Viability and Electron Microscope Studies of Phages T3 and T7 Subjected to Freeze-drying, Freeze-thawing and Aerosolization

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SUMMARY

Phages T3 and T7 were freeze-dried, freeze-thawed or aerosolized. Freeze-drying and aerosolization caused dehydration and resulted in a loss of viability due mainly to one factor, loss of tails. However, the extent of loss of viability and tails was critically dependent upon the manner of rehydration, slow rehydration being best for the preservation of the head–tail complex and viability. Slow freezing (at about 1 °C/min), but fairly rapid thawing (at about 200 °C/min), resulted in marked loss of viability of T7 but only slight loss for T3, through causes other than loss of tails or lysis. Collection of aerosols of T3 and T7 by impinger resulted in much lower viabilities than were obtained by subsonic impaction (e.g. about 10 and 80 % respectively).

The results indicate that the dehydration induced by freeze-drying and aerosolization did not cause loss of viability, but that loss of viability occurred during rehydration. Dehydration of T3 and T7 did not appear to occur during slow freezing (at about 1 °C/min). Mechanisms different from those operating through shear stresses induced by freeze-drying and aerosolization caused loss of viability when T3 and T7 were slowly frozen at about 1 °C/min.

INTRODUCTION

Bacteria subjected to freeze-drying and aerosolization become sensitive to a toxic action of oxygen and it is thought that dehydration induced by these stresses increases the rate of reaction between oxygen and a component essential for bacterial multiplication (Cox & Heckly, 1973; Cox, Baxter & Maidment, 1973). Even in the absence of oxygen, microbial viability is markedly affected by dehydration and rehydration (see, for example, Cox, 1966*a*; Dewald, Browall, Schaeffer & Messer, 1967; Anderson & Cox, 1967). Recently it was suggested that freezing, especially when slow (e.g. 1 °C/min), causes intracellular dehydration (Asahina, 1965; Mazur, 1965; Merryman, 1966). However, Cox & Heckly (1973) showed that oxygen is not toxic for frozen bacteria, so dehydration may not occur during freezing. Comparison of survival of a micro-organism insensitive to oxygen subjected to freeze-drying, freeze-thawing and aerosolization might also indicate whether dehydration and rehydration phenomena occur during all these stresses. This suggested the experiments with phages T3 and T7 presented in this paper. For these organisms shear stresses induced by dehydration and rehydration occur as a consequence of freeze-drying and aerosolization but do not occur during a slow freeze (about 1 °C/min) and a fairly rapid thaw (about 200 °C/min). Phages T3 and T7 are also similar in many other ways (Adams, 1959); they are both susceptible to the dehydration–rehydration stresses (Benbough, 1971; Hatch & Warren, 1969; Warren & Hatch, 1969) but are not killed by oxygen at atmospheric pressure (C. S. Cox, unpublished data).

METHODS

Growth of organisms. Phages T3 and T7 were propagated in shaken, exponential phase cultures of *Escherichia coli* B (1×10^8 /ml) in nutrient broth at 37 °C. Titres of 1 to 3×10^{10} phage/ml of lysate were obtained and the phages were concentrated to a maximum titre of 1×10^{12} plaque-forming units/ml by centrifuging at 80000g for 30 min in a Model L2 65B Centrifuge (Beckman) using a type 30 rotor head. Different batches of a given phage did not always behave similarly and where relevant these findings will be discussed.

Viable counts were made by mixing 0.5 ml of a suitable dilution of phage suspension with 2 ml of 0.5 % (w/v) agar in nutrient broth containing 10^8 *Escherichia coli* B/ml at 42 °C, pouring the mixture on to 1.5 % (w/v) agar in nutrient broth contained in plastic Petri dishes and incubating at 37 °C for 4 h. After this time the plaques were counted and percentage viability calculated, in terms of the viable count of unstressed suspensions being equivalent to 100 % viability.

In aerosol experiments *Bacillus subtilis* var. *niger* spores were used as a tracer (Anderson & Cox, 1967) and percentage viabilities of the test microbes calculated as before (Cox, 1966a).

In all viability assays, 5 agar plates/sample with 50 to 150 plaques or colonies/plate were counted.

Freeze-drying. Portions of the phage suspensions (0.2 ml) were cooled to -55 °C at 1.5 to 2.0 °C/min in serum bottles and then freeze-dried as before (Cox & Heckly, 1973) for 4 h. Suspensions of T3 and T7 in nutrient broth or 0.05, 0.1, 0.2 and 0.4 molal sucrose in nutrient broth were studied. The rehydration procedures used were: (i) addition of 2 ml of nutrient broth; (ii) addition of 2 ml of 2 molal sucrose in nutrient broth; (iii) exposure of the freeze-dried powder to 100 % relative humidity (r.h.) followed by either (i) or (ii). Samples were then removed for viability assay and for examination in the electron microscope (see below).

Freeze-thawing. Samples (0.2 ml) of the phage suspension (as for freeze-drying) were cooled to -70 °C (usually) at 1.5 to 2.0 °C/min. After a total time of 1 h from the start of cooling, the phage suspensions were thawed at 200 °C/min by holding the serum bottles in a water bath at 42 °C. Samples were then taken for viability assay and examination in the electron microscope.

Aerosol experiments. These were performed as before (Cox, 1966a) using a Collison spray and a rotating drum at 40 % r.h. and 25.00 ± 0.05 °C in nitrogen (> 99.999 % pure, British Oxygen Co., London). For this work aerosols were stored for only 3 min because most loss of viability of T3 and T7 occurs within 3 s from spraying (G. S. Cox, unpublished). Aerosol samples were collected with an impactor (May, 1945) on to Formvar-coated electron microscope grids (mounted on glass slides) held directly below the slit of the fourth stage. The impactor was operated subsonically for 3.5 min at a flow rate of 20 l/min, following which an electron microscope grid was rapidly transferred into 1 ml of nutrient broth which then was shaken gently. This suspension was used for viability assay. The samples for electron microscopy were reconstituted by gently bathing one of the grids with a few drops of nutrient broth.

In accordance with the recommendation made at The First International Symposium on Aerobiology in 1963 (Brachman *et al.* 1964) aerosol samples were also collected using impingers (May & Harper, 1957) containing 20 ml of nutrient broth + antifoam (G.E.C. Silicone 60). Additional samples were collected after the aerosol was rehumidified by exposure to 100 % r.h. using the technique of Cox (1966b).

Table 1. Relationship and derived values of percentage viability and percentage tails (see text)

Phage	Procedure	Per- centage viability	Per- centage of tails	k*	% viability ₁ *	% viability ₂ *
T ₃	FD in NB; recon. NB	13·4	9·0	1·49	21·2	63·5
	FD in 0·05 molal Su; recon. 2 molal Su	47·0	34·1	1·38	80·4	58·7
	FD in 0·05 molal Su; rehyd; recon. NB	49·0	32·0	1·53	75·3	65·2
	FT in NB	100·0	45·0	2·22	106·0	94·5
	Aerosol; recon. NB	22·0	9·4	2·34	22·1	99·6
	Aerosol; rehyd; recon. NB	66·0	28·0	2·36	65·8	100·4
	Unstressed suspension	100·0	42·6	2·35	100·0	100·0
T ₇	FD in NB; recon. NB	1·0	2·0	0·50	4·5	22·4
	FD in 0·05 molal Su; recon. NB	3·2	6·7	0·48	14·9	21·5
	FD in 0·05 molal Su; recon. 2 molal Su	13·2	25·5	0·52	56·8	23·3
	FT in NB	21·6	47·6	0·45	106·0	20·2
	Aerosol; recon. NB	35·0	15·9	2·20	35·5	98·7
	Aerosol; rehyd; recon. NB	35·0	15·3	2·28	34·2	102·8
	Unstressed suspension	100·0	44·8	2·23	100·0	100·0

FD = freeze-dried; FT = freeze-thawed; NB = nutrient broth; Aerosol = suspension in NB aerosolized at 40 % r.h., 25 °C, sampled by impactor; Su = sucrose; recon. = reconstituting fluid; rehyd. = exposure to 100 % r.h. before reconstituting. The same batches of T₃ and T₇ were used throughout (see Results and Discussion).

* For definition, see Results and Discussion.

Electron microscope studies. The phage suspensions deposited on Formvar-coated electron microscope grids were drained before being inverted and gently lowered on to the surface of a 2 % (w/v) aqueous solution of ammonium molybdate. The specimens were satisfactorily cleaned and stained after 1 min and were finally coated with carbon.

The specimens were examined in a Philips EM300 electron microscope to determine the percentage of phage having visible tails, the percentage of lysed phage and the degree of penetration of the ammonium molybdate stain.

RESULTS AND DISCUSSION

Experiments were usually performed from two to five times, but results from replicate experiments sometimes varied significantly and the average variation for the data presented below was $\pm 20\%$ of the mean viability value and $\pm 5\%$ of the tails value. On occasions variability was much greater than these average values and where relevant the causes will be discussed.

Freeze-drying

Freeze-drying and reconstitution caused loss of viability. The extent depended upon the phage type and on the freeze-drying suspending fluid, but the overriding factor controlling survival was the manner of reconstituting the freeze-dried powder. Viability was enhanced to a marked extent in all experiments when either 2 molal sucrose was added to the nutrient broth reconstituting fluid or vapour phase rehumidification was used. Figure 1 and 2 are for T₃ freeze-dried in 0·1 molal or 0·2 molal sucrose in nutrient broth. Similar results were obtained with the other suspending fluids examined and with T₇ (Table 1). Table 1 indicates that a low viability was associated with a low percentage of tails and a high viability with a high percentage of tails.

Fig. 1

Fig. 2

Fig. 1. Percentage viability of phage T3 freeze-dried in 0.1 molal sucrose in nutrient broth, as a function of time of exposure to 100% relative humidity before reconstituting with (O) nutrient broth and (●) 2 molal sucrose in nutrient broth.

Fig. 2. Percentage viability of phage T3 freeze-dried in 0.2 molal sucrose in nutrient broth. Other details as for Fig. 1.

Gravimetric studies suggested that increasing the amount of sucrose in the freeze-drying suspending fluid decreased the rate of adsorption of water vapour during vapour phase rehydration. Hence, the difference in the time scale in Fig. 1 and 2 suggests that provided the rate of rehydration is already slow, then the extent rather than the exact rate of water vapour adsorption controls viability of reconstituted phage.

Stresses associated with freeze-drying caused little or no lysis of either T3 or T7, as determined by electron microscopy.

Freeze-thawing

Freezing T3 at 1.5 to 2.0 °C/min and thawing at 200 °C/min caused negligible loss of viability (Table 1). However, severe losses of viability occasionally occurred and 36 freeze-thaw experiments were performed in an attempt to determine the cause(s) of this variation. Several possible factors, such as slight changes in the composition of the suspending fluid and in the freeze and thaw rates, were examined. None of these could be implicated as the cause(s), but since extremely complex suspending media were used (e.g. nutrient broth) the non-reproducibility is possibly due to the variable precipitation of a solid phase; such behaviour has been shown and causes variable results (Silver, 1965). This explanation is supported by the finding that on cooling to -60 °C reproducibility was good, with viability always being close to 100% even when the cooling rate was decreased to an average value of 0.77 °C/min. In 36 experiments, populations of T3 were 100% viable on 17 occasions, approximately 60% viable on 13 occasions and approximately 10% viable on six occasions. These differences are significant because replicate viable counts were usually within $\pm 20\%$ of the arithmetic mean.

Hatch & Warren (1969) also had difficulty in determining the viable count of phage T3 suspensions in nutrient broth cooled to -70 °C and then thawed; the data of Ehrlich, Miller & Idoine (1964) show the same phenomenon.

With T7, consistent results were obtained on cooling to -70°C followed by thawing, and the loss of viability (21.6 % viable) was not caused by loss of tails (Table 1); no appreciable lysis was detected by electron microscopy, as for T3.

Aerosolization

Following aerosolization, storage for 3 min and collection by impactor, T3 and T7 gave results similar to those obtained for freeze-drying, i.e. loss of viability appeared to be related to loss of tails (Table 1). With T3, slow rehydration increased percentage viability and tails, while with T7 viability and tails were the same for both reconstitution procedures (Table 1). Viability of the non-rehumidified T3 sample, unlike the rehumidified sample, varied considerably with different batches of phage and viabilities ranging from about 20 to 65 % were obtained with concomitant increases in percentage of tails. This variation is much greater than the more usual variation of $\pm 20\%$ of the viability value and may indicate that the strength of the head-tail complex can vary between batches of phage T3.

Neither T3 nor T7 appeared to lyse (as determined by electron microscopy) as a result of the aerosolization procedures used. However, it was found that the Collison spray used to generate the aerosols could cause loss of viability of, and a corresponding loss of tails from, both T3 and T7. Collison sprays rely on a refluxing action of the spray suspensions and this, if excessive, produced the above effects. Under the conditions of the experiments (2 ml of spray suspension) the spray pot contents were cycled every 2 s; refluxing (or spray) times of up to 2 min caused negligible loss of viability or tails, but refluxing times of 4 min often caused a 50 % loss in viable and tail counts. In experiments to obtain the data in Table 1, the Collison spray was operated for only 30 s, and after completing aerosol generation the phage suspension left in the spray pot was always checked for possible loss of viability and tails.

Aerosol samples were also collected by raised impinger and viability in these samples was nearly always significantly less than that in samples collected by subsonic impactor. For example, for T3 impactor viabilities were 22.0 % without rehumidification and 66 % with rehumidification (Table 1), while the corresponding impinger viabilities were 0.52 and 11.5 %, agreeing with the corresponding values of 0.1 and 10 found by Hatch & Warren (1969); for T7 the relevant viabilities were 35.0 and 35.0 % (Table 1), and 0.81 and 35.4 %, respectively. Hence, the action of the impinger could cause significant damage to both T3 and T7, probably through shear of tails from phage heads. Phenomena of this nature were considered previously (Anderson & Cox, 1967) and Rechsteiner (1970) showed that the action of sonic impingers caused severe loss of viability of respiratory syncytial virus.

These findings suggest that, if rehumidification were coupled with a very gentle sampling technique, viability of T3 and T7 would probably be unaffected both by aerosolization into, and collection from, rotating drums, and also by the simulated aerosol techniques described by Cox (1965), Silver (1965) and Anderson & Cox (1967).

Stain penetration

No exact correlation was found between viability and degree of penetration of the ammonium molybdate stain. With T3 and T7 stressed by freeze-drying, penetration increased as viability decreased, although freezing and thawing of T3 and T7 did not cause penetration. However, aerosolized T3 and T7 were completely penetrated irrespective of viability.

General discussion

If a stress is applied to a portion of the stock suspension, such that the only action of the stress is to separate tails from the phage head-tail complexes, then the relationship percentage viability/percentage of tails (' k ') obtained with the stressed suspension will be the same as that for the unstressed suspension of phage. However, if the applied stress causes loss of viability by other mechanisms the value of k will differ from that for the unstressed suspension. Hence, calculation of the value of k for differently stressed samples allows the determination of loss of viability caused by loss of tails and that caused by other mechanisms. For this analysis, '% viability₁' will be used to represent the former case and '% viability₂' the latter, while 'viability₁' multiplied by 'viability₂' (corresponding to the observed percentage viability) will be the resultant of the two phenomena.

Values of k and percentage viability obtained as above are given in Table 1. Percentage viability₁ for T3 shows that controlled rehydration prevented significant loss of tails and correspondingly increased viability. Percentage viability₂ indicates that a fairly constant proportion of T3 was inactivated during the processes involved in freeze-drying and that this inactivation was independent of the suspending fluid and the rehydration procedures. The cause of this viability loss is not known but could have been entirely due to freezing. From the results of freeze-thawing experiments this seems possible, but it could also have been because of dehydration only. For aerosolized T3, % viability₂ was close to 100 % (Table 1) and therefore dehydration resulting from exposure to 40 % r.h. did not cause viability loss other than through loss of tails. However, the degree of dehydration produced by freeze-drying would have been much greater than that by aerosolization, because T3 during freeze-drying would have been exposed to almost zero r.h. Hence, the reason why % viability₂ for freeze-dried T3 was less than 100 % (Table 1) is not completely understood.

Percentage viability₁ values for T7 indicate similar behaviour to that for T3, that is, freeze-drying and aerosolization result in loss of viability and tails (Table 1). However, % viability₂ is significantly less than 100 % for freeze-dried and freeze-thawed T7 (Table 1), unlike aerosolized T7 (Table 1). Hence, a similar stress must have been imposed during freeze-drying and freeze-thawing of T7, but not during its aerosolization. The most likely explanation of these results is that the single action of freezing T7 (1.5 to 2.0 °C/min) caused loss of viability independently of loss of tails, or lysis, as determined by electron microscopy.

Following aerosolization and collection by impactor, T3 and T7 both lost viability (Table 1), but this viability loss was caused solely by loss of tails since the value of k is close to that for an unstressed phage suspension and % viability₂ is approximately 100 % (Table 1). T3 and T7 differed slightly in that % viability₁ of T3, unlike that for T7, was sensitive to rehydration. Since no death mechanism other than tail loss was detected, these results are entirely consistent with those of E. J. Dubovi (personal communication) obtained with ϕ X 174. Dubovi found that the viability of ϕ X 174 collected from aerosols at low r.h. decreased by a factor of 10^4 , while DNA extracted from such non-viable phage was as infectious for spheroplasts as that extracted from unstressed ϕ X 174. Hence, as a consequence of aerosolization, storage and collection ϕ X 174, like T3 and T7, is unable to infect its host bacterium. Similarly, the results of Trouwborst & de Jong (1972) can be accounted for. They labelled the DNA in phage T1 with ^{32}P . After aerosolization and rapid rehydration, phage viability exactly paralleled the degree of adsorption of ^{32}P to the host bacterium *Escherichia coli* B. Analysis of the collected T1 on a CsCl gradient showed that the density

of moieties corresponding to maximum radioactivity was 1.67 g/cm³, while that for complete phage was 1.52. Trouwborst & de Jong (1972) suggested that these results were because of release of ³²P from the phage head, i.e. lysis. However, according to *The Handbook of Biochemistry* (1968) the density of T₁ DNA is 1.705 g/cm³, which is considerably greater than the value of 1.67 for the moieties containing ³²P. Therefore, Trouwborst & de Jong (1972) may not have found free T₁ DNA, but DNA contained in T₁ phages without tails, so that loss of tails could also account for their results. Electron microscope studies similar to those performed here should either confirm or eliminate this possibility.

The finding that following dehydration T₇ can lose tails on rapid rehydration provides an explanation for the results of Cox & Baldwin (1964; 1966) which indicate that the aerosol survival of T₇, when adsorbed on to *Escherichia coli* B, increased as the pre-aerosolization time of contact between phage and *E. coli* was increased. With increasing pre-aerosolization contact time, the percentage of host bacteria containing T₇ DNA would have increased up to 100. Once the T₇ DNA was transferred from the phage head via the tail into the host bacterium, separation of phage heads from the head-tail-host complexes would not influence the subsequent development of T₇ progeny. Such infected bacteria would have scored as viable phage and therefore T₇ viability would appear to increase with increased pre-aerosolization contact time until T₇ viability reached 100 %.

The observation that, following dehydration, loss of tails from T₃ and T₇ occurs only during rehydration suggests that water molecules might be involved in the attachment of phage tails to phage heads. On dehydration such water molecules would be removed and result in head-tail complexes with reduced physical strength. The physical integrity of such complexes would therefore become extremely susceptible to the shear forces which occur on the addition of bulk water and to those operating during aerosol sampling by impinger. Both situations resulted in low viability with concomitant loss of tails (Table 1). Furthermore, the phenomenon of tail loss might be expected to increase with increasing dehydration and in fact Warren & Hatch (1969) found that T₃ viability decreased as r.h. decreased.

Results of Warren & Hatch (1969), in conjunction with our own, also indicate that the number of water molecules required for maximum shear resistance of the head-tail complexes could be quite large. Even at 95 % r.h. T₃ loses appreciable viability, yet many biological materials exposed to this r.h. lose only small amounts of water (Bull, 1951; Northcote, 1953; Falk, Hartman & Lord, 1962; 1963; Bateman, Stevens, Mercer & Carstensen, 1962). The situation for T₃ also applies to T₇, because significant viability loss occurs above 80 % r.h. and becomes more extensive as the r.h. is lowered (Benbough, 1971). Extension of studies to include the effects of r.h., together with measurements of water sorption isotherms, now seems to be required.

Loss of viability and tails also can be caused by shear forces other than those considered in our work because Williams & Fraser (1953) showed that phages T₁ to T₇ subjected to high gravitational forces lost viability and tails, as we confirmed in the case of T₇. Williams & Fraser (1953) also indicate that T₃ and T₇, and perhaps other phages, lost tails during the air-drying procedures they used in the preparation of their phage samples for electron microscopy. Hence, loss of tails from T phages appears to be a general result of shear stress. Therefore it is perhaps not surprising that T₃ and T₇ did not lose tails on freezing and thawing, since shear stresses probably were not severe in our freeze-thawing experiments. Extremely rapid thawing rates (well in excess of 200 °C/min) applied to frozen suspensions of T₃ and T₇ may cause shear stresses sufficiently great to result in loss of viability through loss of tails from potentially dehydrated phages T₃ and T₇.

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