Mechanism of Inactivation in Aerosols of Bacteriophage T1

By T. TROUWBORST, J. C. DE JONG AND K. C. WINKLER

Laboratory of Microbiology, State University, Catharijnesingel 59, Utrecht, The Netherlands

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SUMMARY

The inactivation of Escherichia coli bacteriophage T1 was determined after formation of aerosols from solutions in NaCl, NaBr, NaNO3 and Na2SO4. In all cases, survival varied rapidly with relative humidity, with a minimum near the relative humidity corresponding to a saturated solution of the salt. Survival was better when the aerosol was formed at low initial salt concentrations. Broth protected against aerosol-inactivation. Phage particles surviving surface-inactivation by shaking in solution were resistant to aerosol-inactivation.

The inactivation process in the range of humidities where the aerosol particles are fluid seems mainly to be due to surface-inactivation.

INTRODUCTION

Many experiments have been carried out to investigate the survival of viruses in air. Relative humidity (R.H.) has been shown to be an important factor. Furthermore, the inactivation pattern appears to depend on the solution from which the virus is nebulized (Benbough, 1969, 1971; Webb, Bather & Hodges, 1963; Hemmes, 1959) and on the method by which the aerosol samples are collected (Dubovi & Akers, 1970; Hatch & Warren, 1969; Warren, Akers & Dubovi, 1969; Rechsteiner, 1968). The influence of all these variables has not yet been fully understood.

When a salt solution is sprayed as an aerosol water will evaporate until the vapour pressure in the aerosol particle is in equilibrium with the R.H. of the ambient air (Pv). At equilibrium such an aerosol is characterized by three parameters: the vapour pressure of the initial salt solution (Pw), the vapour pressure of the saturated salt solution (Pss) and the vapour pressure at equilibrium (Pv). If Pss < Pv < Pw, the water in the aerosol droplets will evaporate resulting in droplets with increased salt concentration. If Pv < Pss < Pw, evaporation proceeds beyond saturation to the point where the droplets either supersaturate or crystallize and solidify completely. The rate of evaporation and crystallization increases with lower ambient relative humidity (Hidalgo, 1968). The salt concentration and Pss will differ for various salts. This suggested a study of virus inactivation in aerosols, after spraying from different salt solutions.

An important property of aerosols is the extremely large surface to volume ratio. As a result phage particles in the droplets spend a large fraction of their time at or near the air-water interface, where surface inactivation is likely to occur. Surface inactivation has been implicated in the loss of viability which occurs when phage suspensions are shaken (Adams, 1948) and the rate of inactivation increases with salt concentration (Campbell-Renton, 1942). We have therefore compared inactivation of phage by shaking and by aerosol for-
mation, and our results suggest that the same phenomenon is largely responsible for the inactivation of bacteriophage $T_1$ in both systems.

The bacteriophage $T_1$ was chosen for these experiments as it is relatively insensitive to osmotic shock (Anderson, 1953) and our results support the belief that osmotic shock during transfer of phage from the aerosol at high salt concentration to the collecting fluid, is relatively unimportant.

**METHODS**

**Media and propagation of the phage.** Bacteriophage $T_1$ was propagated on *Escherichia coli* B in a synthetic medium described by Adams (1949). The medium contained in 1 l. H$_2$O: 60 g. KH$_2$PO$_4$, 60 g. K$_2$HPO$_4$, 20 g. NH$_4$Cl, 0.5 g. MgSO$_4$ and 0.05 g. FeSO$_4$, to which after sterilization solutions of 1 M-glucose and 1 M-CaCl$_2$ were added up to a final concentration of 0.02 and 0.001 M respectively. Bacteria were removed from the phage-lysate by filtration. The resultant virus suspension had a titre of about $10^{10}$ p.f.u./ml. Just before aerosolization we diluted the virus suspension 1:100 in the required salt solution to dilute out the substances present in the original virus suspension.

**Aerosol equipment.** The aerosols were generated with a spray-gun of the type FK-8, which converted 1 ml. virus suspension to an aerosol in about 4 sec. The aerosol was kept at 20°C in a double walled static system, described by de Jong (1967) and de Jong & Winkler (1968), and was homogenized by a fan. The relative humidity was measured with a LiCl dewcel element (Foxboro) and recorded continuously. The aerosol was collected with a raised Porton impinger, filled with 10 ml. 1% (w/v) peptone solution and 0.1 ml. 10% (v/v) antifoam AF (Dow Corning Corp. U.S.A.). Sampling time was 1 min.; sample size was 11.5 l. of aerosol.

**Titration of the phage and presentation of the results.** After collection of the aerosol, the impinger fluid was titrated in duplicate by the agar-layer method (Adams, 1966). The plaques were counted 18 hr after plating. The phage recovery is defined as $N_t/N_0$ where $N_t$ is the number of p.f.u. found in the impinger fluid of the sample taken at the time $t$ and $N_0$ is the number of p.f.u. expected in the sample as calculated from total number of p.f.u. sprayed, aerosol volume and sample size. In the figures log recovery is plotted.

**Shaking experiments.** The shaking experiments were performed with a flask shaker (Griffin & George Ltd), with a 30 ml. bottle containing 10 ml. phage suspension. The shaking speed was the same in all experiments.

**RESULTS**

**Inactivation in aerosols**

The results of the experiments with phage in solutions of NaCl as spray medium are shown in Fig. 1a, b. The minimum of virus survival was not far from the R.H. where the transition from droplets to solid particles should occur: R.H. = 75% (K.N.C.V., 1962).

Analogous results were found with NaBr (Fig. 2a, b) with a minimum at 55% nearly coinciding with the vapour pressure of the saturated solution (59%). With NaNO$_3$ (Fig. 3a, b), the minimum occurred at 70% R.H. whereas the vapour pressure of the saturated solution corresponded to 75% R.H. (K.N.C.V., 1962). With Na$_2$SO$_4$ survival decreased continuously with increasing R.H. (Fig. 4) in accordance with a vapour pressure at saturation corresponding to 90% R.H. (K.N.C.V., 1962).

The possibility that the minimum was due to toxicity of the concentrated salt solution itself was studied by determining the survival of the phage in these concentrated solutions (Fig. 5). Except in the case of NaBr no inactivation was observed showing that the high salt...
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Fig. 1. Log recovery of bacteriophage T\textsubscript{1} from aerosols after spraying from solutions of (a) 0.1 M-NaCl, (b) 0.003 M-NaCl. Samples were taken at the indicated time during 1 min.

Fig. 2. Log recovery of bacteriophage T\textsubscript{1} from aerosols after spraying from solutions of (a) 0.1 M-NaBr, (b) 0.003 M-NaBr. Samples were taken at the indicated time during 1 min.
concentration itself was not generally the cause. In the case of NaBr the toxicity of the satu-
rated NaBr solution contributed to the minimum but the low recovery at 67% R.H. (Fig. 
2a, b) corresponding to a 6 M solution (Landolt-Börnstein, 1931; Weast, 1968) cannot be 
explained by toxicity because the phage survives well in solutions of this concentration 
(Fig. 5).

The possibility that the minimum was an artefact caused by osmotic shock during collection 
was studied by comparing collection fluids with low and high salt concentration. Table 1 shows that recovery is independent of the salt concentration in the collection fluid suggesting that osmotic shock is of minor importance.

### Inactivation by shaking in solution

Diluted phage suspensions were shaken and strong inactivation was found (Fig. 6). 
Broth protected against this form of inactivation. As the addition of broth to the spray medium also protected against aerosol inactivation (compare Fig. 1b with Fig. 7) a correla-
tion seemed to exist between aerosol-inactivation and inactivation by shaking.

It can be seen in Fig. 6 that shaking phage T1 in 1 M-NaCl for 30 min. left a population resistant to further inactivation. It seemed important to know whether this population would also be resistant to aerosol-inactivation at high R.H. A phage suspension purified by centri-
fugation in a CsCl gradient and diluted $10^{-4}$ in 1 M-NaCl was used. A part of this suspension was shaken for 60 min. and aerosol-inactivation at 90% R.H. was determined before and after shaking (Fig. 8). It appeared that the fraction of phage particles resistant to shaking
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Fig. 4. Aerosol-inactivation of bacteriophage T₁ from aerosols after spraying from a solution of 0.003 M-Na₂SO₄. Samples were taken at the indicated time during 1 min.

Fig. 5. Survival of bacteriophage T₁ in saturated salt solutions and in 6 M-NaBr at 20°C. ×——×, NaNO₃; ×——×, Na₂SO₄; ×——×, NaCl; ○——○, NaBr; ○——○, 6 M-NaBr.

Fig. 6. Inactivation of bacteriophage T₁ by shaking. The stock suspension was diluted 1:10⁴ in the following solution: ×——× 0.1 M-NaCl; ×——× 1 M-NaCl; ×——× 1 M-NaCl; broth (1:10 diluted in 1 M-NaCl); ○——○ 1 M-glucose.
Fig. 7. Protection of bacteriophage T1 against aerosol-inactivation by broth. The suspension medium contained 0.003 M-NaCl and 10 ml. broth/l. Samples were taken at the indicated time during 1 min.

Fig. 8. Aerosol inactivation at R.H. = 90 % of bacteriophage before and after shaking in 1 M-NaCl. During shaking the titre of the original suspension had dropped by a factor of $10^4$.

Table 1. Influence of salt in the collection medium on phage recovery.

**Spray-fluid:** 0.1 M-NaCl; R.H. = 81.7 %, corresponding to 4 M-NaCl.

<table>
<thead>
<tr>
<th>Collection medium</th>
<th>log recovery 10 min</th>
<th>log recovery 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% peptone in 0.1 M-NaCl</td>
<td>-2.36</td>
<td>-2.62</td>
</tr>
<tr>
<td>1% peptone in 5 M-NaCl</td>
<td>-2.41</td>
<td>-2.65</td>
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...was also resistant to aerosol-inactivation. The unshaken suspension was inactivated rapidly within the first minute to a survival of $10^{-4}$. Later on inactivation proceeded much more slowly suggesting another analogy with the shaking experiment.

**DISCUSSION**

The experiments suggest that in the region of high R.H., i.e. when the ambient vapour pressure is higher than that of the saturated salt solution, the inactivation of phage T1 was mainly due to surface-inactivation. This conclusion is based on the resistance against aerosol-inactivation of phage particles which survived shaking and on the protective effect of nutrient broth in both aerosols and shaken suspensions. It is supported by experiments on protection by surface active amino acids, which will be reported separately (Trouwborst,
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The minimum in recovery at the R.H. where the salt solution is saturated correlated with the observations of Campbell-Renton (1942). She reported that the rate of inactivation of phage by shaking increases with increasing salt concentration. Preliminary experiments in this laboratory confirm this for phage $T_1$. The increasing recovery on the wet side of the minimum in the aerosol experiments may then be attributed to the decreasing salt concentration in the droplets at higher humidities.

The kinetics of inactivation at high R.H. also show that not every collision of the phage with the air/water interface will lead to inactivation, as the following calculation demonstrates. With a diffusion constant of $3.5 \times 10^{-5}$ cm$^2$/sec, as will be found for a phage particle like phage $T_1$ (Adams, 1966) we calculate the time necessary to cover the distance between the centre and the surface of the largest aerosol particle (5 $\mu$m.):

$$t = \frac{x^2}{6D} = 1.2 \text{ sec.}$$

where $t$ is the time in sec., $x^2$ is the mean of the square distance covered per sec. and D is the diffusion constant. This time is only a fraction of the time necessary for the inactivation of the phage at high R.H.

When the aerosols are generated from suspensions with low salt concentration, the inactivation was always slower than with higher salt concentrations. This is remarkable as the salt concentration at any R.H. is equal in both cases. However, with low initial salt concentrations more water has to evaporate. Consequently the droplets and the surface are much smaller. We suspect that small quantities of surface active substances (for instance peptides) derived from the lysed bacteria during phage production are protective (Trouwborst, 1970). When spraying from diluted salt solutions such protective substances will be more concentrated and occupy the reduced surface more completely.

The inactivation process at low R.H. is much less clear. It is again apparent from the curves that recovery is much better when low salt concentrations are used (Fig. 1a and 1b). The lower recovery with high salt concentrations could not be attributed to more rapid physical loss of heavier salt particles by sedimentation and settling at the sides of the vessel, as was established with tracer experiments (Trouwborst, 1971). The size of the salt crystals, the situation of the phage-particle in the crystal or the concentration of protective substances might be involved.

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REFERENCES


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