Protection Against Aerosol-inactivation of Bacteriophage T1 by Peptides and Amino Acids

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SUMMARY

Aerosol-inactivation of bacteriophage T1 is prevented by peptone and by apolar amino acids (leucine, phenylalanine). The protecting concentration is related to the salt concentration in the spray-medium, which determines the amino acid concentration in the aerosol particle after evaporation to equilibrium. The protective action of surface active agents supports the hypothesis that inactivation is due to surface inactivation. The surface occupation of the protecting amino acids was calculated with the Gibbs adsorption formula and agrees with amounts necessary to cover the air/water interface, thus preventing the phage from reaching the surface.

The decrease of amino acid concentration in the aerosol particle by surface adsorption is calculated in the appendix.

INTRODUCTION

The survival of a virus in an aerosol is determined by variables like the relative humidity (r.h.) of the ambient air, the composition of the spray-medium and the method of aerosol collection (Hatch & Warren, 1969; Dubovi & Akers, 1970).

The nature and concentration of salts in the spray-medium can influence the survival very markedly (Trouwborst, 1972; Trouwborst, de Jong & Winkler, 1972). Additives like polyhydroxy compounds may protect against inactivation (Benbough, 1969, 1971). Addition of serum albumin can protect arboviruses, especially at high relative humidity (Benbough, 1971). Hemmes (1959) found less inactivation of phage T1 after spraying from broth than after spraying from solutions of NaCl. Webb, Bather & Hodges (1963) noted a protection of the Rous sarcoma virus especially at high relative humidity by the addition of 2% serum.

In many aerosol experiments, the virus is aerosolized from the culture fluid, containing peptides and amino acids. All these substances may influence the survival of the virus.

In this paper the protective activity of some amino acids on the survival of the coliphage T1 is studied in relation to the salt concentration in the medium.

METHODS

Media and propagation of the phage. Bacteriophage T1 was propagated on Escherichia coli B in a synthetic medium described by Adams (1948). The medium contained in l H2O: 60 g KH2PO4, 60 g K2HPO4, 20 g NH4Cl, 0.5 g MgSO4 and 0.05 g FeSO4, to which after sterilization solutions of 1 M-glucose and 1 M-CaCl2 were added to final concentrations of 0.02 and 0.001 M respectively. The phage lysate was made bacteria-free by filtration. The virus suspension had a titre of about 10^6 p.f.u./ml. Just before aerosolization it was diluted
Fig. 1a. Inactivation of phage T1 in aerosols of various ages after spraying from solutions of 0.1 M NaCl. With addition of broth: •—•, t = ½ min; •----•, 10 min; ••••, 30 min. Without broth: x...x, t = 30 min.

Fig. 1b. Inactivation of phage T1 in aerosols of various ages after spraying from solutions of 0.003 M NaCl. With addition of broth: •—•, t = ½ min; •----•, 10 min; ••••, 30 min. Without broth: x...x, t = 30 min.

1:100 in the spray-medium. In experiments with peptone and broth, 0.01 ml was added to 1 ml spray medium. Broth contained in 1 l water: 7.5 g yeast extract, 5.0 g NaCl, 7.5 g Bacto-peptone, 1.0 g Na₂HPO₄, 2.6 ml 5% (w/v) NaOH, pH 7.2. The peptone solution contained 10 g bactopeptone and 5.0 g NaCl in 1 l water pH 7.2.

Aerosol equipment. The aerosols were generated with a spray-gun of the type FK-8, which aerosolizes 1 ml virus suspension in about 4 s. The aerosol was kept at 20 °C in a double walled static system, described by de Jong & Winkler (1968) and was homogenized by a fan. The relative humidity was continuously recorded with a LiCl dewcell element (Foxboro). 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 11.5 l of aerosol.

Measurement of surface tension. The surface tension of the amino acid solutions was determined relative to that of 2.6 M NaCl (77.5 dyn/cm) (Weast, 1968) with a stalagmometer by measuring the weight of the droplets at 20 °C.

Phage titration and presentation of results. After collection of the aerosol, the impinger
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Fig. 2. Aerosol-inactivation of phage T1 at $t = \frac{1}{2}$ min, $\cdots \cdot \cdot \cdot \cdot$; 10 min, $\cdot \cdots \cdot \cdot \cdot \cdot$; 30 min, $\cdot \cdots \cdot \cdot \cdot \cdot$ after spraying from solutions of 0.1 M-NaCl with varying concentrations of peptone. r.h. = 90%.

Fluid was titrated in duplicate by the agar-layer method (Adams, 1966). Plaques were counted 18 h after plating. Phage recovery presented in the figures is defined as $N_t/N_0$, where $N_t$ is the number of p.f.u. in the impinger fluid of the sample taken at time $t$ and $N_0$ is the number of p.f.u. expected in the sample if no biological and physical loss had occurred, as calculated from the total number of p.f.u. sprayed, aerosol volume and sample size.

RESULTS

Inactivation of phage T1 after aerosolization from NaCl solutions is shown in Fig. 1a, b. The addition of broth gives good protection when the spray medium is 0.003 M-NaCl. With peptone, present in broth in a concentration of 0.75% (w/v), analogous results were obtained. When 0.1 M-NaCl is used, the protection is incomplete. This difference can be understood once the differences between the two aerosols are appreciated. Immediately after aerosolization the water of the droplets evaporates to an extent depending on the relative humidity of the ambient air. After spraying from media with low salt concentration more water has to evaporate to obtain the equilibrium concentration and the droplets will be smaller than after spraying from media with a high salt concentration. Consequently, additives like broth and peptone will also be more concentrated. In systems with 0.1 M-NaCl the broth concentration...
presumably is too low to give complete protection. This was tested by increasing the concentration of peptone. The results presented in Fig. 2 show that peptone also protects in systems with 0.1 M-NaCl but that a higher concentration (0.1 %) is required.

Previous experiments with phage sprayed from solutions of NaBr had shown that maximal inactivation occurred at 59 % r.h. at which point the aerosol droplets contain the saturated solution of this salt. Concentrations of NaBr above 6 M, which will be found below 67 % r.h. proved toxic for phage T1 (Trouwborst et al. 1972). Peptone and broth gave also protection in this case (Fig. 3) but the protection was incomplete in the range of toxic concentrations. Thus peptone only protects against inactivation at high relative humidity but not against specific toxicity of NaBr.

**Mechanism of protection by peptone**

In a previous paper it was shown that the inactivation of phage by shaking (Adams, 1948) can be prevented by the addition of broth or peptone (Trouwborst et al. 1972). The parallelism between inactivation by shaking (creating a large air/water interface) and inactivation in aerosols at high r.h. suggested that phage inactivation was due to surface inactivation in both systems.
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Fig. 4a. Aerosol-inactivation of phage T₁ at t = ½ min, •-- •; 10 min, •--- •; 30 min, • • • • after spraying from solutions of 0·1 M-NaCl with varying concentrations of valine, r.h. = 70 %.

Fig. 4b. Aerosol-inactivation of phage T₁ at t = ½ min, •-- •; 10 min, •--- •; 30 min, • • • • after spraying from solutions of 0·1 M-NaCl with varying concentrations of leucine, r.h. = 70 %.

Table 1. Protection against aerosol-inactivation of phage T₁ by an amino acid mixture with the composition of 0·1 % (w/v) caseine hydrolysate

<table>
<thead>
<tr>
<th>Aerosol age (min)</th>
<th>log ( \frac{N_t}{N_0} ) + Amino acid</th>
<th>log ( \frac{N_t}{N_0} ) − Amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>½</td>
<td>−0·58</td>
<td>−2·00</td>
</tr>
<tr>
<td>10</td>
<td>−0·64</td>
<td>−2·55</td>
</tr>
<tr>
<td>30</td>
<td>−0·67</td>
<td>−2·90</td>
</tr>
</tbody>
</table>

Spray-solution 0·003 M-NaCl. r.h. = 74 %.

Peptone is a mixture of peptides and amino acids. To test the effect of the amino acids alone, a mixture of amino acids with the composition of a 0·1 % (w/v) caseine hydrolysate solution was used. The results show that this mixture also protects (Table 1). The protection by the individual amino acids was studied at a concentration of \( 8 \times 10^{-4} \). The amino acids glu, asp, cys, met, lys, arg, ser and ala gave no protection. Only the neutral amino acids with long hydrocarbon chains phenylalanine and leucine gave protection at this concentration. Valine also protected but a much higher concentration was required (Fig. 4a, b).
As with peptone, the protecting amino acid concentration depends strongly on the salt concentration in the spray-medium (Fig. 5). After spraying from solutions with low salt concentrations less amino acid is necessary for optimal protection. The maximal protecting concentration of phenylalanine after spraying from a solution with 0.01 M NaCl is about 10 times lower than after spraying from 0.1 M NaCl (Fig. 5), suggesting that the amino acid concentration in the aerosol particle at equilibrium is of decisive importance for protection.

**Surface activity of amino acids**

Previous experiments suggest that aerosol-inactivation of phage T1 at high relative humidity is due to surface-inactivation. Our experiments show a relation between the protective action of an apolar amino acid and the length of its apolar sidechain (Fig. 4a, b). Substances with apolar moieties accumulate at the air/water interface and might act as a barrier and preventing the phage from reaching the surface.

To support these suppositions the surface tension of solutions of the three amino acids were measured and the surface concentrations calculated with the Gibbs equation

$$\Gamma = -\frac{1}{RT} \frac{d\gamma}{d \ln \alpha}$$

where $\Gamma$ is the amount of adsorbed amino acid, $\alpha$ the activity of the amino acid in the salt solution, $R$ is the gas constant, $T$ the absolute temperature and $\gamma$ is the surface tension of the
amino acid solution at the air/water interface. If the activity coefficient of the amino acid does not vary with its concentration (this supposition is warranted as the salt concentration is much higher than the amino acid concentration) we can calculate from the first formula:

$$\Gamma = \frac{a \Delta y}{RT \Delta a}$$ (2)

where $a$ is the measurable concentration of the amino acid in the salt solution.

The relation between $\gamma$ and $a$ is measured at 20 °C in a salt solution of 2.6 M-NaCl, equivalent to the salt concentration found in aerosol droplets at 90 % relative humidity (Landolt-Börnstein, 1931; Weast, 1968).

The relation proves to be linear in the range of tested concentrations (Fig. 6). The range of measurements is limited by the solubility of leucine and phenylalanine, which is exceeded at concentrations of $6 \times 10^{-2}$ and $8 \times 10^{-2}$ M respectively.

The slopes of the curves ($\Delta y/\Delta a$) for leucine, phenylalanine and valine are respectively 85.8, 55.6 and 12.5 dyn/cm × L/mol. The amino acid concentrations inside the aerosol particles at optimal protective concentrations can be calculated from Fig. 5. Phenylalanine gives protection at a concentration of $5 \times 10^{-3}$ M in the spray-fluid (0.1 M-NaCl). At 90 % r.h. the equilibrium concentration of NaCl is 2.6 M and the amino acid concentration will be $26 \times 5 \times 10^{-3} = 13 \times 10^{-2}$ M. Using the adsorption formula, and extrapolating the $\gamma/a$ relation in Fig. 6 to $13 \times 10^{-2}$ M this corresponds to a surface per amino acid molecule of 57 Å². This surface occupation could prevent the phage from reaching the air/water interface. The calculation is only exact when the concentration of the amino acid in the aerosol droplet is not markedly reduced by surface adsorption (see appendix) and if the extrapolation to a supersaturated solution is permitted. If the amino acid in the droplet crystallizes, the surface concentration would be a factor 8/13 lower, corresponding to an area of 93 Å² per amino acid molecule.

Protection is not only given by amino acids. The surface active substance OED, a mixture of oxyethylene docosyl ether ($C_{22}H_{46}OC_2H_4OH$) and oxyethylene octadecyl ether ($C_{18}H_{37}-OC_2H_4OH$) (Mihara, 1966) also protects against inactivation at high relative humidity (Fig. 7).
DISCUSSION

Broth and peptone protect against aerosol-inactivation of phage T1. This protecting action can be ascribed to the amino acids. The most apolar amino acids give the best protection against inactivation, suggesting a protection by accumulation at the surface, in accordance with previous results which have shown that the inactivation is a surface dependent process (Trouwborst et al., 1972; Trouwborst, 1971). In accordance with this, protection is also given by the surface active agent OED. In the case of the amino acids, an estimate of the mean separation of molecules in the adsorbed layer suggests that one amino acid molecule every 57 Å² of surface area is sufficient to protect the bacteriophage in the droplet. Adsorption of bacteriophage may depend on there being sufficient available free surface, which requires a low surface occupation and a low surface pressure. James & Augenstein (1966) reported prevention of protein adsorption on a Ba-stearate monolayer at pressures of 3 to 4 dyn/cm. McRitchie & Alexander (1963) calculated the free surface necessary to start adsorption of bovine serum albumin as 135 Å². However, adsorption may also involve interactions between specific sites on the adsorbed structure and components of the monolayer (Quinn & Dawson, 1970 a, b). While nothing is known about the adsorbing sites of the phage, and the theories of adsorption are not yet very well established, we have to suffice with these remarks.

Protection against inactivation might also be caused by interaction of the amino acids with essential phage components. Walwick, Brady & Kay (1967) found protection of phage T3 against freeze-drying by certain tryptone fractions, suggesting a possible interaction of peptides with the phage, resulting in increased stability, though also in this case surface inactivation might be involved.

Peptone also protects against inactivation at low relative humidity. This protection will at
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least partially be due to protection against surface inactivation during the stage of evaporation from droplet to droplet nucleus. However, peptone protects also against inactivation after evaporation, because the inactivation from $\frac{1}{2}$ min to 30 min is also reduced (compare with previously reported results Trouwborst et al. 1972). This might also be protection against surface inactivation if the phage permeated to the surface of the aerosol particle during evaporation.

At high concentrations amino acids may be toxic (Fig. 5). Toxicity is only found when the amino acid solution in the aerosol droplet is supersaturated or crystals are present.

The results presented here might explain results obtained by other authors. Dubovi & Akers (1970) found reduction in phage recovery of the phage MS-2 after pre-humidification. This reduction could be counteracted by addition of peptone, suggesting a dilution of protecting substances in the aerosol particles during pre-humidification.

The recovery of other viruses, like phage $T_3$ and phage S-13, mainly inactivated at low relative humidity (Hatch & Warren, 1969; Dubovi & Akers, 1970), is increased by pre-humidification, probably by a reduction in osmotic shock during collection. The recovery of mengovirus-37A and vesicular stomatitis virus, which are also unstable at low relative humidity, is however not influenced by pre-humidification, suggesting another mechanism of inactivation (Warren, Akers & Dubovi, 1969). At least three different mechanisms for the aerosol inactivation of viruses have been detected: (1) surface inactivation, mainly at high relative humidity; (2) inactivation by drying of the virus particle which is not influenced by pre-humidification at collection; (3) inactivation by drying, influenced by pre-humidification.

**APPENDIX**

**Decrease of amino acid concentration in the aerosol particle by surface adsorption**

The aerosol particles are characterized by a large surface/volume ratio. Adsorption of surface active substances at the relatively large air/water interface may result in a decrease of the concentration of these substances inside the droplets, resulting in a possible error of previous adsorption calculations.

The total amount of the surface active substance, for instance the amino acid is given by:

$$4\pi r_e^3 \times \Gamma + \frac{4}{3} \pi r_e^2 \times a_e = \frac{4}{3} \pi r_0^3 \times a_0$$  \hspace{1cm} (3)

where $\Gamma$ is the surface adsorption (mol/cm²), $r_e$ is the radius of the aerosol particle after evaporation to equilibrium (cm), $r_0$ is the radius of the particle before evaporation (cm), $a_e$ is the amino acid concentration in the aerosol particle at equilibrium (mol/cm³) and $a_0$ is the amino acid concentration in the spray medium (mol/cm³).

The radius of the aerosol particle at evaporation to equilibrium may be calculated from

$$r_e^3 \times c_e = r_0^3 \times c_0$$  \hspace{1cm} (4)

where $c_e$ is the salt concentration in the aerosol particle at equilibrium as determined by the relative humidity and $c_0$ is the salt concentration in the spray medium.

The adsorption at the interface is related to the concentration inside the droplet by the adsorption formula (form 2). When the relation between $\gamma$ and $c$ is linear (and this is true for the measurements shown in Fig. 6) we may rearrange formula (2):

$$\Gamma = \frac{k}{RT} \times a_e$$  \hspace{1cm} (5)

where $k = \frac{d\gamma}{da}$.
Fig. 8. Factor for correction of the amino acid concentration in the aerosol droplet, on account of decrease of concentration by surface adsorption, calculated for phenylalanine

\[
\frac{d\gamma}{da} = -55.6 \frac{\text{dyn/cm}}{\text{mol/L}}
\]

and the values of \(c_e/c_0\): 30 (--): 300 (----): 1000 (-----). \(r_0\) = radius of aerosol particle before evaporation, \(c_e\) = salt concentration in the aerosol particle after evaporation. \(c_0\) = salt concentration in the spray fluid.

After substitution of formula (4) and (5) in formula (3) we may write after rearrangement:

\[
a_e = a_0 \times \frac{c_e}{c_0} \times \frac{r_0}{r_0 + 3 \frac{k}{RT} \times \left(\frac{c_e}{c_0}\right)^\frac{1}{3}}.
\]

If no adsorption and no depletion took place, the amino acid concentration would be given by the first two factors.

The third factor

\[
\frac{r_0}{r_0 + 3 \frac{k}{RT} \times \left(\frac{c_e}{c_0}\right)^\frac{1}{3}}
\]

may thus be seen as a correction factor for the decrease in concentration. It becomes important when

\[
3 \frac{k}{RT} \times \left(\frac{c_e}{c_0}\right)^\frac{1}{3} \sim r_0.
\]

The correction factor is calculated for phenylalanine, with \(k = 55.6 \text{ dyn/cm} \times \text{L/mol}\) (Fig. 6), and is graphically represented in Fig. 8 for different values of \(c_e/c_0\).

The depletion proves to be important for small (< 1 \(\mu\)m) aerosol droplets, particularly after spraying from solutions with low salt concentration (where \(c_e/c_0\) is large). The depletion is also strongly dependent on the value of \(k\), thus on the hydrophobicity of the adsorbing substances. In our experiments most phage-containing particles have a radius larger than 1 \(\mu\)m before evaporation, and the depletion may be neglected in the calculation of surface...
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adsorption. The phenomenon described may be of importance in other experiments where small aerosol particles are produced and protection is given by substances with a high degree of surface activity. Protection will then be better in the large droplets, resulting in selective inactivation of a virus in small droplets.

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REFERENCES


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