

## Osmotic injury in rapidly thawed T4 bacteriophage

By P. R. M. STEELE

*University Department of Pathology, Cambridge, England\**

(Received 24 January 1972)

### SUMMARY

Osmotic injury in frozen-thawed T4 phage was caused by the sudden and large fall in the electrolyte concentration of the unfrozen aqueous phase during rapid thawing of frozen samples. In accordance with the classical interpretation of osmotic shock it was found that DNA was quantitatively liberated from T4 phage inactivated by the osmotic injury of rapid thawing.

The degree of inactivation of osmotically shocked T4 phage was temperature dependent, being much increased by lowering the temperature, but was independent of the pH of the suspending medium. The T4Bo osmotic shock-resistant phage was refractory to the osmotic injury of rapid thawing.

### INTRODUCTION

Previous studies on the effects of freezing and thawing suspensions of T4 and T4Bo bacteriophages (Greaves, Davies & Steele, 1967; Steele, Davies & Greaves, 1969*a, b*) showed that the phage were inactivated by three different mechanisms of injury. These can conveniently be termed 'osmotic injury', 'ionic injury' and 'eutectic injury'.

Osmotic injury to the T4 phage was believed to be responsible for most of the inactivation of rapidly thawed samples. It was avoided by slow thawing of frozen samples. Leibo & Mazur (1969) have also implicated osmotic injury as a cause of inactivation of frozen-thawed T4 phage.

Ionic injury resulted from the dual effects of electrolyte concentration and lowered temperature in frozen samples, while eutectic injury occurred when phage suspensions were frozen to temperatures below the eutectic temperature of the suspending medium.

Ionic and eutectic injuries were dealt with in the earlier studies. It is the purpose of this communication to clarify the correlation between osmotic injury in frozen-thawed phage and the phenomenon of 'osmotic shock' which is demonstrable in unfrozen phage suspensions.

\* Present address, Middlesex Hospital Medical School, Cleveland Street, London W. 1.

## MATERIALS AND METHODS

The methods of phage preparation and purification, and the techniques used for freezing, thawing and titre determinations were the same as those described previously. Phosphate buffer was added to the suspending media of samples subjected to sub-zero treatments to prevent the ionic injury due to electrolyte concentration (Steele *et al.* 1969*b*).

*DNA determinations*

DNA determinations were obtained using the ethidium bromide fluorimetric technique of Le Peck & Paoletti (1966). When the dye ethidium bromide (2,7-diamino-9-phenylphenanthridine-10-ethyl bromide) binds to nucleic acids, for a suitably chosen wavelength of fluorescence excitation the intensity of fluorescence emission increases by a factor of 50-fold to 100-fold. This increase in fluorescence is directly proportional to the nucleic acid concentration and is not affected by the presence of protein.

In this study fluorescence measurements were obtained with an Aminco-Bowman Spectro-fluorimeter with the wavelengths set at  $\lambda_{\text{Ex.}}$  524  $m\mu$  and  $\lambda_{\text{Emm.}}$  587  $m\mu$ .

A preliminary experiment showed that there was no increase in the fluorescence of ethidium bromide solutions when intact T4 phage were added at a concentration of  $2 \times 10^{12}$  p.f.u./ml. Presumably the ethidium bromide molecule is too large to penetrate the phage head membrane and thus cannot bind to the internal DNA.

T4 phage DNA was prepared and purified by the phenolic extraction procedure (Kaiser, 1960) and standardized in terms of 'phage equivalents DNA/ml.' by colorimetric comparison with intact purified phage of known plaque titre using the diphenylamine reaction (Burton, 1956).

A calibration graph was then constructed by adding known concentrations of purified T4 phage DNA in phosphate buffer ( $\text{KH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$  0.13 M - pH 7) to an equal volume of ethidium bromide solution (10  $\mu\text{g./ml.}$  in phosphate buffer), and plotting the fluorescence readings of the mixtures.

Experimental frozen-thawed or osmotically shocked phage samples were added to an equal volume of ethidium bromide solution (10  $\mu\text{g./ml.}$  in phosphate buffer) and their fluorescence intensities determined. The concentrations of liberated DNA as 'phage equivalents DNA/ml.' were then read off the calibration graph.

## RESULTS AND DISCUSSION

*Osmotic injury during freezing and thawing*

Samples of T4 phage or T4Bo (osmotic-shock-resistant) phage suspended in phosphate-buffered saline ( $\text{KH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$  0.13 M, NaCl 0.15 M - pH 7) were frozen and equilibrated at  $-5^\circ\text{C.}$  for 10 min. and then cooled at  $1^\circ\text{C./min.}$  to temperatures down to  $-20^\circ\text{C.}$  On reaching the desired temperature the samples were immediately rewarmed either rapidly ( $450^\circ\text{C./min.}$ ) or slowly ( $5^\circ\text{C./min.}$ ) until thawing was complete, and the viable titres determined (Fig. 1).

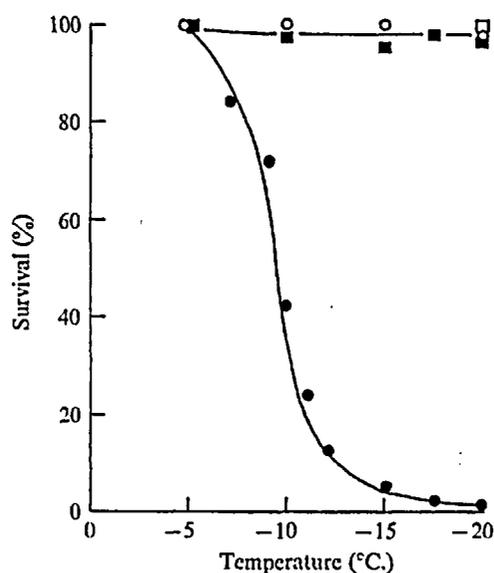


Fig. 1. The percentage survival of frozen-thawed T4 and T4Bo phages suspended in 0.13 M phosphate buffer + 0.15 M-NaCl. Samples were cooled at 1° C./min. ○, T4 phage, slow thaw; ●, T4 phage, rapid thaw; □, T4Bo phage, slow thaw; ■, T4Bo phage, rapid thaw.

The only significant inactivation which occurred was in the T4 phage samples which were rewarmed rapidly. In the classical osmotic inactivation of T4 phage – ‘osmotic shock’ – first described by Anderson (1953) and later by Leibo & Mazur (1966), suspensions of phage in concentrated salt solutions are rapidly diluted with distilled water. The resulting inactivation of the phage is believed to occur because the diffusion of water into the phage head is more rapid than electrolyte loss. This transient osmotic pressure gradient across the head membrane causes its subsequent rupture. Rapid thawing of frozen samples produces a similar rapid dilution of electrolytes, which are concentrated by freezing.

#### *Effect of pH and temperature*

Before identifying the osmotic injury of rapid thawing with osmotic shock, two important factors must be considered. First, there is a pH difference since phosphate-buffered saline becomes acidic during freezing (Van den Berg, 1959) and secondly there is the temperature difference.

Table 1 shows that osmotic shock sensitivity (T4 phage) and osmotic shock-resistance (T4Bo phage) are not affected by the pH of the suspending medium.

The effect of temperature was investigated by rapid 100-fold dilution in distilled water of T4 phage suspended in concentrated NaCl solutions, the NaCl solutions and distilled water being maintained at 25° C. or 4° C. (Fig. 2). There was a considerable decrease in the viability of the samples osmotically shocked at 4° C. compared with 25° C. The experiment was repeated using NH<sub>4</sub>Cl suspending media and osmotically shocking the phage at 56° C., 25° C. or 4° C. (Fig. 3). Once again a definite temperature effect was observed. There was little inactivation of the

Table 1. *The effect of pH on osmotic shock and osmotic shock-resistance. Samples were rapidly diluted 100-fold from 3 M-NaCl at adjusted pH (using N-HCl or N-NaOH) into distilled water*

pH	T4 phage survival (%)	pH	T4Bo phage survival (%)
3.7	1.3	4.6	102
4.6	1.9	7.0	96
5.5	1.6	7.6	93
6.5	0.7	10.1	95
7.9	1.3	—	—
8.8	0.9	—	—

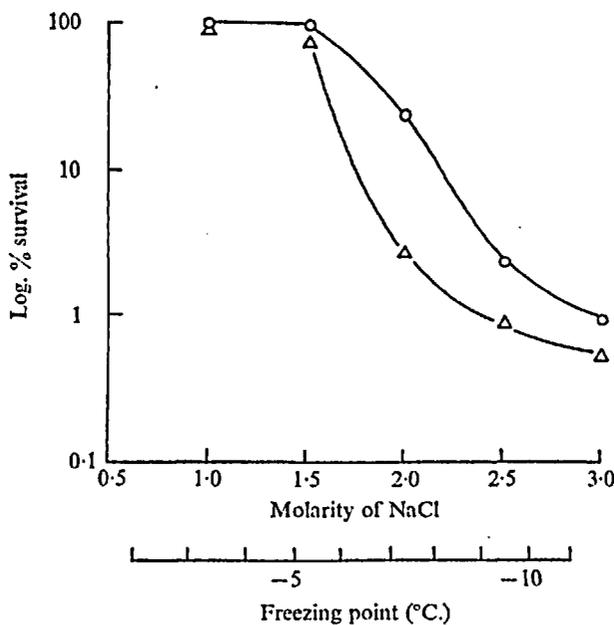


Fig. 2. The effect of temperature on the survival of osmotically shocked T4 phage. Samples were osmotically shocked by a rapid 100-fold dilution from NaCl solutions at the indicated molarity into distilled water. O, 25° C.; Δ, 4° C. The lower scale shows the freezing points of the solutions for comparison with Fig. 1.

phage samples osmotically shocked at 56° C. Obviously the osmotic transients last longer and are more severe at lower temperatures owing to the slower rates of diffusion of the ions.

This decreased survival of phage osmotically shocked at lower temperatures explains why the degrees of inactivation produced by osmotic shock at 25° C. and osmotic injury during rapid thawing are very similar for a given salt concentration even though rapid thawing is a slower dilution process than the osmotic shock procedure.

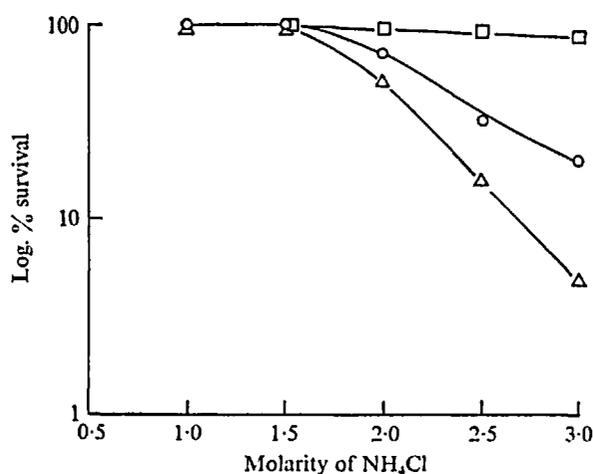


Fig. 3. The effect of temperature on the survival of T4 phage osmotically shocked from  $\text{NH}_4\text{Cl}$  solutions. The procedure was the same as for Fig. 2. □, 56° C.; ○, 25° C.; △, 4° C.

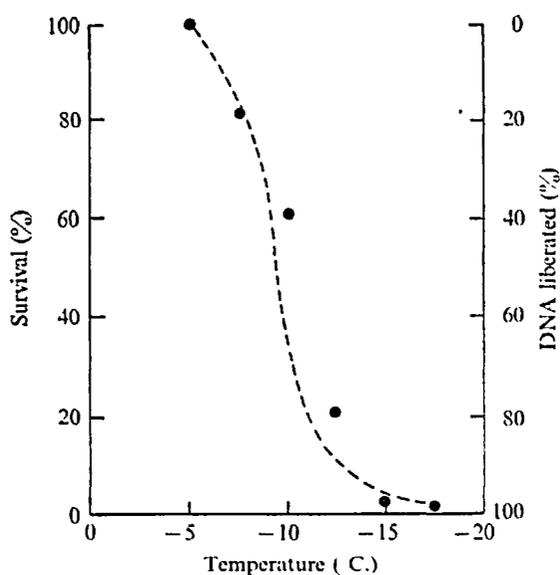


Fig. 4. Samples of T4 phage suspended in 0.13 M phosphate buffer + 0.15 M NaCl were cooled at 1° C./min. and thawed rapidly. The percentage DNA liberated (●) was compared with the viability as determined by plaque assay (dotted line – taken from Fig. 1).

### Liberation of DNA

It has been demonstrated both microscopically (Kleinschmitt, Lang, Jacherts & Zahn, 1962) and chemically (Hershey & Chase, 1952) that DNA is released from the heads of osmotically shocked T-even phage.

Using the ethidium bromide estimation it was found that 99% of the T4 phage DNA was liberated after osmotic shock by rapid 100-fold dilution of a suspension in 3 M NaCl with distilled water, at 25° C. This exactly correlated with the 1%

viability as determined by plaque assay. Fig. 4 shows the excellent agreement between the viability of rapidly thawed T4 phage and the percentage DNA liberated.

Leibo & Mazur (1966) found that approximately half of their T4B phage inactivated by osmotic shock retained their DNA, as measured by CsCl density gradient profiles. They stored their purified phage stocks in 1.5% nutrient broth, whereas the stocks used in the present experiments were stored in phosphate buffer. We have previously shown (Steele *et al* 1969*a*) that compounds, probably peptides, present in broth media substantially alter the sensitivity of T4 phage to the effects of freezing and thawing. This raises the interesting possibility that peptides adsorbed onto the T4 phage might partially prevent the release of DNA during osmotic shock without altering the degree of inactivation. Such an explanation is especially likely in the light of the results of Panijel, Huppert & Barbu (1957) who showed that the proportion of T2r phage which liberated their DNA during freezing and rapid thawing could be significantly increased by purifying the phage with trypsin (1.5  $\mu\text{g./ml.}$ ) thereby removing proteins and peptides adsorbed from their broth medium, although this treatment did not alter the sensitivity of the phage to freezing and thawing as measured by plaque assay.

I wish to thank Professor R. I. N. Greaves and Dr J. D. Davies for their interest and encouragement.

This work has been supported in part by a grant from the Medical Research Council.

#### REFERENCES

- ANDERSON, T. F. (1963). The morphology and osmotic properties of bacteriophage systems. *Cold Spring Harbor Symposia on Quantitative Biology* 18, 197.
- BURTON, K. (1956). A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochemical Journal* 62, 315.
- GREAVES, R. I. N., DAVIES, J. D. & STEELE, P. R. M. (1967). The freeze-drying of frost-sensitive organisms. *Cryobiology* 3, 283.
- HERSHEY, A. D. & CHASE, M. (1952). Independent functions of viral protein and nucleic acid in the growth of bacteriophage. *Journal of General Physiology* 36, 39.
- KAISER, A. D. (1960). The transformation of *Escherichia coli* with deoxyribonucleic acid isolated from bacteriophage lambda-dg. *Journal of Molecular Biology* 2, 392.
- KLEINSCHMITT, A. K., LANG, D., JACHERTS, D. & ZAHN, R. K. (1962). Darstellung und Langenmessungen des gesamten Deoxyribonucleinsäure-inhalts von T2 Bakteriophagen. *Biochimica et Biophysica Acta* 61, 857.
- LEIBO, S. P. & MAZUR, P. (1966). Effect of osmotic shock and low salt concentration on survival and density of bacteriophages T4 and T4Bo. *Biophysical Journal* 6, 747.
- LEIBO, S. P. & MAZUR, P. (1969). Freezing of bacteriophage T4: Temperature and rate effects as a function of salt concentration. *Virology* 38, 558.
- LE PECK, J. D. & PAOLETTI, C. (1966). A new fluorimetric method for RNA and DNA determination. *Analytical Biochemistry* 17, 100.
- PANIJEL, J., HUPPERT, J. & BARBU, E. (1957). Effets de la congélation sur les bactériophages. *Annales de l'Institut Pasteur* 93, 183.
- STEELE, P. R. M., DAVIES, J. D. & GREAVES, R. I. N. (1969*a*). Some factors affecting the viability of freeze-thawed T4 bacteriophage. *Journal of Hygiene* 67, 107.
- STEELE, P. R. M., DAVIES, J. D. & GREAVES, R. I. N. (1969*b*). Some factors affecting the viability of freeze-thawed T4 bacteriophage. II. The influence of certain electrolytes on the degree of inactivation. *Journal of Hygiene* 67, 679.
- VAN DEN BERG, L. (1959). Effects of additions of NaCl and KCl on the system of  $\text{KH}_2\text{PO}_4$ ,  $\text{Na}_2\text{HPO}_4$ - $\text{H}_2\text{O}$  on pH and composition during freezing. *Archives of Biochemistry and Biophysics* 34, 305.