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Some factors affecting the viability of freeze-thawed T 4 bacteriophage

II. The influence of certain electrolytes on the degree of inactivation

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Steele, Davies & Greaves (1969) reported that when preparations of T 4 bacteriophage, suspended in phosphate-buffered salt solutions, were subjected to freezing and thawing, the resulting loss of viability was due to two different mechanisms. First, T 4 phage which had been frozen to temperatures *above* the eutectic temperature of the suspending medium were inactivated if the frozen samples were thawed rapidly. This inactivation did not occur when the frozen samples were thawed slowly, and it appeared to be due to 'osmotic shock', i.e. rapid dilution of a concentrated suspending medium (Anderson, 1953; Leibo & Mazur, 1966). Secondly, when osmotic damage was avoided by slow thawing there was inactivation of T 4 phage samples which had been cooled to temperatures *below* the eutectic temperature of the suspending medium. This relationship between eutectic temperature and freeze-thawing damage suggested that damage was due to the removal of the last traces of 'unbound' water as ice.

However, it was later observed that when the phosphate buffer was absent from the suspending medium, considerable inactivation of frozen-thawed T 4 phage occurred above the eutectic temperature, even when frozen samples were thawed slowly (i.e. avoiding osmotic shock). The extent of this inactivation appeared to be dependent on the type of ions in the suspending medium. The purpose of the present study was to investigate these observations, especially with regard to the accepted theories of injury to cells during freeze-thawing.

MATERIALS AND METHODS

Host bacteria

The host organism *Escherichia 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 of the defined salt medium of Adams (Adams, 1959), with aeration, for phage preparation. Stocks were maintained on 1·5% nutrient agar plates.

Bacteriophage

The T 4 phage and T 4 Bo osmotic shock-resistant phage (a gift from Dr S. P. Leibo) were prepared from 500 ml. lysed cultures of *E. coli* B. The methods used for phage preparation, purification and titre determination were the same as those described previously (Steele *et al.* 1969).

Experimental procedure

An experimental stock suspension of the T 4 or T 4 Bo phage at a concentration of 2×10^9 p.f.u./ml. in phosphate buffer (or 2×10^{11} p.f.u./ml. when very low experimental survival was anticipated) was diluted 1000-fold into the experimental suspending medium. Samples of 0·1 ml. were cooled at 1° C./min. (unless otherwise stated). At −5° C. ice formation was induced by touching the surface of each sample with a fine wire cooled in liquid nitrogen. Samples were thawed slowly by placing them in a thick block of polystyrene maintained at 4° C. The samples were diluted 10-fold, 100-fold or 1000-fold with phosphate buffer prior to being assayed.

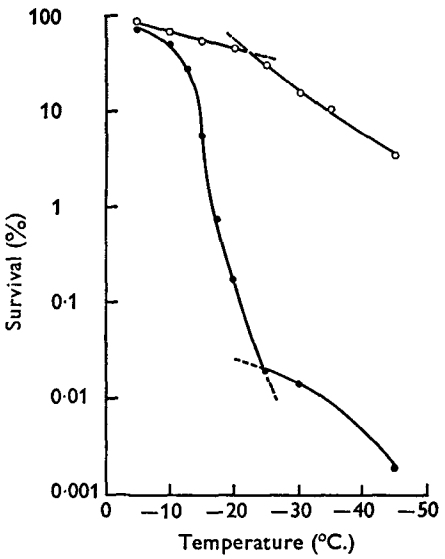

Fig. 1

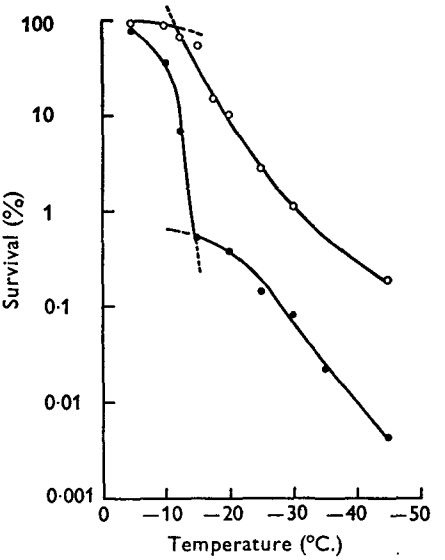

Fig. 2

Fig. 1. The survival of frozen-thawed T 4 phage suspended in either 0·1 molal NaCl (○), or 0·1 molal NaBr (●). Samples were cooled at 1° C./min. to the indicated temperatures and then thawed slowly.

Fig. 2. The survival of frozen-thawed T 4 phage suspended in either 0·1 molal KCl (○), or 0·1 molal KBr (●). Samples were cooled at 1° C./min. to the indicated temperatures and then thawed slowly.

RESULTS AND DISCUSSION

Inactivating effect of neutral salts

Samples of the T 4 phage suspended in 0·1 molal solutions of NaCl, NaBr, KCl and KBr were cooled to temperatures from 0° to −45° C. and then thawed slowly. The resulting percentage viabilities are shown in Figs. 1 and 2. There was a marked alteration of slope in each inactivation curve which occurred very close to the eutectic temperature of the respective salt (NaCl −21·5° C., NaBr −28° C., KCl −11° C., KBr −13° C.). Inactivation above the eutectic temperature was much greater in the presence of Br^- compared to Cl^-. Below the eutectic tempera-

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ture the rate of inactivation was slightly greater with potassium salts than with sodium salts, presumably because of the higher eutectic temperatures of the potassium salts.

Since the extent of inactivation above the eutectic temperature was clearly dependent on the species of ions in the suspending medium, the inactivating effect of other neutral salts above their eutectic temperatures was investigated (Fig. 3). The effectiveness of different anions in causing inactivation of frozen-thawed T 4 phage increased in the order $Cl^- < Br^- < I^-$. Lithium salts caused more inactivation than the corresponding sodium or potassium salt, but the differences were less than those between the effects of different anions.

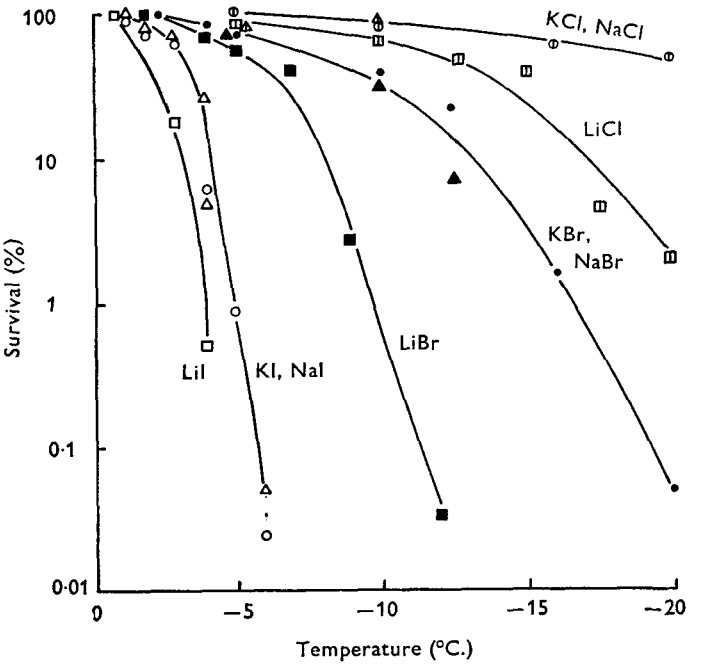

Fig. 3. Samples of the T4 phage, suspended in 0·1 molal solutions of various neutral salts, were cooled at 1° C./min. to the indicated temperatures and thawed slowly. ⌽, NaCl; ●, NaBr; ○, NaI; ⧋, KCl; ▲, KBr; △, KI; ▥, LiCl; ■, LiBr; □, LiI.

When a salt solution is frozen, the formation of ice causes the salt to become increasingly concentrated in the unfrozen aqueous phase. At any particular sub-zero temperature the molal concentration of the unfrozen solution is fixed and independent of the initial salt concentration. The volume of unfrozen solution however is directly proportional to the initial salt concentration. The influence of the volume of unfrozen solution on the survival of frozen-thawed T 4 phage was investigated by freeze-thawing samples of T 4 phage in NaBr solutions of different initial concentrations (0·1, 0·5 and 1·0 molal). It was found that the different initial concentrations of NaBr had no effect on the subsequent survival of frozen-thawed T 4 phage.

Effect of phosphate buffer

It was reported in a previous communication (Steele *et al.* 1969) that T 4 phage, suspended in phosphate-buffered salt solutions, were not inactivated by freeze-thawing at temperatures above the eutectic temperature, so long as osmotic shock was avoided by slow thawing. This protective effect of phosphates was investigated by freeze-thawing T 4 phage and T 4 Bo phage suspended in mixtures of phosphates and NaBr or KBr (Table 1). The addition of mixtures of phosphates gave good protection in all cases. Added K_2HPO_4 gave almost complete protection, whereas added KH_2PO_4 or NaH_2PO_4 raised survival to only 50%. Added Na_2HPO_4 was

Table 1. *Percentage survival of T 4 or T 4 Bo phage in sodium or potassium bromide with the addition of various phosphates*

(The suspensions were cooled at 1° C./min. to the indicated temperatures and thawed slowly.)

Added phosphate, 0·1 molal	T4 phage in 0·1 molal NaBr, −17·5° C.	T4 phage in 0·1 molal KBr, −12·5° C.	T4 Bo phage in 0·1 molal NaBr, −17·5° C.
None	0·8	9·3	0·9
KH_2PO_4–Na_2HPO_4	98	102	93
KH_2PO_4–K_2HPO_4	88	98	85
NaH_2PO_4–Na_2HPO_4	77	98	101
KH_2PO_4	51	51	43
K_2HPO_4	93	102	102
NaH_2PO_4	52	49	42
Na_2HPO_4	64	65	81

not as effective as K_2HPO_4, but this is undoubtedly a reflexion of its high eutectic temperature (−0·5° C.). It is very interesting that dihydrogen phosphates were only half as effective as K_2HPO_4. It might at first appear that the difference was due to the pH of the solution. However, added KH_2PO_4–Na_2HPO_4 was almost completely protective and yet produces an acidic eutectic mixture (Van den Berg, 1959).

In other experiments it was found that $NH_4.OCOCH_3$ and $(NH_4)_2SO_4$ were also very effective in prevention of inactivation of frozen-thawed T 4 phage.

Inactivation in the absence of ice

The relative effectiveness of different salts in inactivating or protecting T 4 phage during freeze-thawing is of the same order as has been reported for their effects on the conformational stability of a large variety of macromolecules (Whitaker & Tapel, 1962; Von Hippel & Wong, 1964; Jencks, 1965; Bello, 1966). Although the mechanism whereby concentrated solutions of different salts exert their characteristic effects on macromolecules is not yet known, their action may be mediated, at least in part, through salt-induced changes in the structure of water (Bello, Riese & Vinograd, 1956; Simpson & Kauzmann, 1953; Von Hippel & Wong, 1964; Jencks, 1965).

It would not be correct, however, to state that T 4 phage are inactivated during freeze-thawing solely owing to the great increase in the concentration of salt in the unfrozen aqueous phase, because in this simple explanation a notable discrepancy is overlooked. It is known, from studies of the effect of osmotic shock on T 4 phage at room temperature, that if osmotic shock is avoided by slow dilution of the suspending medium T 4 phage are not inactivated by exposure to 3 molal solutions of NaCl, NaBr or LiCl (Anderson, 1953; Leibo & Mazur, 1966). During freezing such salts reach a concentration of 3 molal at approximately $-12°$ C., at which

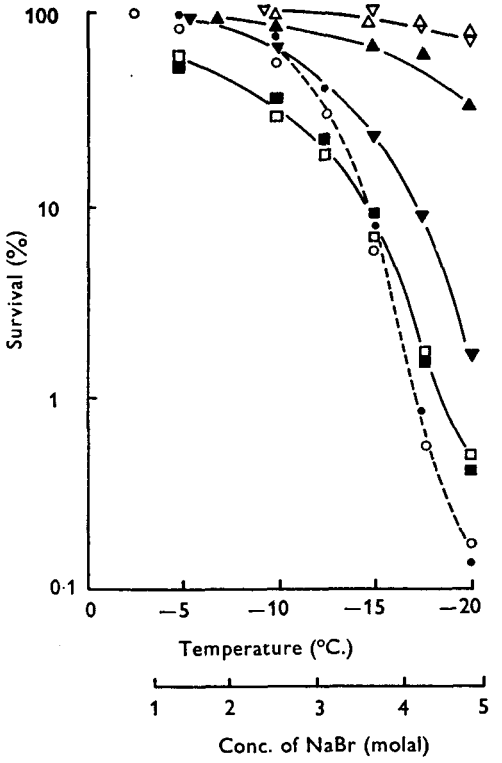

Fig. 4

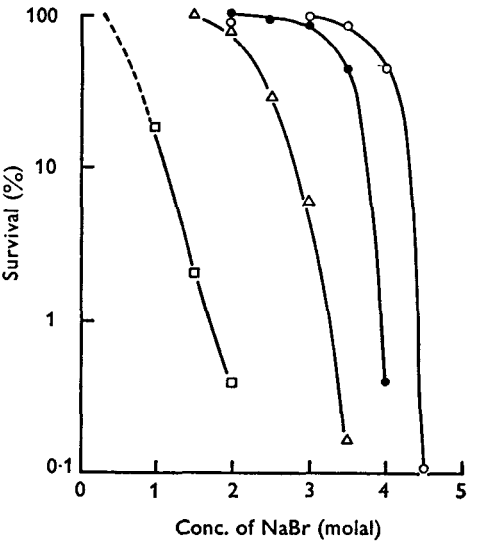

Fig. 5

Fig. 4. Inactivation of phage in the absence of ice. Samples of the T4 and T4Bo phages, suspended in NaBr solutions of different initial molalities, were cooled at 1° C./min. to the indicated temperatures and rewarmed slowly. These samples were not seeded with a cold wire and remained unfrozen throughout the experiment. The dotted line shows the control inactivation curve for samples of T4 and T4Bo phages, suspended in 0·1 molal NaBr, which were cooled with freezing and thawed, as in the normal experimental procedure. The lower scale shows the molal concentration of NaBr in the unfrozen aqueous phase in equilibrium with ice at subzero temperatures. ▽, 1·0 molal NaBr (T4 phage); △, 1·0 molal NaBr (T4Bo phage); ▲, 2·0 molal NaBr (T4Bo phage); ▼, 2·5 molal NaBr (T4Bo phage); □, 3·5 molal NaBr (T4 phage); ■, 3·5 molal NaBr (T4Bo phage); ○, 0·1 molal NaBr (T4 phage, frozen control); ●, 0·1 molal NaBr (T4Bo phage, frozen control).

Fig. 5. The effect of lowered temperature on the degree of inactivation of T4Bo phage. The figure shows the survival of T4Bo phage following 30 min. exposure to unfrozen NaBr solutions of the indicated molalities at 20° C. (○), 0° C. (●), $-10°$ C. (△), and $-20°$ C. (□).

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temperature they all produced marked inactivation of T 4 phage and T 4 Bo phage. This inactivation was in no way due to the increase in phage concentration during freezing, since different initial titres of phage in experimental samples were found to have no effect on the subsequent percentage viabilities after freeze-thawing.

In order to investigate this discrepancy, a modified experimental technique was adopted. Samples of T 4 phage and T 4 Bo phage, suspended in concentrated NaBr solutions, were cooled at 1° C./min. to temperatures from $-5°$ to $-20°$ C. and rewarmed slowly. The samples were *not* seeded with a cold wire and remained unfrozen throughout the experiment. The cooled and rewarmed samples of T 4 phage were diluted slowly in a stepwise fashion to avoid osmotic shock during the assay of viability. Samples of the osmotic shock-resistant T 4 Bo phage were diluted directly into phosphate buffer before being assayed. Control inactivation curves were determined for samples of T 4 phage and T 4 Bo phage suspended in 0·1 molal NaBr. These latter samples were seeded at $-5°$ C. as in the normal experimental procedure. The results of these experiments are shown in Fig. 4.

The susceptibilities of the T 4 and T 4 Bo phages were identical, and at any temperature the extent of the inactivation was related to the salt concentration whether ice was present or not. The most interesting observation, however, was that in the absence of ice and change in salt concentration there was still a considerable increase in inactivation as the temperature was lowered. This was not an effect of prolonged exposure of the phage to a concentrated salt solution, since at room temperature a 3·5 molal NaBr solution caused almost no inactivation of phage over a period of several hours.

The experiments indicated that the temperature of the experimental samples was as important as the salt concentration in influencing the degree of inactivation of the T 4 and T 4 Bo phages.

Further experiments were carried out to investigate the effect of temperature. Samples of the T 4 Bo phage were exposed to unfrozen concentrated solutions of NaBr for 30 min. at 20°, 0°, $-10°$ and $-20°$ C. The results are shown in Fig. 5. Clearly the effect of lowering the temperature was to produce a very marked increase in the degree of inactivation of phage exposed to identical salt concentrations.

Storage above the eutectic temperature

In the experiments described so far, samples were cooled to the desired temperature and immediately thawed. A further series of experiments was performed in which frozen samples of the T 4 phage, suspended in various salt solutions, were stored at subzero temperatures for periods of 10 min. to 5 hr. before thawing. In all cases it was observed that inactivation of the phage continued for the first 20–30 min. of storage at a rate comparable to that observed during cooling, but thereafter the inactivation rate decreased by a factor of more than 50-fold. It appeared that a proportion of the phage was in some way protected against the injurious effect of the concentrated salt in the unfrozen aqueous phase. For each suspending medium the fraction of phage which was protected was lower the lower the storage temperature, and at any particular storage temperature was

dependent on the species of ions in the suspending medium, being decreased by different ions in the same order of effectiveness as had been observed for inactivation during freezing and thawing without storage. These observations were investigated further using NaBr solutions as the suspending medium; $-15°$ C. was arbitrarily chosen as the storage temperature.

At first it was thought that the effect might be due to the very low concentration of phosphate buffer in the suspending medium (samples were prepared by a 1000-fold dilution of a stock suspension of T 4 phage, in phosphate buffer, into the experimental suspending medium). Subsequent experiments (Fig. 6) showed,

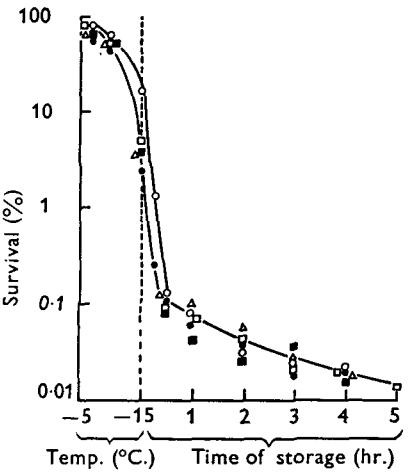

Fig. 6

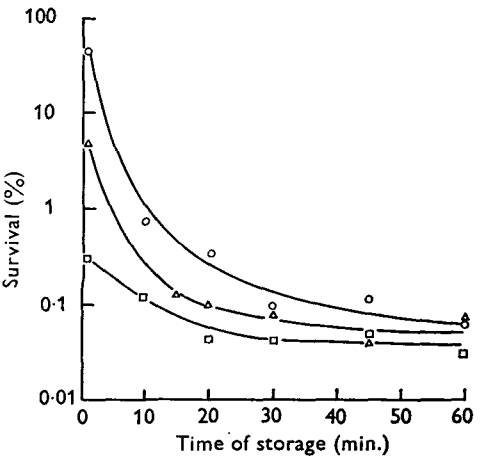

Fig. 7

Fig. 6. The effect of storage on the viability of T4 phage. Samples of T4 phage, suspended in 0·1 molal NaBr, were cooled at 1° C./min. to $-15°$ C. and stored at that temperature for the indicated periods, and then thawed slowly. The figure shows the inactivation during both pre-freezing and after storage at $-15°$ C. ○, 100-fold dilution from phosphate buffer; □, 1000-fold dilution from phosphate buffer; ●, 10,000-fold dilution from phosphate buffer; ■, initial T4 phage titre of 2×10^8 p.f.u./ml.; △, two freeze–storage–thaw cycles.

Fig. 7. The effect of freezing rate. Samples of T4 phage, suspended in 0·1 molal NaBr, were cooled at 100° C./min. (○), 1° C./min. (△) or 0·3° C./min. (□) to -15 °C. and stored at that temperature for periods of 0–60 min. before thawing slowly.

however, that the same results were obtained whether the experimental samples had been prepared by a 100-fold, 1000-fold or 10,000-fold dilution of the stock suspension. (Stock suspensions with T 4 phage titres of 2×10^8, 2×10^9 and 2×10^{10} p.f.u./ml. respectively were used, so that the initial experimental titre was 2×10^6 p.f.u./ml. in each case.) Moreover, the protected fraction was independent of the initial phage concentration (Fig. 6) and freezing rate (Fig. 7). One set of samples of T 4 phage, suspended in 0·1 molal NaBr, were stored at $-15°$ C. for 60 min. and thawed, and then refrozen and stored at $-15°$ C. a second time. The protected fraction observed during the second period of storage (expressed as percentage of the surviving plaque forming units remaining after the first period of storage) was identical with that observed for a single freeze–storage–thaw cycle (Fig. 6).

Very significant results were obtained when experiments were carried out using different initial concentrations of NaBr in the suspending medium. The initial survival at −15° C. of frozen-thawed T 4 phage had been shown to be independent of the initial NaBr concentration. During storage, however, large differences were observed (Fig. 8). The protected fraction of phage was highest in those samples initially suspended in 0·01 molal NaBr, being about 1% of the original titre, and was considerably lowered when higher initial concentrations of NaBr were used. There were no protected phage when T 4 or T 4 Bo phage suspended in 3·5 molal NaBr were stored at −15° C. in the absence of ice.

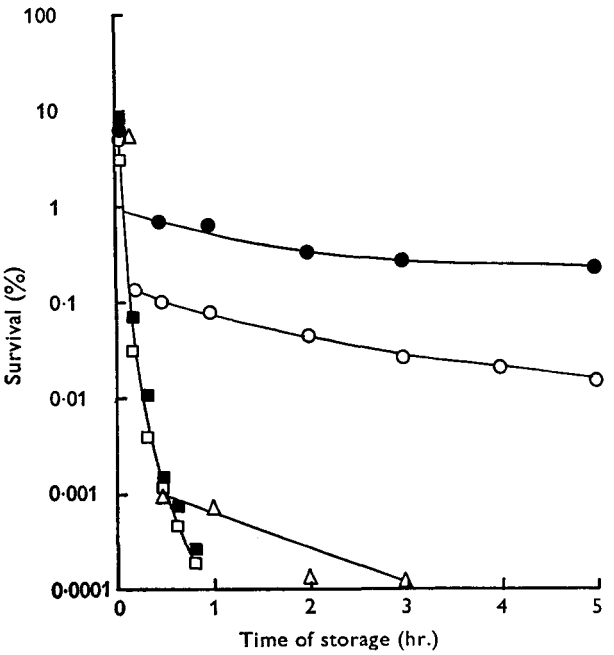

Fig. 8. Effect of initial NaBr concentration. Samples of T4 and T4Bo phages, suspended in NaBr solutions of different initial molalities, were cooled at 1° C./min. to −15° C. and stored at that temperature for the indicated times, and then thawed slowly. ●, 0·01 molal NaBr (T4 phage); ○, 0·1 molal NaBr (T4 phage); △, 1·0 molal NaBr (T4 phage); ■, 3·5 molal NaBr (T4 phage, unfrozen); □, 3·5 molal NaBr (T4Bo phage, unfrozen).

When the initial concentration of salt in the suspending medium is raised, the width of unfrozen channels of liquid between the ice crystals at any subzero temperature is greater (Nei, 1968). The results therefore suggest that during freezing phage particles which are trapped in channels of unfrozen liquid of less than a critical maximum width are in some way protected against inactivation. There is evidence that water in narrow channels may possess a higher degree of structural order than bulk liquid (Bangham & Bangham, 1968; Willis, Rennie, Smart & Pethica, 1969). Perhaps such a 'structuring' of the water protects the phage against inactivation. It is indeed paradoxical to find that ice can act as a protective against freezing injury!

Effect of D_2O

In recent years the work of several investigators, notably Kauzmann (1959), Némethy & Scheraga (1962*a*, *b*), Némethy, Steinberg & Scheraga (1963), and Tanford (1962) and their co-workers, has shown the great importance of hydrophobic bonds in the conformational stability of proteins. Hydrophobic bonds become weaker when the temperature is lowered, and this fact may explain the effect of lowered temperature on the degree of inactivation of T 4 and T 4 Bo phages. Hydrophobic interactions between apolar amino acid side-chains are slightly stronger in D_2O than H_2O (Kresheck, Schneider & Scheraga, 1965), and it has been observed that ribonuclease (Hermans & Scheraga, 1959), gelatin (Harrington & Von Hippel, 1961) and catalase (Guild & Van Tubergen, 1957) are more stable to thermal modification in D_2O than H_2O. Similarly, denaturation of ovalbumin by urea is slower in D_2O than H_2O (Maybury & Katz, 1959).

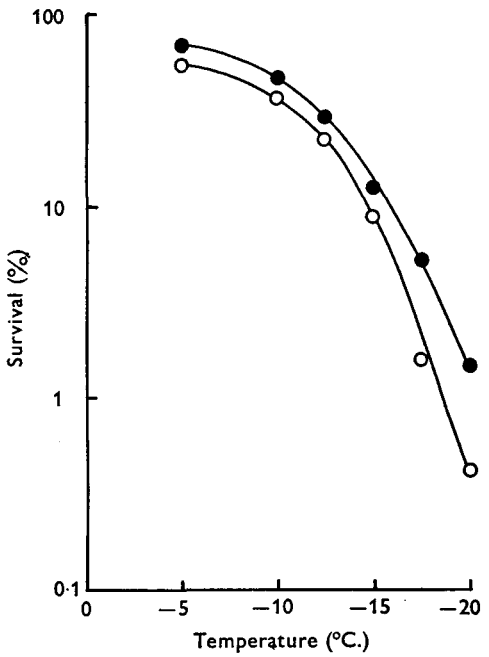

Fig. 9

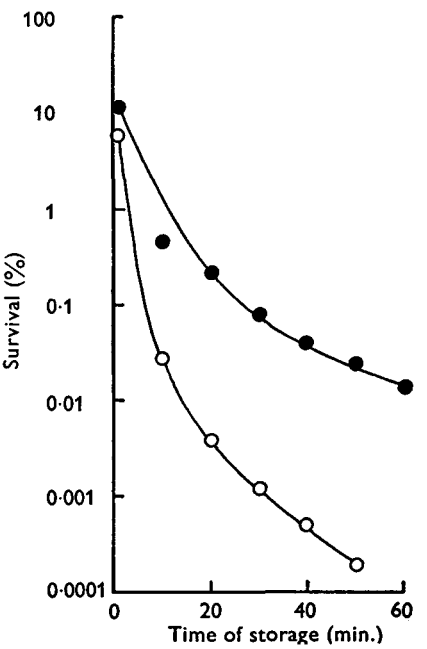

Fig. 10

Fig. 9. Effect of D_2O. Samples of T 4 Bo phage suspended in 3·5 molal NaBr–H_2O (○), or 3·15 molal NaBr–D_2O (●) were cooled at 1° C./min. without freezing to the indicated temperatures and rewarmed slowly.

Fig. 10. Effect of D_2O during storage. Samples of T 4 Bo phage suspended in 3·5 molal NaBr–H_2O (○), or 3·15 molal NaBr–D_2O (●) were cooled at 1° C./min. without freezing to −15° C. and stored at that temperature for periods of 0–60 min. before rewarming slowly.

In order to investigate a possible stabilizing effect of D_2O on phage, T 4 Bo phage suspended in 3·15 molal NaBr in D_2O (99·7 % D_2O, Koch-Light Labs., England) were cooled to subzero temperatures, without freezing, and rewarmed,

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and the results compared with those previously obtained using 3·5 molal NaBr in H_2O (Fig. 9). (Since molality is defined as 'moles of solute per 1000 g. of solvent', the *mole fraction* of NaBr is the same in 3·5 molal NaBr–H_2O as in 3·15 molal NaBr–D_2O, i.e. 0·059.) The percentage viability of T 4 Bo phage cooled and rewarmed in the D_2O solution was clearly greater than in the H_2O solution, the difference being fourfold at $-20°$ C. The difference was considerably magnified during storage at $-15°$ C., reaching a factor of over 100-fold after 50 minutes of storage (Fig. 10).

Protective effect of glycerol

Glycerol has been extensively used as a protective additive against freeze-thawing injury to cells since the discovery of its protective effect by Polge, Smith & Parkes (1949). Lovelock (1953) demonstrated that glycerol lowers the concentration of solutes in equilibrium with ice at any temperature, whilst Rey (1960),

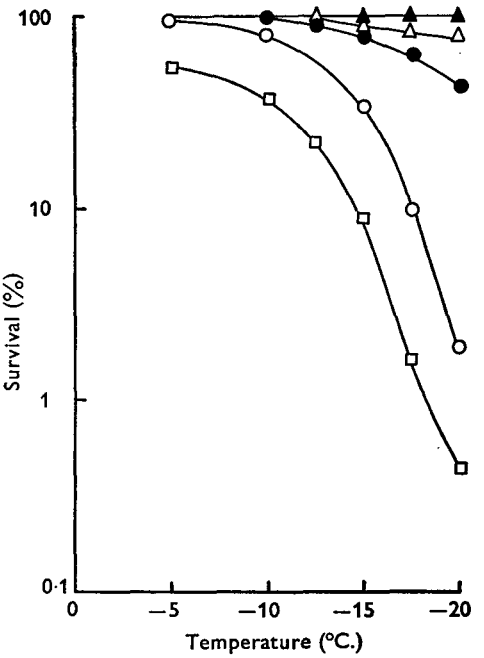

Fig. 11. The protective effect of glycerol in the absence of ice. T 4 Bo phage were suspended in 3·5 molal solutions of NaBr containing 1 % glycerol (○), 5 % glycerol (●), 10 % glycerol (△), 20 % glycerol (▲), or no glycerol (□). Samples were cooled at 1° C./min. without freezing, to the indicated temperatures and rewarmed slowly.

and Greaves & Davies (1965) have shown that the presence of glycerol eliminates salt eutectics during freezing. These two effects of glycerol have usually been regarded as the basis of its protective action against freeze-thawing injury (Smith, 1961; Meryman, 1966). It was therefore of interest to determine the effect of added glycerol on the survival of phage cooled to subzero temperatures in the *absence* of ice.

Samples of T 4 Bo phage were suspended in 3·5 molal NaBr solutions containing 1, 5, 10 and 20 % glycerol. The samples were cooled at 1° C./min., without freezing,

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and rewarmed slowly. The resulting viabilities are shown in Fig. 11. The addition of glycerol protected against inactivation although there was no ice present in the experimental samples. At a concentration of 20% the added glycerol was completely protective. An interesting parallel to this result is the observation of Simpson & Kauzmann (1953) that addition of glycerol prevents denaturation of ovalbumin by urea.

SUMMARY

The effects of various salts on the viability of T 4 and T 4 Bo phages during freezing and thawing have been studied. The effectiveness of different salts in causing inactivation or protection of phage during freeze–thawing was of the same order as has been observed for their effects on the conformational stability of macromolecules. There were two important contributory factors causing inactivation of phage during freeze–thawing: (i) concentration of solutes during freezing, and (ii) lowering the temperature, without change in salt concentration.

The viability of phage following storage at subzero temperatures was dependent on the storage temperature, the species of ions in the suspending medium and the initial salt concentration. Viability was greatest when phage had been initially suspended in dilute solutions.

Survival of T 4 Bo phage, following cooling to subzero temperatures and rewarming, was greater in D_2O solutions than in H_2O solutions.

It was found that 20% glycerol completely protected against inactivation of T 4 Bo phage, suspended in 3·5 molal NaBr, which were cooled to subzero temperatures and rewarmed in the absence of ice. Without added glycerol the viability of T 4 Bo phage suspended in 3·5 molal NaBr, which were cooled to $-20°$ C and rewarmed was less than 1%. This protective effect of glycerol is in contradiction to the accepted views of its mode of action in prevention of freezing injury.

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