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Prevention of low temperature denaturation injury in T4Bo phage by low concentrations of traditional cryoprotective additives

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SUMMARY

The well known cryoprotective additives glucose, sucrose, glycerol, dimethyl sulphoxide, polyvinylpyrrolidone, dextran and ammonium acetate have been found to prevent inactivation of T4Bo phage frozen in sodium bromide solutions. Their protective effects in this experimental system could not be accounted for by a colligative mechanism. It is proposed that they may act by modifying the structure of the unfrozen aqueous phase rather than by direct interaction with the phage.

INTRODUCTION

A large variety of additives have for many years been used alone or in mixtures to prevent inactivation of frozen and freeze-dried suspensions of viruses and micro-organisms. A central problem in elucidating the mode of action of these cryoprotective additives has been the lack of precise knowledge of the mechanisms which cause inactivation during freezing and drying. Using the T4 and T4Bo bacteriophages of *Escherichia coli* as a model system it has been possible to demonstrate three distinct mechanisms of inactivation during freezing and thawing, namely osmotic shock, low temperature salt denaturation and eutectic crystallization (Leibo & Mazur, 1969; Steele, Davies & Greaves, 1969*a*, *b*; Steele, 1972*a*). Such a definitive model system is valuable for investigating the effects of cryoprotective additives. Using this system powerful cryoprotective effects of very low concentrations of small basic peptides have recently been demonstrated (Steele, 1972*b*). This paper reports the effects of several commonly used cryoprotective additives on inactivation of T4Bo phage by concentrated neutral salts during freezing. Sodium bromide was used as the test neutral salt as it causes a sufficiently large degree of inactivation to make any protective actions of additives very obvious. High concentrations of glycerol have been shown to be protective in this system (Steele *et al.* 1969*b*). The other additives investigated were sucrose, glucose, dimethyl sulphoxide (DMSO), the polymers polyvinylpyrrolidone and dextran, and ammonium acetate which has been shown to protect frozen red blood cells (Meryman, 1968).

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MATERIALS AND METHODS

Purified phage stocks suspended in 0·1 M phosphate buffer (KH_2PO_4-Na_2HPO_4, pH 7) were prepared from 500 ml. lysates of *E. coli* B grown in Adams' medium. The methods of phage preparation, purification and titre determination were the same as those described previously (Steele *et al.* 1969*a*). Phage stocks were stored at 4° C.

Cooling apparatus

Samples in glass freeze-drying tubes (Johnson & Jorgensen Ltd) were placed in an aluminium rack with a 7 mm. deep base-plate into which 0·5 cm. diameter holes were countersunk to closely accommodate the sample tubes. The rack rested centrally in a bakelite tube, whose lower end was submerged to a depth of 20–30 cm. in liquid nitrogen in a Linde LNR25B refrigerator, and was cooled by cold nitrogen vapour generated by a 700 Watt heating element fixed in the lower end of the tube. The cooling rate was adjusted by a variable transformer connected in series to the heating element. Temperatures were measured by a copper-constantan thermocouple connected to a potentiometric Kipp & Zonen BD5 micrograph recorder.

Experimental procedure

A stock suspension of T4Bo phage at a concentration of $2{\cdot}5 \times 10^9$ p.f.u./ml. was diluted 1000-fold in the experimental suspending medium. Samples of 0·1 ml. were cooled at 1° C./min and seeded at $-3°$ C. by touching their surface with a fine wire cooled in liquid nitrogen. The samples were equilibrated for 15 min. after seeding at $-3°$ C. before starting cooling. At the desired subzero temperature samples were transferred to a thick polystyrene block maintained at 4° C. to accomplish slow thawing. Samples were suitably diluted 1/10, 1/100 or 1/1000 in phosphate buffer, and plaque assays performed. Survivals were expressed as a percentage of the control plaque counts.

Freezing point determinations

Samples of NaBr or NaBr/sucrose mixtures of approximately 5 ml. were cooled in boiling tubes in an insulated alcohol bath at approximately 0·25° C./min. Repeated attempts were made to seed the samples with a small ice crystal in a Pasteur pipette. The mean temperature at which ice began to form was taken as the freezing point.

Determination of critical inactivating NaBr concentrations

T4Bo phage samples of 0·1 ml. suspended in solutions of NaBr of several concentrations were stored at 0° C. (melting ice bath), $-10°$ C. and $-15°$ C. (precooled, insulated alcohol baths) for 20 min. The samples were unseeded and remained unfrozen. After storage they were diluted in phosphate buffer and assayed for percentage survivals as described. The critical inactivating NaBr

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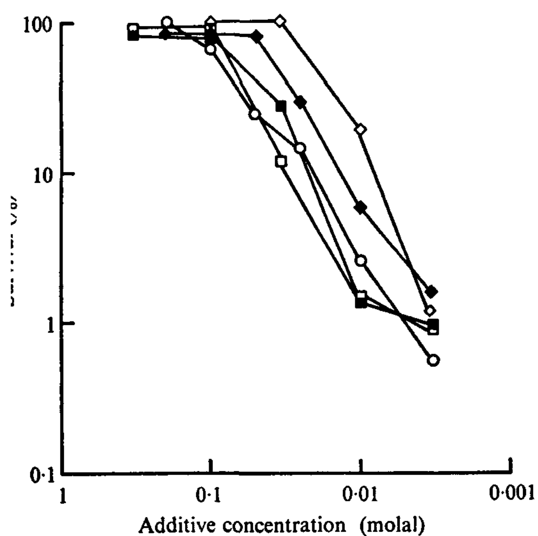

Fig. 1

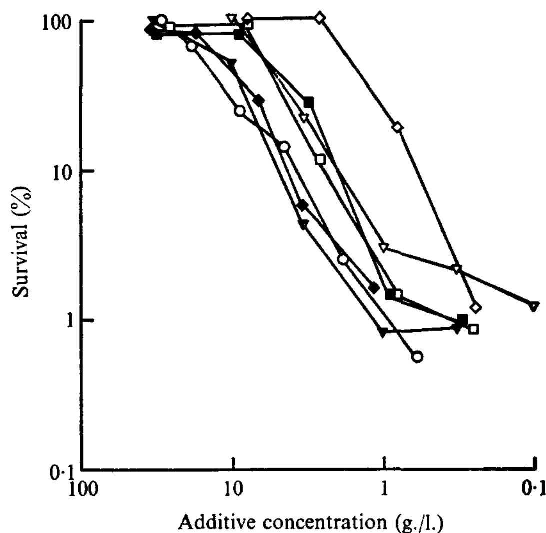

Fig. 2

Fig. 1. The effect of additives at the indicated concentrations on the survival of T4Bo phage. Samples of T4Bo phage suspended in 0·1 molal NaBr plus additive were cooled at 1° C./min. to −17·5° C. and thawed slowly. ◇, ammonium acetate; □, DMSO; ■, glycerol; ◆, sucrose; ○, glucose. The control survival (no additive) was 0·4%.

Fig. 2. The effect of additives at the indicated concentrations on the survival of T4Bo phage. The test system was the same as in Fig. 1. ◇, ammonium acetate; □, DMSO; ■, glycerol; ◆, sucrose; ○, glucose; ▽, PVP; ▼, dextran.

concentration for each temperature was taken as that which caused 50% loss of plaque forming units during the 20 min. of storage.

Polymers

The two polymers investigated were polyvinylpyrrolidone (PVP-40, Pharmaceutical Grade, Sigma Chemical Company, average mol. wt. 40,000) and dextran (Dextran-70, Pharmacia Fine Chemicals, average mol. wt. 70,000).

RESULTS

The effect of cryoprotective additives

Samples of T4Bo suspended in 0·1 molal NaBr plus a series of increasing molal concentrations of the additives DMSO, glycerol, glucose, sucrose and ammonium acetate were cooled to −17·5° C. and thawed slowly. The percentage survivals are shown in Fig. 1. All conferred almost complete protection on the frozen T4Bo phage at concentrations of 0·1 molal. At lower concentrations their order of effectiveness was glucose, DMSO, < glycerol < sucrose < ammonium acetate. In Fig. 2 these results are expressed in terms of additive concentration by weight and compared to those obtained with the polymers PVP and dextran. Here their order of effectiveness was glucose, sucrose, dextran < PVP, DMSO,

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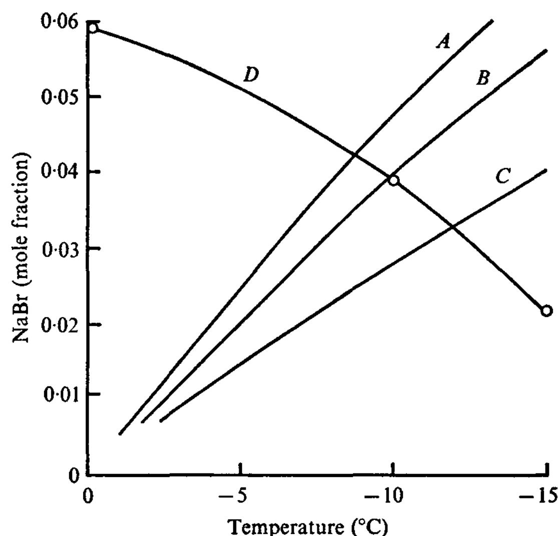

Fig. 3. Freezing point curves for NaBr (*A*), and sodium bromide/sucrose mixtures in molal ratios of 4/1 (*B*) and 1/1 (*C*). The critical inactivating concentrations of sodium bromide for T4Bo phage at 0° C., −10° C. and −15° C. are shown in curve (*D*).

glycerol ⩽ ammonium acetate. The ratios of weight-effective of the additives in raising survivals from the control of 0·4 % to 50 % were ammonium acetate 1:PVP, DMSO, glycerol 3·5:glucose, sucrose, dextran 10.

Test for a colligative mechanism of action

It has long been believed that one of the main mechanisms of action of traditional cryoprotective additives is that they lower the concentration of other solutes in equilibrium with ice at any temperature – the so-called colligative mechanism (Lovelock, 1953). A direct test for a colligative mechanism of protection in this model system was made using sucrose as an example. Fig. 3 shows the NaBr concentrations during freezing compared with those where sucrose was present in the freezing mixtures in NaBr:sucrose molal ratios of 4:1 and 1:1. Also shown are the experimentally determined critical concentrations of NaBr. From the results the predicted temperatures at which significant inactivation of T4Bo phage should begin during freezing, if the colligative mechanisms were the only effect of the added sucrose, are NaBr, −9° C., NaBr 4:sucrose 1, −10° C., NaBr 1:sucrose 1, −12·5° C. To determine the actual temperatures, samples of T4Bo phage were frozen in 0·1 molal NaBr, and 0·1 molal NaBr solutions with added sucrose concentrations of 0·025 molal and 0·1 molal (Fig. 4). The temperatures at which significant inactivation (50–60 % survival) of T4Bo phage first occurred were NaBr, −10° C., NaBr 4:sucrose 1, −17·5° C., NaBr 1:sucrose 1, −30° C.

As a further test unfrozen samples of T4Bo phage were cooled in concentrated additive mixtures to −20° C. at 1° C./min without freezing and rewarmed slowly

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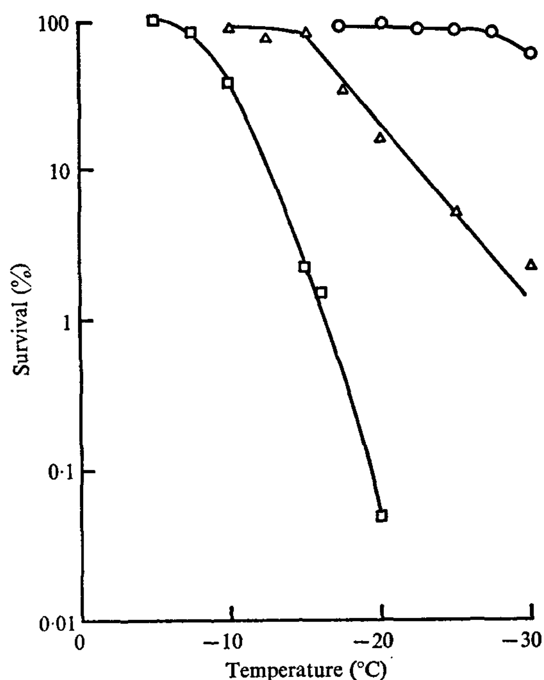

Fig. 4. Inactivation of T4Bo phage when frozen in NaBr and NaBr/sucrose media. Samples were cooled at 1° C./min to the indicated temperatures and thawed slowly. The suspending media were 0·1 molal NaBr (□); 0·1 molal NaBr + 0·025 molal sucrose (△); 0·1 molal NaBr + 0·1 molal sucrose (○).

Table 1. *The survivals of T4Bo phage cooled, without freezing, at 1° C./min. to −20° C. and thawed slowly at 4° C. in concentrated suspending media containing sodium bromide + additive. The control survivals (no additive present in suspending medium) were* < 1 %.

Sodium bromide concentration	Additive	Additive concentration	Survival (%)
3·5 molal	Sucrose	1 molal	44
3·5 molal	Sucrose	2 molal	90
3·5 molal	Glucose	1 molal	100
3·0 molal	PVP	10 % (w/v)	100
3·0 molal	Dextran	10 % (w/v)	42
3·5 molal	Glycerol	10 % (v/v)	81
3·5 molal	DMSO	10 % (v/v)	40

(Table 1). The additives gave considerable or complete protection to the T4Bo phage against lethal concentrations of NaBr in the absence of freezing and thus were not protecting by a colligative mechanism.

DISCUSSION

The effects of neutral salts on macromolecules are believed to result from changes in the solubility of the internal hydrophobic groups probably mediated

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by effects of the salts on the solution, together with their affinity for polar amide groups (von Hippel & Schleich, 1969). Sodium bromide represents a salt which causes denaturation, whereas ammonium acetate is a stabilizing salt (von Hippel & Wong, 1964). When used as a mixture in this study the effect of ammonium acetate was dominant as it completely protected frozen-thawed T4Bo phage when the molal ratio of the salts in the suspending medium was NaBr 3 : ammonium acetate 1 (Fig. 1).

The protective actions of the other additives studied were shown not to be due to a colligative mechanism. They are all strong hydrogen-bonding compounds and thus also modify the structure of the solution. Perhaps it is in this way that they are able to neutralize the inactivating effect of sodium bromide during freezing. Alternatively they may act through direct interactions with the T4Bo phage although their great diversity of molecular weights makes this a far less likely possibility.

The experimental freezing unit described in this paper was designed by Professor R. I. N. Greaves.

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