Interaction of Some Factors in the Mechanism of Inactivation of Bacteriophage MS2 in Aerosols

T. TROUW Borst AND J. C. DE JONG

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

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The mechanisms involving inactivation of bacteriophage MS2 in aerosols and the effect of protective substances in the spray-medium were studied after spraying from various NaCl solutions. Results with aerosols generated from the salt solutions showed that with higher salt concentration in the spray-medium higher concentrations of protective substances were needed to protect phage MS2 against aerosol inactivation. Phenylalanine, which has a protective action at low concentration, produced less protection in aerosol droplets that were super-saturated solutions of this substance or in which crystals of phenylalanine can be expected to form. Our results suggested that protection by peptone and phenylalanine was related to the concentration in the aerosol droplet after evaporation to equilibrium, whereas protection by the surface active agent OED (a commercial mixture of oxyethylene docusylether and oxyethylene octadecylether) was related to the concentration at which a monolayer is formed around the aerosol particle. Inactivation of phage MS2 was maximal in the aerosol particle in fluid phase and became less at lower relative humidity where aerosol particles are expected to be in the solid state. It is suggested that inactivation of bacteriophage MS2 in aerosols could be explained by surface inactivation at the air-water interface.

The inactivation of viruses in aerosols is known to be dependent on the relative humidity (RH) of the ambient air, on the composition of the spray-medium (4, 10, 12, 16, 22) and on the method of aerosol collection (9, 11, 20).

Dubovi and Akers (9) found a decrease in recovery of phage MS2 after prehumidification of the aerosol sample before collection, if no peptone was added to the spray-medium. Such an effect was not found with many other viruses (9, 11, 20) and the significance of this phenomenon has been left unexplored. For this reason the mechanism of inactivation in aerosols of phage MS2 has been studied. Salt concentrations in the spray-medium and protecting concentrations of other additives could be correlated; therefore, the inactivation of phage MS2 and the effect of small amounts of additives were studied after spraying from various NaCl solutions.

If an aerosol is produced from a solution of NaCl, the salt within the aerosol particles can be in the solid phase below 75% RH (15) at 20°C, because the vapor pressure of a saturated NaCl solution at 20°C corresponds with 75% RH (21). At an RH higher than 75% the aerosol particles will be droplets of a NaCl solution, which has a concentration determined by the RH of the ambient air (15). As droplets have a relatively large air-water interface, surface inactivation could play a role, as was found for phage T1 (1, 18, 19). The sensitivity of phage MS2 against surface inactivation was therefore studied in correlation to inactivation in aerosols at high RH.

MATERIALS AND METHODS

Media, virus propagation, and purification. Bacteriophage MS2 was propagated in cells of Escherichia coli strain KA 81. The bacterial cells were grown in broth at 37°C to an optical density (OD) of 0.7 cm⁻¹ at 530 nm (4 × 10⁶ cells/ml) with vigorous aeration. Phage MS2 was then added at an input multiplicity of 5. After incubation for 6 h, chloroform was added and the lysate was centrifuged at 12,000 × g for 10 min. The phage in the supernatant fluid was purified by precipitation with (NH₄)₂SO₄ and treatment with Freon-11 as described by Strauss and Sinsheimer (17). The phage suspension was further purified by centrifugation in a CsCl gradient with the two-layer method of Brunck and Leick (5). The upper half of the 5-ml tube contained 2.5 ml of phage suspension with 0.825 g of CsCl, the lower half 2.5 ml
of phage suspension with 1.925 g of CsCl. Centrifugation was performed in an SW-40 rotor (MSE-65) at 35,000 rpm (100 x g) for 18 h at 4 C. Phage-containing fractions were pooled and dialyzed against 0.01 M tris(hydroxymethyl)aminomethane (Tris)-buffer (pH 7.6) with 0.1 M NaCl. The phage titer obtained was 5 \times 10^{18} plaque-forming units (PFU)/ml.

**Induction of surface inactivation.** Samples of 10 ml of a diluted MS2 phage suspension were shaken by means of a flask shaker (Griffin and George Ltd.) in a 30-ml bottle at a constant speed.

**Aerosol equipment and procedures.** The stock suspension of phage MS2 was diluted 1:10^4 in the spray-medium just before spraying with a direct spray apparatus (FK8, Fort Detrick, U.S.A.), which converts 1 ml of fluid into an aerosol in 4 s. Before evaporation the particle size medium diameter was about 2 \mu m and the mass median radius was about 9 \mu m (T. Trouwborst, thesis, Rijksuniversiteit Utrecht, 1971). The aerosol was kept at 20 C in a double-walled static system of 2,000 liters, as previously described (7), and was homogenized by a fan. The RH was measured with a LiCl dewcell element (Foxboro) and was continuously recorded. The aerosol was collected with a raised Porton impinger filled with 10 ml of 1% (wt/vol) peptone in saline. Samples were collected for 1 min at 11.5 liters per min.

**Titration of phage MS2.** Phage MS2 was titrated in duplicate by Adams' agar layer method (2) by using *E. coli* KA 81 as the indicator strain. The recovery of infectious virus particles is expressed as \( N_t / N_e \), where \( N_t \) is the number of PFU collected at time \( t \) and \( N_e \) is the expected number of PFU calculated from the total number of PFU aerosolized and the volume of the aerosol collected.

**RESULTS**

The survival at different relative humidities of phage MS2 after spraying from 0.1 M NaCl is shown in Fig. 1. Inactivation was maximal at 70 to 75% RH and was less at lower RH. No loss of phage titer was found if phage MS2 was kept in solutions with various concentrations of NaCl. These results suggest that the high concentrations of NaCl in aerosol-droplets were not toxic to this phage (Table 1). No effect was found on the recovery of phage MS2 after raising the osmotic strength of the collection medium (Table 2), indicating that osmotic shock during collection, if it occurs at all, plays an unimportant role in aerosol. Some inactivation might occur during generation of the aerosol. Therefore, phage MS2 was sprayed directly in a peptone solution, reducing the time between aerosol production and collection. By this method, no significant inactivation was found, suggesting that inactivation during aerosol generation is probably of minor importance.

Phage MS2 in NaCl suspensions is rapidly inactivated by shaking as shown in Fig. 2a. Bubbling nitrogen gas through the suspension also causes inactivation. Inactivation by shaking is more pronounced at a higher salt concentration. The processes of shaking and bubbling expose the phage to a large continuously changing air-water interface. Such a large air-water interface is also found with aerosol particles at

![Fig. 1. Aerosol-inactivation at varied relative humidities of phage MS2 after aerosolization from 0.1 M NaCl. Samples are taken at 0.5, 10, and 30 min after spraying.](image)

| TABLE 1. Influence of concentrated salt solutions on viability of phage MS2* |
|-----------------|-----------------|-----------------|
| Media           | \( \log N_t/N_e \) at | \( t = 3 \) h | \( t = 8 \) h |
| 1 M NaCl        | -0.06            | -0.16           |
| 2.6 M NaCl      | -0.10            | -0.20           |
| Saturated NaCl  | 0.00             | -0.08           |

*The phage stock suspension was diluted 1:10^4 in the salt solutions and incubated at 20 C for the periods indicated.
TABLE 2. Influence of salt in the collection medium on recovery of aerosolized phage MS2 at the indicated relative humidities

| RH (%) | Collection media (1%) | Log \(N_t/N_0\) at 1/2' | Log \(N_t/N_0\) at 30' |
|--------|-----------------------|---------------------------|------------------------|
| 82     | Peptone solution      | -2.69                     | -3.98                  |
| 82     | Peptone solution + 4.2 M NaCl | -2.69                     | -4.21                  |
| 95     | Peptone solution      | -2.54                     | -3.21                  |
| 95     | Peptone solution + 1.6 M NaCl | -2.32                     | -3.14                  |

*Spray fluid: 0.1 M NaCl.

high RH. Phage particles which are more resistant to shaking are also more resistant to inactivation in aerosols (Fig. 2b), indicating a further correlation between inactivation by shaking and inactivation in aerosols at high RH. Peptone, which protects against inactivation by shaking (Fig. 2a) protects also against inactivation in aerosols. Protection in aerosols is strongly dependent on the salt concentration in the spray-medium (Fig. 3). Peptone (0.01%) protects the virus in aerosols sprayed with 0.003 M NaCl, but at a higher salt concentration protection is less. A good protection can, however, also be obtained at a higher salt concentration but then the concentration of peptone has to be raised (Fig. 3). Protection was also given by the single amino acid phenylalanine (Fig. 4). Here again protection was less after spraying with a higher salt concentration. Figure 4 shows that at a 10 times higher salt concentration, about 10 times more phenylalanine has to be added for protection. At high phenylalanine concentration protection, however, decreased. At high concentrations of phenylalanine the solubility of phenylalanine in the aerosol droplet can be exceeded. The salt concentration in the droplet amounts to 2.6 M NaCl at 90% RH as can be calculated from the vapor pressure lowering of NaCl solutions (21). The solubility of phenylalanine in 2.6 M NaCl was therefore determined and this was found to be exceeded at concentrations higher than 0.06 M.

The surface active substance OED, which is a commercial mixture of oxyethylene doco-

![Fig. 2. a, Inactivation of phage MS2 by shaking. The stock phage suspension was diluted 1:10⁴ in the indicated media and then subjected to surface-inactivation by shaking 10 ml of suspension in a 30-ml bottle by means of a flask shaker. b, Inactivation in aerosol at 90% RH of the fraction of phage MS2 surviving in a shaking experiment. A sample of phage was subjected to shaking in 1 M NaCl until a more resistant fraction appeared and then sprayed. As a control a non-shaken sample was sprayed directly from 1 M NaCl.](image-url)
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95

0.1 M NaCl + 0.1 % peptone

0.003 M NaCl + 0.01 % peptone

0.1 M NaCl + 0.01 % peptone

0

-1

-2

-3

-4

0 20 40 60 80 100 % RH

FIG. 3. Aerosol inactivation at varied relative humidities of phage MS2 after aerosolization from the indicated media. Samples were taken at 30 min after spraying.

sely ether and oxyethylene octadecylether (14), protects very well against inactivation by shaking (Fig. 2a) and also protects against inactivation in aerosols. In aerosols generated from 2.6 M NaCl at 90% RH nearly maximal protection was found at about 1.1% (wt/vol) OED in the spray-medium (Fig. 5). To show whether the protective concentration might be determined by the surface occupation of OED on the droplet surface, the surface occupation by OED was determined in a separate experiment by spreading a known amount of OED emulsion in a Langmuir trough on the air-water surface. A dense monolayer of OED was found at an OED concentration of about $4.5 \times 10^{-7}$ to $6.5 \times 10^{-7}$ g/cm$^2$ (Fig. 6). The protective concentration of OED at 1.1% (wt/vol) (Fig. 5) results in a surface concentration of $5 \times 10^{-7}$ g/cm$^2$ for particles with a radius of 1.4 μm after spraying from 2.6 M NaCl at 90% RH. The particle size median diameter of the aerosol was found at 2 μm (Trouwborst, thesis, State University, Utrecht, 1971). As a first approximation these data seem to correspond sufficiently.

FIG. 4. Aerosol inactivation of phage MS2 at $t = 30$ min after spraying from 0.1 and 1.0 M NaCl supplemented with varying amounts of phenylalanine in the spray medium. RH, 90%.

FIG. 5. Aerosol inactivation of phage MS2 at 90% RH after spraying from 0.1 and 2.6 M NaCl supplemented with varying amounts of the surface active agent OED in the spray medium. Samples are collected at $t = 30$ min.
could be toxic. Crystal formation, which is strongly dependent on the total amount of phenylalanine in the droplets because of a relatively high surface energy of small crystals (15) will occur earlier in larger droplets. This could explain the lesser protection at 1.0 M NaCl compared to 0.1 M NaCl (Fig. 7). The protective concentration of OED is also dependent on the salt concentration but to a lesser extent. OED differs from phenylalanine in that it is insoluble and accumulates at the air-water interface. Surface size then will be important.

Phage MS2 is most strongly inactivated at high RH when the aerosol particles are fluid. Inactivation is not likely due to toxicity of the concentrated salt solution in the aerosol droplet, because no inactivation in concentrated salt solutions is found. Inactivation was also not caused by shear forces nor by an enhanced oxidation because a high velocity of the liquid in the spraying system did not affect the virus and because inactivation was the same in a nitrogen atmosphere. By eliminating these possible inactivation processes we get the indication that inactivation is related to the air-water interface. By shaking, a large continuously renewing air-water interface is formed. Such an interface itself can be a cause of inactivation (1, 18, 19), because a virus in such an interface is subjected to unbalanced forces. Because the

**DISCUSSION**

Peptone and phenylalanine protect phage MS2 against inactivation in aerosol. Higher concentrations are needed if the salt concentration in the spray-medium is raised. The effect of the salt concentration on the protective action is explained by the difference in droplet size after evaporation. At 90% RH the NaCl concentration in the droplet after evaporation to equilibrium will be 2.6 M. After spraying from 0.1 M NaCl the concentration factor is 2.6 and after spraying from 1.0 M NaCl this amounts to 2.6. In the latter case 10 times more phenylalanine or peptone will have to be added to the spray medium to obtain the same concentration in the droplet. The consequence of this is illustrated in Fig. 7 where the phage survival is plotted as a function of the final phenylalanine concentration in the droplet. For both salt concentrations, protection is maximal at about the same final phenylalanine concentration, indicating that the final phenylalanine concentration determines the protection. The protective effect of phenylalanine disappears at concentrations higher than 0.06 M (Fig. 7). We found that the solubility of phenylalanine in 2.6 M NaCl is exceeded at concentrations higher than 0.06 M. The supersaturated solution, the crystals, or the process of crystallization then

**Fig. 6.** Surface pressure of a monolayer of OED at varying surface concentrations. A 0.03-ml amount of 1% OED in 0.1 M NaCl was spread above a solution of 2.6 M NaCl, whereafter the monolayer was gently compressed.

**Fig. 7.** Aerosol inactivation of phage MS2 at $t = 30$ min after spraying from 0.1 and 1.0 M NaCl with phenylalanine, represented as a function of the phenylalanine concentration in the aerosol droplets after evaporation at 90% RH ($C_r = 2.6$ M NaCl). The experimental data were obtained from Fig. 4.
aerosol-system has also a large air-water interface, surface inactivation is probably an important mechanism of inactivation in aerosols for phage MS2. This is supported by the parallel between resistance to surface inactivation and aerosol inactivation (Fig. 2b). Peptone and OED which protect against inactivation by shaking also protect against inactivation in aerosols. The concentration of OED that exerts protection in aerosols is found at a concentration in which just a dense monolayer of this substance is formed at the air-water interface. This supports the concept of surface inactivation, because permeation of molecules in the air-water interface can be affected by the presence of a monolayer with surface pressures of 3 to 4 dyn/cm (13), preventing these molecules from surface inactivation.

The decrease of recovery of phage MS2 after prehumidification, reported by Dubovi and Akers (9) can also be explained on the basis of our observations. By prehumidification the particle size is increased and the concentration of protecting substances is decreased. Under these circumstances the conditions for surface inactivation are present.

The minimum in the curves of Fig. 1 at 75% RH can be the result of several factors. In the first place aerosol droplets can be in solid state at a RH below 75%. This will occur more rapidly at lower RH, resulting in a shorter time for surface inactivation. Secondly, the droplets are smaller at 75% RH than at higher RH, resulting in a shorter distance to the droplet surface, increasing the number of collisions with the surface. In addition, in the shaking experiments we found that the rate of inactivation by shaking increases at higher salt concentrations (Fig. 2a). At low salt concentrations ( \( \leq 0.01 \) M NaCl) no inactivation was found (manuscript in preparation). This could explain the results of Webb (23) who found that the MS2 related phage f\( _5 \) was stable in aerosol after spraying from water.

The importance of surface processes for the inactivation of phage MS2 in aerosols at high RH is remarkable, as it differs in this respect from a small animal virus like encephalomyocarditis virus (EMC). Though both viruses seem superficially of analogous structure (small lipid-free RNA viruses with icosahedral shape), the EMC virus survives well at high RH (6). As EMC virus survives well in aerosols at high RH, it can be expected that this virus is not sensitive to surface inactivation by shaking and this is what we found (manuscript in preparation). These results suggest that only for viruses inactivated at high RH, i.e., lipid-containing viruses, surface inactivation can be an important mechanism.

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LITERATURE CITED

1. Adams, M. H. 1948. Surface inactivation of bacterial viruses and of proteins. J. Gen. Physiol. 31:417-431.
2. Adams, M. H. 1966. The bacteriophages. Interscience Publishers Inc., New York.
3. Anderson, T. F. 1953. The morphology and osmotic properties of bacteriophage systems. Cold Spring Harbor Symp. Quant. Biol. 18:197-203.
4. Benbough, J. E. 1971. Some factors affecting the survival of airborne viruses. J. Gen. Virol. 10:209-220.
5. Bruneck, C. F., and V. Leich. 1969. Rapid equilibrium isopycnic CsCl gradients. Biochim. Biophys. Acta 179:136-140.
6. de Jong, J. C. 1970. Data presented on the 3rd International Symposium on Aerobiology, Brighton 1969, p. 210. In I. H. Silver (ed.), Aerobiology. Academic Press Inc., London.
7. de Jong, J. C., and K. C. Winkler. 1968. The inactivation of poliovirus in aerosols. J. Hyg. Camb. 66:557-565.
8. Dubovi, E. J. 1971. Biological activity of the nucleic acids extracted from two aerosolized bacterial viruses. Appl. Microbiol. 21:761-762.
9. Dubovi, E. J., and T. G. Akers. 1970. Airborne stability of taillless bacterial viruses S-13 and MS-2. Appl. Microbiol. 18:624-629.
10. Harper, G. J. 1963. Some observations on the influence of suspending fluids on the survival of airborne viruses, p. 335. Proceedings of the 1st international symposium on aerobiology, Berkeley, Calif.
11. Hatch, M. T., and J. C. Warren. 1969. Enhanced recovery of airborne T\(_4\) coliphage and Pasteurella pestis bacteriophage by means of a presampling humidification technique. Appl. Microbiol. 17:685-689.
12. Hemmes, J. H., K. C. Winkler, and S. M. Kool. 1960. Virus survival as a seasonal factor in influenza and poliomyelitis. Nature (London) 188:430-431.
13. James, L. K., and L. G. Augenstein. 1966. Adsorption of enzymes at interfaces. Advan. Enzymol. 28:1.
14. Mihara, Y. 1966. Frost protection by fog droplets coated with monomolecular films. Nature (London) 212:602-603.
15. Orr, C., F. K. Hurd, and J. Corbett. 1958. Aerosol size and relative humidity. J. Colloid Sci. 13:472-482.
16. Songer, J. R. 1967. Influence of relative humidity on the survival of some airborne viruses. Appl. Microbiol. 15:34-42.
17. Strauss, J. H., and R. L. Sinheimer. 1963. Purification and properties of bacteriophage MS-2 and of its ribonucleic acid. J. Mol. Biol. 7:43-54.
18. Trouwborst, T., J. C. de Jong, and K. C. Winkler. 1972. Mechanism of the inactivation in aerosols of the bacteriophage T\(_4\), J. Gen. Virol. 15:235-242.
19. Trouwborst, T., and K. C. Winkler. 1972. Protection against aerosol inactivation of bacteriophage T\(_4\) by peptides and amino acids. J. Gen. Virol. 17:1-11.
20. Warren, J. C., T. G. Akers, and E. J. Dubovi. 1969. Effect of prehumidification on sampling of selected airborne viruses. Appl. Microbiol. 18:893-896.
21. Weast, R. C. 1968. Handbook of chemistry and physics, 49th ed. The Chemical Rubber Co., Ohio.
22. Webb, S. J., R. Bather, and R. W. Hodges. 1963. The effect of relative humidity and inositol on air-borne viruses. Can. J. Microbiol. 9:87-93.
23. Webb, S. J., and J. L. Walker. 1969. The influence of cell water content on the inactivation of RNA by partial dessication and ultraviolet light. Can. J. Microbiol. 14:565.