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The preservation of bacteriophage H 1 of *Corynebacterium ulcerans* U 103 by freeze-drying

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Corynebacteriophage H 1 was found to be extremely labile when stored in suspension at 4° C. and also when stored at low temperatures after conventional slow rates of cooling. This seemed a suitable organism to use for a detailed investigation of its sensitivity to freezing and drying under different conditions with the intention of applying the results, in the light of recent research work on the factors influencing survival during and after freeze-drying, to maintain maximum survival in the freeze-dried product. Bacteriophages, though comparatively simple in structure, represent well-defined organized biological entities, and it is hoped that the result of such studies will eventually prove to be useful in enabling the damage which occurs during freezing, thawing and drying to be localized at a structural or biochemical level.

MATERIALS AND METHODS

Preparation and maintenance of phage

The initial sample of bacteriophage H 1 of *Corynebacterium ulcerans* U 103 was obtained in the form of a broth lysate which had been frozen rapidly and stored at −70° C. After thawing rapidly at 37° C., 0·5 ml. of this lysate was used to inoculate a 10 ml. volume of a susceptible culture of *C. ovis* E 1144 B in digest broth, which had been incubated on an inclined revolving stage for 2 hr. at 37° C. (to prevent clumping), and lysis was allowed to proceed for 5 hr. at 37° C.

This lysate was then used to inoculate 20 ml. of a 2 hr. culture of *C. ovis* prepared as before. A further 5 hr. was allowed for complete lysis to occur. Bacterial debris was removed by centrifugation, and 2 ml. volumes of the supernatant were dispensed into neutral glass tubes, cooled rapidly by direct immersion in liquid nitrogen and stored at −196° C.

Preparation of experimental samples

When required, a tube containing 2 ml. frozen lysate was thawed rapidly by agitation in a water bath at 37° C., and diluted 1/10 with digest broth. Equal volumes of this suspension and of each double-strength additive were thoroughly mixed, and 0·1 ml. samples of each mixture dispensed into 0·5 ml. freeze-drying tubes (Johnson and Jorgensen, London). Double-strength solutions (%, w/v) of additives were prepared in distilled water and sterilized by autoclaving, except solutions containing peptone, which were Seitz-filtered.

Assay

The bacteriophage was assayed by a top agar technique similar to that described by Adams (1959). The indicator bacteria (*C. ovis* E 1144 B) were taken from an overnight culture and subcultured for 2–3 hr. on digest agar slopes at 37° C. The culture was then transferred to 5 ml. of digest broth and placed in a Mickle shaker for 5 min. at the minimum speed. Any remaining clumps of bacteria were removed by light centrifugation.

Experimental samples of 0·1 ml. and 0·15 ml. of the indicator suspension were added to 2·5 ml. of 0·7 % digest agar which was melted and maintained at 46° C., care being taken during the mixing to prevent any bubble formation. The mixture was then poured as a thin layer on top of nutrient agar in a Petri dish. The number of plaques produced were counted after 24 hr. incubation at 28° C. Triplicate samples were used for each experimental condition and triplicate counts were made on each sample so that each result given represents the mean of nine counts.

Rate of cooling

Rates of cooling were used varying from 1 to 900° C./min. and different ranges of rate required different methods.

A cooling rate of 1° C./min. was obtained by cooling the sample in 0·5 ml. freeze-drying tubes in an aluminium block placed on the thermo-electrical refrigerated stage of the freeze-drying apparatus (Greaves & Davies, 1965).

For rates between 1 and 5° C./min. the method described by Nagington & Greaves (1962) was convenient. The apparatus consists of a hollow cylinder of polystyrene with a polystyrene base, fitting into the neck of a Linde LNR-25 B liquid nitrogen refrigerator. Sample tubes are placed in the cylinder, and the rate of cooling is determined by varying the depth of insertion of the cylinder in the refrigerator neck.

Rates of 5–40° C./min. were obtained by a modification of the previous method, the sample tubes being placed in a large Pyrex tube (3 cm. × 15 cm.) insulated externally with cotton wool. This tube was placed in a Linde storage cylinder with perforated base, which was lowered to about 1 in. above the surface of the liquid nitrogen in a Linde LNR-25 B refrigerator. The rate of cooling was adjusted by altering the thickness of the cotton-wool insulation. For rates of 40–70° C./min. the insulated tube was dispensed with, and sample tubes were placed directly in the Linde storage cylinder which was held at varying distances above the liquid nitrogen surface in the Linde LNR-25 B refrigerator.

In the higher ranges, 100–350° C./min. was obtained by immersing the lower halves of the sample tubes in ethanol which had been cooled to temperatures between −60 and −100° C. by pouring liquid nitrogen on the surface of the ethanol contained in a Dewar bowl and stirring until the nitrogen had boiled off. For a rate of 450° C./min. the lower halves of the sample tubes were immersed directly in liquid nitrogen, and for the highest rate, 900° C./min., the sample tubes were insulated externally with a coating of kieselguhr in a glycerol–methanol mixture as adhesive before immersing the lower halves of the tubes in liquid nitrogen. The

kieselguhr coating acted by preventing the formation of an insulating layer of gaseous nitrogen, and so enabled a rapid rate of freezing to be achieved.

Temperature measurement was by means of a 'Virtis' thermistor probe (10–700–7 p) attached to a Virtis (Gardiner, New York) sample temperature recorder (10–702–1). The probe was placed in a control sample situated between the experimental samples. Using the freezing techniques described, the rate of cooling was not constant over the whole range of temperatures and for this reason the rate of cooling was measured over the linear range $-10°$ to $-25°$ C.

In all cases samples were thawed rapidly by immersing and shaking in a water-bath maintained at 37° C. unless otherwise stated.

Freeze-drying

The apparatus used was that described by Greaves & Davies (1965). This consisted of a two-stage thermoelectric refrigerator mounted on a thick brass base plate which was water-cooled on the opposite side. A recessed 'O' ring in this plate gave a vacuum seal for the 'bell jar' top of the desiccator. The desiccator chamber was connected to a 'Megavac' pump, via a vacuum valve and a phosphorus pentoxide trap. The tubes containing samples to be dried were placed in aluminium blocks on the second stage and a fixed temperature was maintained throughout the drying period. Samples were dried under a pressure reaching 0·002 mm. Hg after 48 hr. (72 hr. for those dried at $-35°$ C.) After primary drying the cooling stage was switched off and the sample allowed to warm slowly to room temperature. Air was then admitted slowly and the samples were either immediately rehydrated with 1 ml. digest broth or were submitted to a secondary drying overnight on a vacuum manifold at room temperature. After secondary drying all samples were sealed *in vacuo* (0·01 mm. Hg) and kept in the dark at room temperature until required.

RESULTS

Preliminary tests showed that the survival of corynebacteriophage H 1 suspended in digest broth dropped by four log units every 5 days when stored at 4° C. Rapid cooling (450° C./min.) by immersion in liquid nitrogen followed by a rapid thaw at 37° C. gave 66 % survival, whilst cooling slowly (1° C./min.) to $-40°$ C. followed by a rapid thaw gave 26 % survival. The higher survival obtained after rapid freezing was in agreement with that obtained in a study on the freeze-thaw sensitivity of the coliphage T 4 (Steele, Davies & Greaves, 1969). However, with the T 4 bacteriophage it has been found that osmotic effects mask damage at slower cooling rates (Leibo & Mazur, 1967). It has also been suggested that osmotic damage was one of the main factors in preventing the successful freeze-drying of the coliphage T 4 (Davies, 1967). It was therefore necessary to investigate the sensitivity of the corynebacteriophage to osmotic damage before attempting to interpret any freeze-thaw or freeze-drying results.

Osmotic sensitivity

Samples of 0·1 ml. of bacteriophage suspension in broth were added to 10 ml. volumes of saline solutions of increasing strength, mixed and allowed to stand at

room temperature for 15 min. Samples were then taken for assay, after suitable dilution.

The results (Table 1) show that the corynebacteriophage H 1 is resistant to osmotic shock. As osmotic shock is considered to be involved in rehydration

Table 1. *Survival of the corynebacteriophage after exposure to varying concentrations of saline*

Concentration of NaCl	Percentage survival
0 (distilled water)	71
0·175 M	93
0·25 M	102
0·5 M	101
1 M	92
2 M	99
3 M	99
Saturated	98

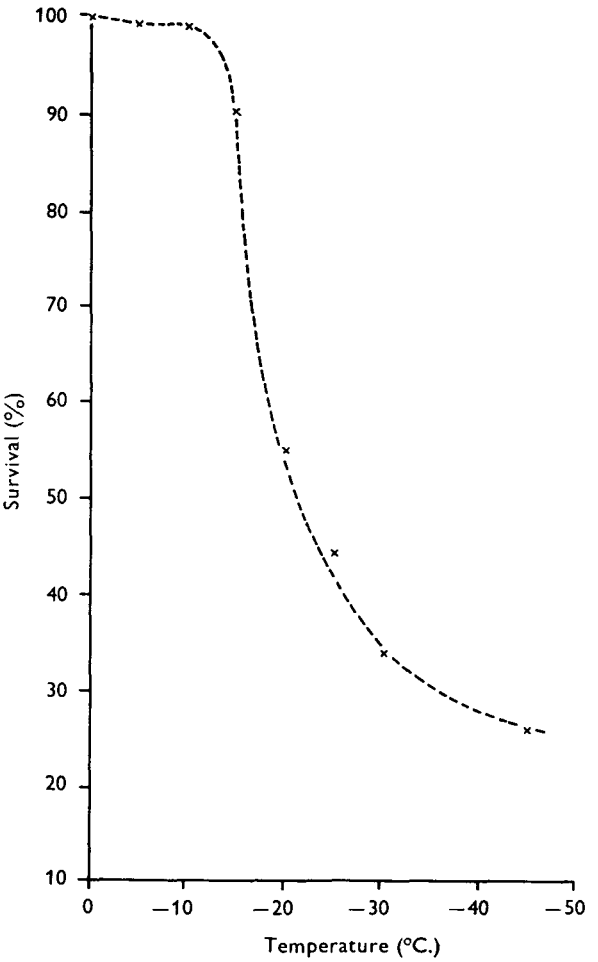

Fig. 1. The effects of cooling broth suspensions of corynebacteriophage at 1° C./min. to different subzero temperatures.

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damage it seemed reasonable that if freezing damage could be eliminated it might be possible to freeze-dry the corynebacteriophage and still obtain a high survival.

Freezing

A preliminary experiment had shown that a slow rate of cooling was more damaging to the corynebacteriophage than rapid cooling. It was therefore of interest to determine whether there was in fact a critical temperature at which

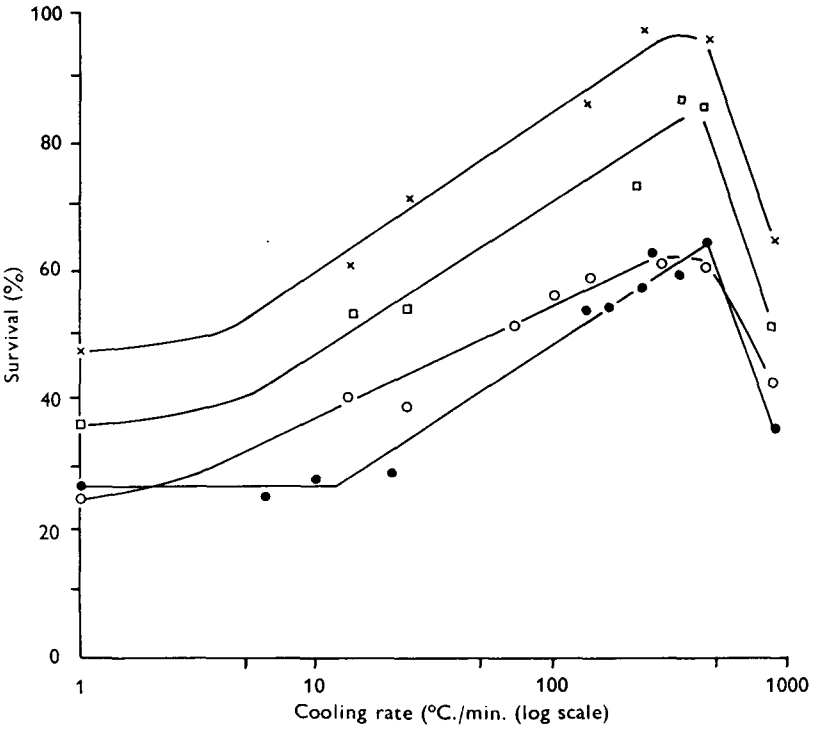

Fig. 2. The effects of different cooling rates on suspensions of corynebacteriophage in various additives. Suspending media: ×, 20 % peptone; □, 10 % peptone; ○, 10 % sucrose; ●, broth lysate.

damage started. Samples were cooled at 1° C./min. and ice formation induced at −5° C. The temperature was held at −5° C. for 10 min. to allow equilibration before continuing to cool at 1° C./min. Samples were removed at temperatures between −10° and −45° C., thawed rapidly, and viability assessed. The results (Fig. 1) showed that the damage began to occur at −14° C. and this increased with further lowering of the temperature. It is probable that such damage is caused by an increasing concentration of salt in the suspending medium, the effects being somewhat greater during cooling (Davies, 1969) and it is therefore likely that the physical or chemical consequences of such an increase in ion concentration at each temperature are time-dependent and should therefore be reduced by increasing the rate of cooling (Fig. 2). An increase in survival was found to occur with increasing rates of cooling; the maximum survival being obtained at

450° C./min. At 900° C./min., however, there was a drop in the survival, and it is thought that this can be explained by the formation and presence of ice crystals within the organism or by the removal of structural water from the protein or nucleic acid components at the high rate of cooling used. Adding various protective agents to the suspension increased the survival and this was particularly noticeable when the concentration of peptone was increased until at 20% it was possible to achieve 95% survival when cooling at 450° C./min. It was subsequently found that the addition of 10% sucrose and 2% sodium glutamate to 20% peptone did not affect the survival of corynebacteriophage obtained with peptone alone when cooled at 450° C./min. and further studies were made with mixtures of these three additives.

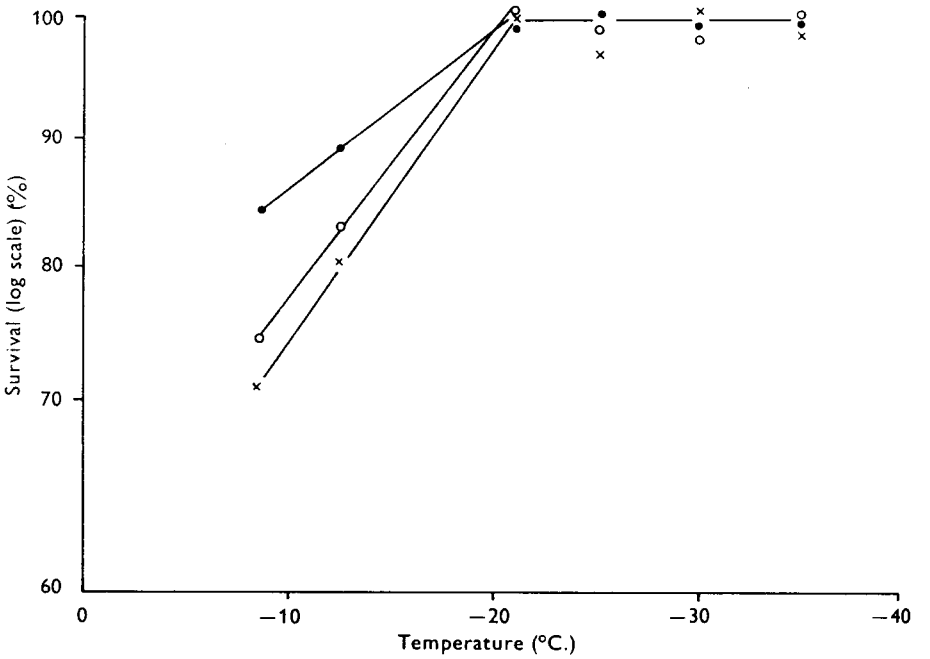

Fig. 3. The effects of cooling at 450° C./min. and subsequent equilibration at different subzero temperatures over a period of 15 min. followed by a rapid thaw. Suspending media: ×, 20% peptone; ○, 20% peptone + 10% sucrose; ●, 20% peptone + 10% sucrose + 2% sodium glutamate.

Freeze-drying

After cooling at 450° C./min. to −196° C. samples suspended in 20% peptone, 20% peptone + 10% sucrose, and 20% peptone + 10% sucrose + 2% sodium glutamate, were transferred to the drying block which was maintained at a fixed temperature. The samples were allowed to equilibrate for 15 min. On studying the effects of different temperatures it was found that provided the temperature was below −21° C. there was no damage during the warming to a suitable drying temperature (Fig. 3). The damage which occurred at temperatures above −21° C. may have been caused by the growth of ice crystals or alternatively by a high concentration of a solute at a low temperature.

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After drying at the various temperatures it was found that in the suspending medium 20% peptone + 10% sucrose + 2% glutamate survival was relatively constant (Fig. 4). On the other hand, if the glutamate was absent the survival in 10% peptone or 10% peptone + 10% sucrose increased logarithmically with decreasing temperatures, both media giving over 97% survival after drying at −35° C.

Storage

The survival immediately after drying is not in itself a sufficient guide to the survival likely to be achieved after long-term storage. The results of storing samples, which had been freeze-dried at −25° C., in the dark at room temperature are shown in Table 2. Survival after 3 months storage did not alter significantly in

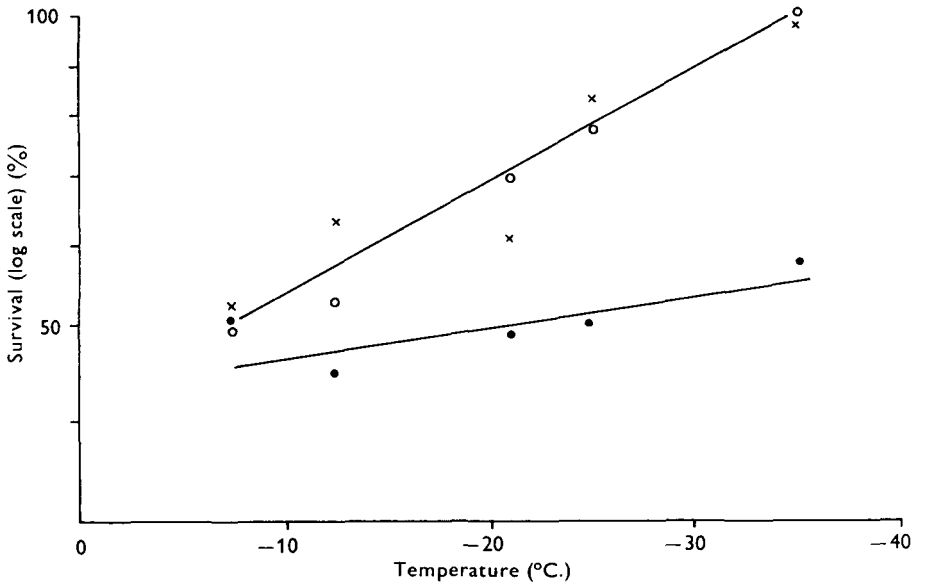

Fig. 4. The survival of corynebacteriophage after cooling at 450° C./min., equilibration at a particular temperature for 15 min. followed by drying from this temperature for 48 hr. (72 hr. for the samples dried at −35° C.) with a final pressure of 0·002 mm. Hg. Samples were allowed to warm to room temperature and were rapidly rehydrated with digest broth. Suspending medium: ×, 20% peptone; ○, 20% peptone + 10% sucrose; ●, 20% peptone + 10% sucrose + 2% sodium glutamate.

Table 2. *Percentage survival of the corynebacteriophage after freeze-drying at −25° C. and after storage of the dried product at room temperature in the dark*

	Suspending fluid used for freeze-drying		
Time of storage (days)	20% peptone	20% peptone, 10% sucrose	20% peptone, 10% sucrose, 2% sodium glutamate
0	84	80	53
40	82	85	55
92	75	82	54

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those samples dried in 20% peptone + 10% sucrose or 20% peptone + 10% sucrose + 2% sodium glutamate. There was, however, some evidence of a drop in survival in samples dried in 20% peptone alone.

In order to investigate the significance of this drop in survival, samples which had been dried at −35° C. and sealed *in vacuo* were subjected to heat-stability tests. Samples were tied in weighted muslin bags and immersed in water maintained at different temperatures for periods of 1 hr. (Fig. 5). In 20% peptone

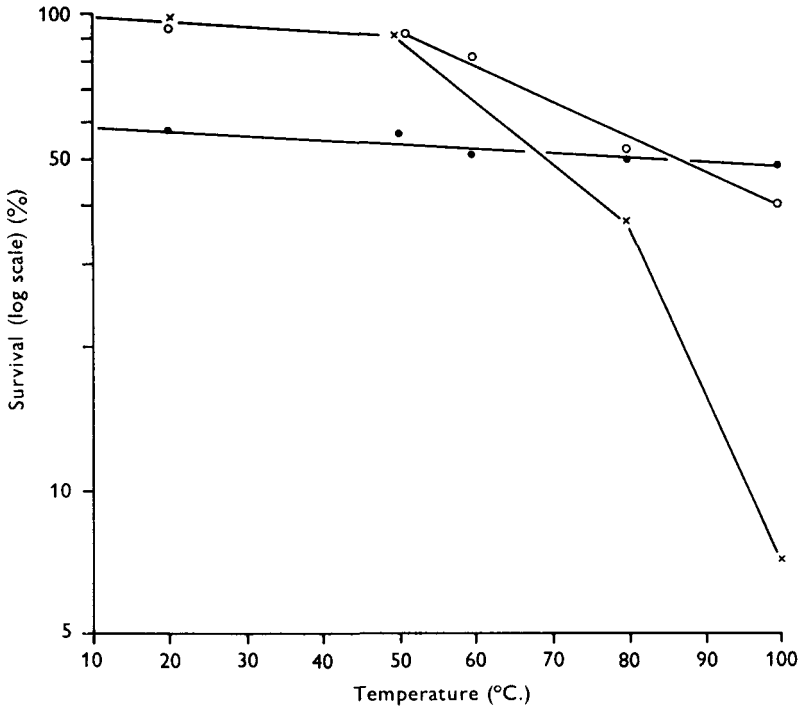

Fig. 5. The effects of heating for 1 hr. at different temperatures on the survival of suspensions of corynebacteriophage freeze-dried at −35° C. and sealed *in vacuo* (0·01 mm. Hg). Suspending media: ×, 20% peptone; ○, 20% peptone + 10% sucrose; ●, 20% peptone + 10% sucrose + 2% sodium glutamate.

survival remained constant at temperatures up to 60° C. Above this temperature survival began to drop, the rate of decline being even more evident at temperatures above 80° C. The curve appears to consist of two components, suggesting that two types of mechanism are involved in the drop in survival at high temperatures. With the addition of 10% sucrose there appears to be only one cause of damage, which again occurs at temperatures above 50° C. The addition of 2% glutamate to the mixture, however, whilst decreasing the immediate survival after drying, seems to stabilize the dried organisms against further damage brought about by increasing temperatures.

Maintaining the samples at 100° C. for different periods of time gave similar results to those described above (Fig. 6). Suspensions in 20% peptone showed a

steep drop in survival over the first 30 min., with a more gradual logarithmic decline during the following 4–5 hr. The initial drop was reduced by the addition of 10 % sucrose but the slope of the second part of the curve remained unaltered. The addition of 2 % sodium glutamate to the mixture stabilized survival throughout the heat treatment and seemed in particular to reduce the second type of damage.

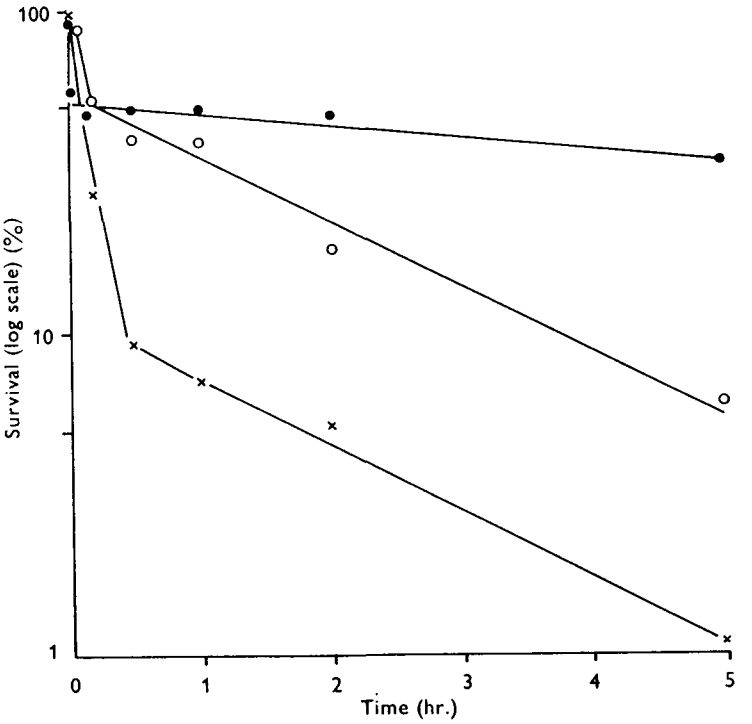

Fig. 6. The effects of storage at 100° C. for different periods of time on the survival of corynebacteriophage suspension freeze-dried at −35° C. and sealed *in vacuo*. Suspending media: ×, 20 % peptone; ○, 20 % peptone + 10 % sucrose; ●, 20 % peptone + 10 % sucrose + 2 % sodium glutamate.

DISCUSSION

It has proved possible to eliminate freezing damage in the corynebacteriophage H 1 by selecting a suitable concentration of protective agent and adjusting the cooling rate so as to prevent damage from increasing salt concentration whilst also preventing damage caused by the formation of minute crystals at the high rate of cooling. The concentration of peptone required to give maximum survival suggests that the protective action was the result of the formation of glass-like structure (Davies, 1966) though recent work has shown that the peptides present in the solution may also exert a specific effect on the protein membrane (Davies, 1969).

The addition of sucrose to the freeze-drying medium followed the suggestion by Fry & Greaves (1951) that sugars help to bind water and prevent over-drying.

This suggestion has received further support in the results of the heat-stability testing where peptone alone gives a rapid drop in survival. Sucrose also has the advantage that it does not interfere in the protection afforded by sodium glutamate (Muggleton, 1958). It is not yet known why the addition of sodium glutamate should cause a drop in the survival immediately after drying, though the fact that it stabilizes the organisms against the secondary damage during heat treatment suggests that this damage is caused by carbonyl-amino group interaction.

Although it is impossible to predict long-term storage without detailed accelerated storage tests, it is reasonable to assume from our heat-stability tests that, for storage at room temperature (20° C.) for reasonable lengths of time, 20 % peptone + 10 % sucrose would give satisfactory results. If, however, higher temperatures were likely to be encountered 20 % peptone + 10 % sucrose + 2 % glutamate mixture would give the optimal survival over long-term storage though it must be emphasized that this would be at the expense of an immediate high survival. The final choice of media thus depends upon the conditions to be encountered in the dried state.

The high survival obtained after drying at low temperatures has confirmed that drying in the absence of glutamate should take place from as low a temperature as practical (Greaves & Davies, 1965). Research is now in progress to determine whether drying at lower temperatures determines the removal of water from ice crystals before the removal of water bound by the suspending medium. This in turn may influence the stability of the structural water within the macromolecules of the organism.

The successful preservation of the corynebacteriophage H 1 by freeze-drying has served to emphasize that by adjusting such factors as suspending medium, cooling rate and drying temperature to suit different species of organisms it is now possible to achieve maximum survival over prolonged periods in the dried state.

SUMMARY

This paper describes an investigation into the successful preservation at room temperature of the bacteriophage H 1 of *Corynebacterium ulcerans* U 103 which was extremely labile when in suspension at 4° C.

Cooling at a rate of 1° C./min. showed that the survival decreased logarithmically at temperatures between −14 and −45° C. Survival of broth suspensions of the corynebacteriophage were found to increase proportionally with an increase in the rate of cooling though there was a marked drop in survival at rates of approximately 900° C./min. The addition of peptone solutions was found to increase the survival over the range studied, whereas the addition of sucrose solutions had only a slight effect.

By avoiding freezing damage by cooling at rates of 450° C./min. in (*a*) 20 % peptone solution, (*b*) 20% peptone and 10% sucrose, and (*c*) 20% peptone, 10 % sucrose and 2 % sodium glutamate, a study was made of the drying stage of the freeze-drying process. On drying at controlled temperatures it was found that there was no damage on rewarming to temperatures below −21° C.

after cooling to −196° C., but that the survival immediately after drying in the absence of glutamate, showed a logarithmic relationship with the temperature of drying, lower temperatures giving better survival.

On storage for a period of 3 months at room temperature *in vacuo* and darkness, there was no appreciable loss in survival in the mixtures though suspensions in peptone alone showed a slight decrease. At higher temperatures this decrease in survival could be differentiated into two types of damage, each of which could be influenced by the presence of sucrose or glutamate.

We are grateful to Professor H. R. Carne for providing the initial sample of bacteriophage H 1 of *Corynebacterium ulcerans* U 103, and to Professor R. I. N. Greaves for his interest and encouragement during the course of this investigation.

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