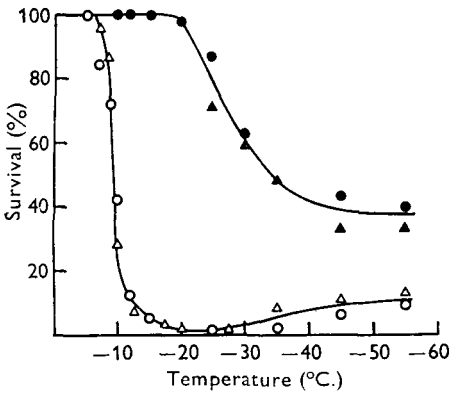


Fig. 3

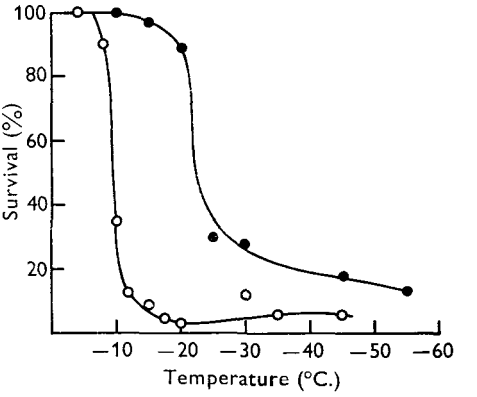


Fig. 4

Fig. 3. The percentage survival of frozen-thawed T 4 phage suspended in 0·13 M phosphate buffer + NaCl. Samples were cooled at 1° C./min. to the indicated temperatures. ○, Buffer + 0·15 M-NaCl, rapid thaw; ●, buffer + 0·15 M-NaCl, slow thaw; △, buffer + 0·5 M-NaCl, rapid thaw; ▲, buffer + 0·5 M-NaCl, slow thaw.

Fig. 4. The percentage survival of frozen-thawed T 4 phage suspended in 0·13 M phosphate buffer + 0·15 M-KCl. Samples were cooled at 1° C./min. and thawed either rapidly (○) or slowly (●).

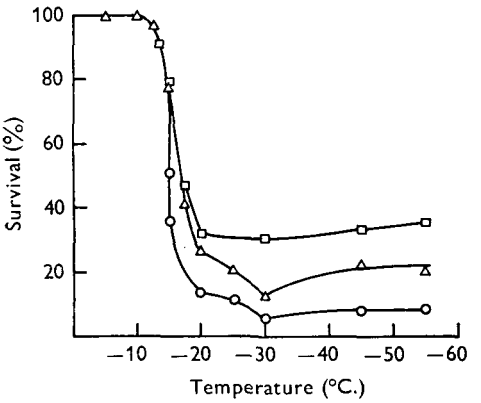


Fig. 5

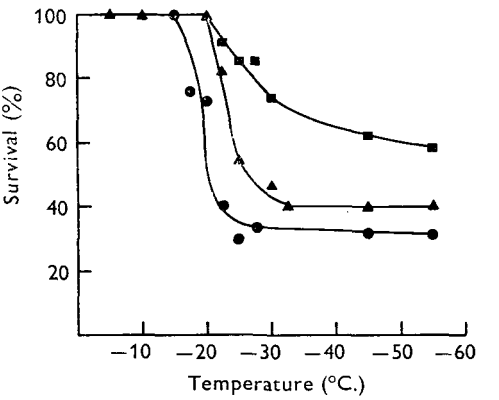


Fig. 6

Fig. 5. The effect of addition of peptone on the survival of frozen-thawed T 4 phage. Samples were cooled at 1° C./min. to the indicated temperatures and thawed rapidly. The concentrations (w/v) of added peptone were: ○, 1 %; △, 5 %; □, 10 %.

Fig. 6. The effect of addition of peptone on the survival of frozen-thawed T 4 phage. Samples were cooled at 1° C./min. to the indicated temperatures and thawed slowly. The concentrations (w/v) of added peptone were: ●, 1 %; ▲, 5 %; ■, 10 %.

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than with buffer alone, but the gradient of the viability curve was identical. Between $-15°$ C. and $-25°$ C. there was a dip in the viability curve. The presence of NaCl greatly increased the survival of slowly thawed T4 phage, inactivation occurring between $-20°$ and $-45°$ C.

Table 1. *The effect of amino acids at a concentration of* 0·1 M *on the survival of T4 phage cooled at* 1° C./*min. to* $-45°$ C. *and thawed either rapidly* (*RT*) *or slowly* (*ST*)

| Amino acid | Survival RT (%) | Survival ST (%) | Amino acid | Survival RT (%) | Survival ST (%) |
|---|---|---|---|---|---|
| (Buffer) | 1·0 | 10 | Methionine | 1·0 | 15 |
| Glycine | 1·0 | 14·8 | Phenylalanine | 0·5 | — |
| Alanine | 2·8 | 9·4 | Histidine | 0·8 | 22 |
| Valine | 3·2 | 5·8 | Arginine | 0·2 | 1·7 |
| Leucine | 1·3 | 15·5 | Lysine | 2·4 | 1·3 |
| Iso-leucine | 1·0 | 14·5 | Aspartic acid | 3·7 | 8·8 |
| Serine | 1·0 | 25 | Glutamic acid | 4·0 | 22 |
| Threonine | 0·5 | 12 | Proline | 0·7 | 6·2 |
| Cystine | 1·2 | — | | | |

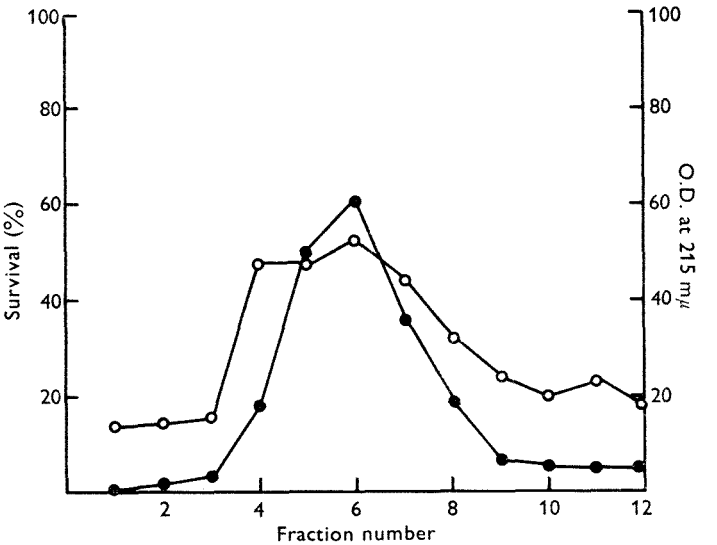


Fig. 7. The effect of G 25 Sephadex fractions of peptone on the percentage survival T 4 phage cooled at 1° C./min. to $-45°$ C. and thawed slowly. The phage were suspended in 0·13 M phosphate buffer + peptone fraction. For details of fractionation procedure see text. ○, % survival; ●, O.D.

*Addition of potassium chloride*

The effect of added 0·15 M-KCl on the viability of freeze-thawed phage (Fig. 4) was similar to that observed with NaCl. The survival of slowly thawed phage was increased, but to a lesser degree than with NaCl, most of the inactivation occurring between $-15°$ and $-35°$ C.

*Effect of peptone*

Peptone was added to T4 phage samples in concentrations of 1%, 5% and 10% (w/v). The addition of peptone progressively increased the survival of rapidly

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thawed phage, although the gradient of the inactivation curve was not appreciably altered (Fig. 5). The temperature range of inactivation was lowered by 2–5° C.

Peptone gave significant protection to slowly thawed phage (Fig. 6), markedly altering both the gradient and temperature range of inactivation.

*Amino acids*

Separation of a 1 % solution of peptone by two-dimensional paper electrophoresis showed that it contained most of the common amino acids together with small peptides of all charges. Since there were so many amino acids present in peptone, pure amino acids were tested separately for protection against freeze-thaw injury at

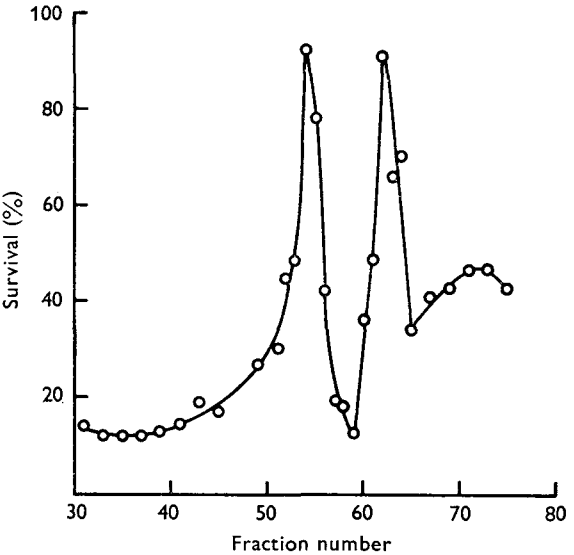


Fig. 8. The effect of twice-fractionated peptone. T 4 phage suspended in 0·13 M phosphate buffer + peptone fraction were cooled at 1° C./min. to −45° C. and thawed slowly. There are two well-defined protective peaks.

a concentration of 0·1 M (Table 1). None of them had a significant effect on rapidly thawed T4 phage. There was some variation in their effect on slowly thawed samples: arginine and lysine decreased survival, whereas serine, histidine and glutamic acid were slightly protective. However, there was no indication that the significant protection afforded by peptone was due to its amino-acid constituents.

*Sephadex fractionation*

A preliminary separation of peptone was carried out by fractionating 20 mg. of peptone through a 40 cm. column of G25 Sephadex (50 ml. bed-volume). The resulting 5 ml. fractions were concentrated five-fold by freeze-drying and tested for protective effect (Fig. 7). The position and relative concentration of peptide were estimated by absorption at 215 m$\mu$. The middle fractions gave good protection to slowly thawed T4 phage, moreover the maximum survival was increased by fractionation. The fractions had no significant effect on the viability of rapidly thawed phage.

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Following this preliminary experiment, 800 mg. of peptone was fractionated through a wide G25 Sephadex column; the middle peptide fraction was collected and freeze-dried. The resulting 450 mg. of material was refractionated through a

Table 2. *The effect of initial T4 phage concentration on survival*

(The samples were cooled at 1° C/min. to −45° C. and thawed either rapidly or slowly.)

| Suspending medium | Initial conc. of phage p.f.u./ml. | Survival rapid thaw (%) | Survival slow thaw (%) |
|---|---|---|---|
| 0·13 M buffer | $3{\cdot}7 \times 10^8$ | 1·2 | 44 |
| | $1{\cdot}2 \times 10^8$ | 1·3 | 33·5 |
| | $1{\cdot}2 \times 10^7$ | 2·6 | 12 |
| | $1{\cdot}2 \times 10^6$ | 1·5 | 13 |
| | $1{\cdot}2 \times 10^4$ | 1·8 | 11·7 |
| 0·13 M buffer + 0·15 M-NaCl | $3{\cdot}9 \times 10^8$ | 15·5 | 82·5 |
| | $1{\cdot}3 \times 10^8$ | 14·4 | 74 |
| | $1{\cdot}3 \times 10^7$ | 11·7 | 60 |
| | $1{\cdot}3 \times 10^6$ | 13·4 | 41 |

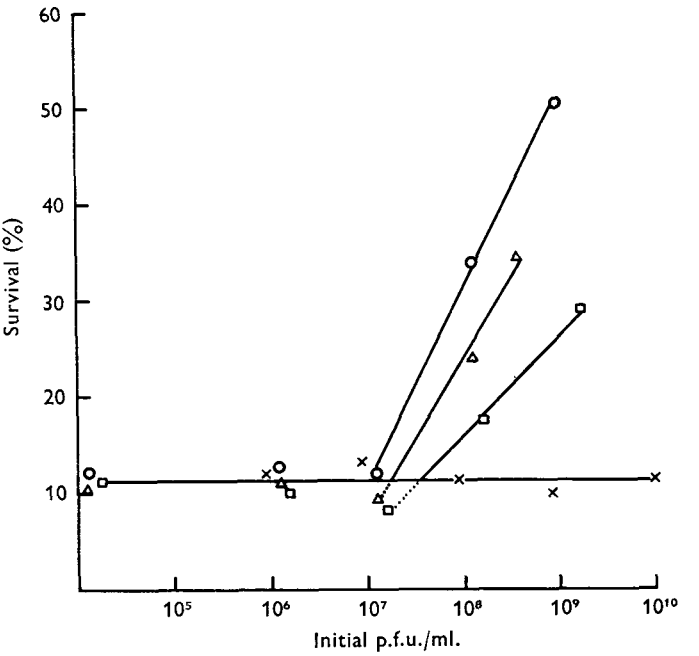


Fig. 9. The effect of initial phage concentration and 'washing' on the survival of T 4 phage cooled at 1° C./min. to −45° C. and thawed slowly. The suspending medium was 0·13 M phosphate buffer. T 4 phage purified from broth lysates of *Escherichia coli* B were washed with distilled water while adsorbed on to magnesium pyrophosphate gel. This treatment considerably lowered the high survival obtained with concentrated suspensions. No concentration effect was observed with phage purified from a lysate of *E. coli* B grown in Adams' medium. ○, Washed 3 times; △, washed 7 times; □, washed 12 times; ×, phage prepared from a lysate of *E. coli* B grown in Adams' medium.

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160 cm. G 25 Sephadex column (800 ml. bed-volume) and the 10 ml. fractions concentrated tenfold by freeze-drying and tested for protective effect, against inactivation of slowly thawed T 4 phage (Fig. 8). There were two well-defined peaks of protection with maxima of 90% survival, followed by a broad peak with a maximum of 45% survival.

Unfortunately further investigation of the protective fractions proved to be very difficult. Electrophoretic separation of the fractions at pH 3·5 showed that they contained a large range of peptides which did not fall into discrete bands.

### *Effect of the initial T 4 phage concentration*

The initial phage titre routinely used in experimental samples was $1–2 \times 10^6$ p.f.u./ml. When higher concentrations of T 4 phage were freeze-thawed a marked concentration effect was observed (Table 2); the survival of slowly thawed phage was considerably increased, although no such effect was observed with rapidly thawed samples: possibly the phage had absorbed protective compounds from the original broth lysate. As a test of this, the phage were adsorbed on to magnesium pyrophosphate gel (Schito, 1967) and washed several times with distilled water, before being eluted off the gel with 0·13 M phosphate buffer. The effect of washing is shown in Fig. 9. Clearly a large part of the protection can be 'washed off' the phage. As a further test, T 4 phage were prepared by lysing *E. coli* B grown in the defined salt medium of Adams (Adams, 1959) instead of in nutrient broth. These phage showed no increase in survival with increase in concentration.

## DISCUSSION

It is significant that most of the inactivation of rapidly thawed T 4 phage occurred in a narrow temperature range, which was only slightly altered by addition of salts or peptone. Rapid thawing produces a sudden dilution of concentrated solutes and thus a sharp fall in osmotic pressure of the suspending medium. The T 4 phage used in the present experiments was inactivated to 1% viability by rapid 100-fold dilution from 3 M-NaCl into distilled water, but remained 100% viable if the dilution was carried out slowly. Anderson (1953) termed this phenomenon of inactivation caused by a rapid fall in salt concentration 'osmotic shock'. Slow thawing effectively produces a slow dilution of the solutes concentrated during freezing. Little inactivation of slow-thawed samples occurred in the temperature range in which most of the rapid-thaw inactivation was observed. It therefore seems likely that osmotic damage was responsible for most of the inactivation of rapidly thawed T 4 phage. Leibo & Mazur (1967) also implicated osmotic damage as a cause of inactivation in freeze-thawed T 4 phage.

The eutectic temperatures of the suspending media used in the experiments are: phosphate buffer $-4·5°$ C.; buffer + NaCl $-23·5°$ C.; buffer + KCl $-12·5°$ C. (Van den Berg, 1959). Slowly thawed T 4 phage were inactivated only below the eutectic temperature of the respective suspending medium. Furthermore viability was increased by lowering the eutectic temperature. This relationship between eutectic temperature and freeze-thaw damage indicates that the damage is due to removal of

the last traces of unbound water as ice, and that damage is most severe when this water is removed at a relatively high freezing temperature.

Peptone strongly protected against inactivation of slowly thawed T 4 phage. The results obtained by fractionation of peptone showed that the constituents largely responsible for this protection could be separated as two peptide fractions. Unfortunately the unspecific degradation of protein by papaine produces a range of innumerable small peptides and amino acids. Such a mixture is clearly unsuitable for future investigation into the nature of the protection afforded by peptides. What is required is a specific enzymic digest of a pure protein of known amino acid sequence. Work along these lines is now in progress using a tryptic digest of rabbit globin.

It was shown in the experiments that the increase in survival of slow-thawed T 4 phage produced by increasing the initial phage titre was greatly diminished by washing the phage with distilled water, and was entirely prevented by preparing the T 4 phage stock in a salt medium instead of nutrient broth. In the light of the results obtained with the peptide fractions of peptone it seems possible that the concentration effect may have been caused by protective peptides adsorbed on to the phage from the original broth lysate.

## SUMMARY

Some effects of freeze-thawing on the T 4 bacteriophage have been studied. The results indicated that most of the inactivation of rapidly thawed samples was due to osmotic damage, whereas inactivation of slowly thawed samples appeared to be correlated with the eutectic temperature of the suspending medium. Peptone significantly increased the survival of slowly thawed T 4 phage. The particular constituents of peptone largely responsible for this protection were separated as two distinct peptide fractions, using G 25 Sephadex.

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