Inactivation of some Bacterial and Animal Viruses by Exposure to Liquid–air Interfaces

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SUMMARY

Surface inactivation of the bacteriophages T1, T3, T6, MS2, of EMC virus and Semliki Forest virus was studied, exposing the viruses to a large air/water interface by aeration or by rotating the fluid in a spherical flask. EMC virus in 1 M-NaCl was not sensitive to this treatment, phage T3 and T6 were only little affected, but the phages T1 and MS2 and Semliki Forest virus were rapidly inactivated by bubbling air or nitrogen gas through the suspension. In salt solutions at rest no inactivation of these viruses was observed. Inactivation by aeration was prevented by addition of peptone, by apolar carboxylic acids and by the surface active agent OED. If a large solution/glass interface is present, some loss of virus occurs by adsorption to the glass surface. Phenylalanine protected against adsorption to the glass surface, but protected less effectively against inactivation by aeration. The rate of surface inactivation was strongly dependent on the salt concentration in the medium. At low NaCl concentration (0.01 M) nearly no inactivation was found for phage T1 and MS2 and phage T3 was not sensitive to aeration in 1 M-NaCl but was rapidly inactivated in 2.6 M-NaCl. The rate of inactivation decreased with time of shaking and in the case of phage T1 a nearly completely resistant fraction of 10^-4 of the original particles remained. The resistance against surface inactivation was a non-heritable property. Resistance against thermal inactivation was not correlated with resistance to surface inactivation, suggesting that the mechanism of inactivation differs in these processes.

INTRODUCTION

Shaking of a virus suspension can result in a decrease of the virus titre. Inactivation by shaking has first been reported for a phage by Campbell-Renton (1942) and some other results suggest that animal viruses are also sensitive to this treatment (MacLimans, 1947). More extensive work about the cause of this loss of viable virus has been done by Adams (1948), who demonstrated that loss of viable phage by shaking of suspensions of the phages T1–T7 was correlated with the generation of a large continuously renewing air/water interface. Inactivation at an air/water interface was also found to be important for viruses in aerosols stored at high relative humidity and the parallelity between inactivation of

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viruses in aerosols and by shaking was striking. (Trouwborst, 1971; Trouwborst, de Jong & Winkler, 1972; Trouwborst & Winkler, 1972; Trouwborst & de Jong, 1973a).

Much work on inactivation of macromolecules at air/water interfaces has been done with enzymes and inactivation is generally attributed to extrusion of the hydrophobic parts of the molecule into the air phase and subsequent unfolding of the protein chain (James & Augenstein, 1966). However, only little is known about the factors governing the inactivation of a virus at the air/water interface. Campbell-Renton (1942) reported less surface inactivation in salt-free media. This shows that the salt concentration has an important role in this process.

In this paper, the process of surface inactivation, the action of protecting substances and the influence of the salt concentration is studied for the bacteriophages T1, T3, T5, MS2 and with encephalomyocarditis virus and Semliki Forest virus. Some thermoresistant phage mutants were also tested in order to examine whether a relation existed between resistance to surface inactivation and temperature inactivation.

**METHODS**

*Propagation and assay of the viruses.* The phage T1 and its thermoresistant mutants T1<sub>18-11</sub> and T1<sub>18-22</sub> (obtained from Dr D. A. Ritchie) were propagated on *Escherichia coli* B in a synthetic medium containing per litre: 60 g K<sub>2</sub>HPO<sub>4</sub>, 60 g K<sub>2</sub>HPO<sub>4</sub>, 20 g NH<sub>4</sub>Cl, 0.5 g MgSO<sub>4</sub>, 0.05 g FeSO<sub>4</sub> and 5.6 g NaCl, pH 6.8. After sterilization solutions of 1 M-glucose and 1 M-CaCl<sub>2</sub> were added up to a final concentration of 0.02 and 0.001 M, respectively. A 16 h old culture of *E. coli* B was diluted 1:30 in the synthetic medium and was incubated for 2 h under vigorous aeration at 37 °C. Then phage was added up to a titre of 10<sup>6</sup> p.f.u./ml. After 2 h of incubation, the bacterial debris was removed from the lysate by low-speed sedimentation.

Bacteriophage T5, its temperature-resistant mutant T5<sub>mr</sub> and the host *Escherichia coli* F were obtained from Dr Lanni. These strains and bacteriophage T3 were propagated as described for phage T1.

Bacteriophage MS2 and its host *Escherichia coli* KA 81 were propagated in broth as described by Strauss & Sinsheimer (1963), with the addition of 0.05 M-tris buffer, pH 7.2, to the growth medium.

EMC-virus and Semliki Forest virus (SFV) were grown in L cells, suspended in Eagle's minimal essential medium without serum or bicarbonate and buffered with 0.01 M-HEPES. After 20 h incubation the infected cell suspension was freeze-thawed and the cell debris was removed by low-speed sedimentation.

The phage suspensions were titrated for viable phage according to the agar layer method of Adams (1966), using the host as indicator strain.

EMC and SFV were titrated by conventional plaque techniques on L cells.

*Purification of the viruses.* The bacteriophages T1, T3, T5 and their mutants were purified by sedimentation at 60,000 g (19,000 rev/min) for 3 h in a 6 × 250 ml rotor (MSE-65). The sediment was resuspended in the synthetic medium and was further purified by sedimentation in a CsCl gradient according to the two layer method of Brunck & Leick (1969). The lower layer contained 1.8 ml phage suspension to which 2.7 g solid CsCl was added. The upper layer consisted of 2.5 ml phage suspension and 2.8 g of CsCl. Sedimentation was performed in a 3 × 5 ml rotor (SW 40, MSE-65) at 32,000 rev/min (110,000 g) at 10 °C for 24 h. After sedimentation the phage containing fractions were dialysed against 0.1 M-NaCl.

Phage MS2 was purified by precipitation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and treatment with Freon-11,
Surface-inactivation of viruses

as described by Strauss & Sinsheimer (1963). Further purification was achieved by CsCl sedimentation according to the two layer method. The layers contained each 2.5 ml phage suspension with respectively 0.825 and 1.925 g CsCl. Sedimentation was performed at 4 °C in a 3 × 5 ml SW-40 rotor (MSE-65) at 32000 rev/min (110000 g) for 24 h. The phage containing fractions were dialysed against 0.01 M-tris-buffer, pH 7.6, with 0.1 M-NaCl. The final phage titre was 5 × 10^{13} p.f.u./ml.

EMC virus and SFV were concentrated and purified by sedimentation at 105000 g in a type 30 rotor (Spinco, Beckmann) during 2 h. The sediment was suspended in Hanks’s balanced salt solution without bicarbonate, pH 7.2. SFV was thereafter purified by passage through a column of Sepharose 4B and elution with 0.1 M-NaCl + 0.01 M-PO_{4}-buffer, pH 7.6.

Radioactive labelling of bacteriophage T1 and radioactivity determinations. A 1:30 dilution of a 16 h old culture of Escherichia coli B was inoculated in broth and grown at 37 °C during 2 h. The bacterial cells were centrifuged and the pellet was suspended in synthetic medium, whereafter 1 μCi [14C] of a purified protein hydrolysate (the Radiochemical Centre, Amersham) was added per ml together with phage T1 up to a titre of 10^{8} p.f.u./ml. The phage was propagated and purified as described for the unlabelled phage T1. Radioactivity in aqueous samples of [14C]-labelled phage was counted with a liquid scintillation counter (Nuclear Chicago, Mark II) in a toluene-Triton X-100 scintillation liquid mixture.

Surface inactivation experiments. The following two methods were used. (a) Ten ml of the virus suspension was put in a spherical flask (φ = 13 cm) which rotated at a speed of 170 rev/min at an angle of 30° with the horizontal level in a waterbath of 20 °C. (b) A stream of air or nitrogen gas was bubbled through 10 ml virus suspension in a cylindrical bottle (φ = 2.5 cm). The air was forced through a capillary tube (φ = 0.15 cm), fixed just above the bottom of the flask. The gas flow rate was 4.7 l/min. For determination of virus survival 0.1 ml samples were taken at standard intervals.

RESULTS

The decrease of virus titre of the purified suspensions in a rotating flask is demonstrated in Fig. 1 (a to d). A rapid decrease was found in 1 M-NaCl with phage T3, MS2 and SFV, whereas the titre of EMC virus and phage T3 was nearly not affected. To examine whether the EMC virus was somehow protected by impurities from the medium, this virus was again tested in the presence of phage MS2. As shown in Fig. 1 d the phage titre decreases rapidly, suggesting that no protecting substances (at least for phage MS2) are present in the EMC suspension.

The rate of decrease of virus titre of phage T1, T3 and MS2 is strongly dependent on the salt concentration. In 0.1 M-NaCl no loss of phage MS2 is detectable (Fig. 1 c) and phage T3 was only markedly lost at salt concentrations higher than 1.0 M-NaCl (Fig. 2). The effect of the salt concentration cannot be ascribed to toxicity of the salt alone, as the titres of virus suspensions in such salt solutions were not reduced without rotation.

In the rotating flask, the virus suspension fluid obtains a large air/water interface which could induce surface inactivation (Adams, 1948). However, a large glass/solution interface is also present and some virus loss could occur as a consequence of adsorption to the vessel wall. In order to examine the importance of adsorption to the vessel wall, a purified [14C]-labelled suspension of phage T1 was put into the rotating flask and the amount of label left in the fluid after rotation was determined. As Table 1 (a) shows, a rapid loss of [14C] label was found. The decrease of [14C] label was lower than the decrease of p.f.u., suggesting
Fig. 1. Loss of virus in the rotating flask. (a) Phage T\textsubscript{1}. Purified phage diluted 1:10\textsuperscript{4} in ○—○, 0.001 M-NaCl; ×—×, 0.01 M-NaCl; □—□, 0.1 M-NaCl; ■—■, 1.0 M-NaCl. (b) Phage T\textsubscript{3} 1:10\textsuperscript{4} diluted in ○—○, 0.1 M-NaCl; ×—×, 1.0 M-NaCl; ■—■, phage T\textsubscript{3} and ○—○, phage T\textsubscript{5} at 1:10\textsuperscript{5} diluted in 1 M-NaCl. (c) Phage MS\textsubscript{2}, 1:10\textsuperscript{5} ———, diluted in 0.01 M-NaCl; ×—×, 0.1 M-NaCl; ○—○, 1.0 M-NaCl; □—□, 2.6 M-NaCl. (d) □——□, EMC virus; ×—×, SFV; ■——■, phage MS\textsubscript{2}; 1:10\textsuperscript{5} diluted in 1.0 M-NaCl.
that adsorption to the vessel wall was not the only cause of loss of virus and that non-viable but radioactive particles are present in the fluid. Inactivation could be due to adsorption to the vessel wall, supposing elution of inactivated particles, but might also be caused by surface inactivation at the air/water interface. To differentiate between the two an agitation device was needed with a small glass surface and a large air/water interface. This was easily found by bubbling gas through the suspension in a small vessel. With this method, adsorption to the wall is much less (Table 1b) and the loss of p.f.u. is increased.
Fig. 2. Loss of viable phage T₃ by bubbling air through the virus suspension. Phage T₃ was 1:10⁴ diluted in 1.0 M-NaCl (—), 2.6 M-NaCl (×), saturated NaCl (○—○).

Table 1. Loss of viable phage T₃ (p.f.u.) and of radioactivity ([¹⁴C]-label) (a) in rotating flask, (b) by bubbling air through the virus suspension

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Δ p.f.u.</th>
<th>Δ radioactivity</th>
<th>Δ p.f.u. — Δ radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) 5</td>
<td>0.71</td>
<td>0.54</td>
<td>0.17</td>
</tr>
<tr>
<td>10</td>
<td>1.38</td>
<td>0.84</td>
<td>0.54</td>
</tr>
<tr>
<td>30</td>
<td>2.50</td>
<td>1.00</td>
<td>1.50</td>
</tr>
<tr>
<td>(b) 5</td>
<td>1.42</td>
<td>0.27</td>
<td>1.15</td>
</tr>
<tr>
<td>10</td>
<td>1.92</td>
<td>0.43</td>
<td>1.49</td>
</tr>
<tr>
<td>30</td>
<td>3.80</td>
<td>1.09</td>
<td>2.71</td>
</tr>
</tbody>
</table>

The large number of non-viable, but radioactive particles in the suspension suggest that, at least in this case, the air/water interface is a more important site of inactivation than the glass surface.

Decrease of virus titre and inactivation could be prevented by addition of peptone and broth (Fig. 3). Protection was also obtained by addition of an apolar carboxylic acid (caproic acid) and the non-ionic surface active agent OED (which is a commercial mixture of oxyethylene docosylether and oxyethylene octadecylether). Previously it was reported (Trouwborst & Winkler, 1972), that protection by peptone against surface inactivation in aerosols could be ascribed to the apolar amino acids. In the bubbling system protection by phenylalanine is incomplete however (Fig. 4), although loss of virus is less than in the control without phenylalanine.

As shown in Fig. 1(a), the virus population was not homogeneous with regard to the response to surface-exposure. From phage T₃ a nearly completely resistant fraction was obtained after inactivation with a factor of 10⁴. This resistant phage fraction was propagated and tested for sensitivity to surface-inactivation. Six cycles of propagation of the resistant
Surface-inactivation of viruses

Fig. 3. Protection against loss of virus in the rotating flask in 1.0 M-NaCl by 0.03 M-caproic acid (□ --- □), 0.1% (w/v) peptone (○ ○○), 0.1% (w/v) OED (× ××).

Fig. 4. Effect of phenylalanine on loss of viable phage and of [14C]-label by aeration. Phage T1 was diluted 1:500 in 1.0 M-NaCl and 1.0 M-NaCl + 0.06 M-phenylalanine. ○○○, decrease of p.f.u. in 1.0 M-NaCl; × --- ×, decrease of p.f.u. in 1.0 M-NaCl + 0.06 M-phenylalanine; ○ --- ○, decrease of [14C] label in 1.0 M-NaCl; × --- ×, decrease of [14C] label in 1.0 M-NaCl + 0.06 M-phenylalanine.
Fig. 5. (a) Inactivation by shaking (rotation) of phage T₁ and two temperature-resistant mutants. ○—○ T₁; ⋄—⋄ T₁₁; x—x T₁₂. (b) Temperature-inactivation of phage T₁, T₁₁ and T₁₂ in 1.0 M-NaCl. T = 70°C.
fractions did not result in a raised resistance of the phage progeny, demonstrating that the
resistance is not a heritable property of the phage particle.

A resistant population of phage was also found after thermal inactivation of phage T_1,
T_3, T_5 and \( \lambda \) (Adams & Lark, 1950; Hertel, Marchi & Muller, 1962; Rubinstein, 1968;
Ritchie & Malcolm, 1970; Parkinson & Huskey, 1971; Yamagishi & Ozeki, 1972). This
property was shown in the case of phage T_5 and \( \lambda \) to be correlated with a lower DNA
content and raised resistance to chelating agent shock (Yamamoto, Fraser & Mahler, 1968).
Therefore we tested whether a relation existed between heat resistance and resistance against
surface inactivation. The experiments were performed with the heat-resistant mutants of
phage T_3 and T_5. As shown in Fig. 5(a), the mutants had no raised resistance to surface
inactivation, whereas a considerable difference was found in sensitivity to temperature inacti-
vation (Fig. 5b). In the reversed situation the phage fraction resistant to surface inactivation
was not more resistant to temperature inactivation, suggesting that the mechanism of
inactivation in these processes differs. This is supported by the inverse effect of salt which
protects against heat inactivation and enhances surface inactivation.

**DISCUSSION**

The phages T_1, T_3, T_5 and MS2 and SFV are sensitive to surface forces in 1 M-NaCl,
whereas EMC virus is stable. This corresponds with the results found with viruses in
aerosol droplets at high relative humidity, in which case also a relatively large air/water
interface is present. In aerosols EMC virus is stable at high relative humidity, whereas phage
T_1, MS2 and SFV are rapidly inactivated (Benbough, 1969; de Jong, 1970; Trouwborst
et al. 1972; Trouwborst & de Jong, 1973b). The substances peptone and OED which
protect against surface inactivation protect also against inactivation in aerosols. On the
contrary, 0.06 M-phenylalanine which gives good protection in aerosols (Trouwborst &
Winkler, 1972) does not protect very well in the bubbling/rotating experiments. The
bubbling system differs from the aerosol by the continuous renewal of the air/water
interface so that some time is needed before the surface can be occupied by the amino acid.
In the rotating system the glass/solution interface remains constant and adsorption to this
surface is prevented by phenylalanine, in parallel with the protection at a resting air/water
interface. These results suggest that protection by phenylalanine against surface inactiva-
tion during aeration is not due to interaction with the phage but to occupation of the
air/water interface as suggested earlier (Trouwborst & Winkler, 1972), because the amount
of phenylalanine adsorbed by phage will not be changed by aeration.

Inactivation of a virus at the air/water interface will depend on adsorption of the virus
in the air/water interface and on the tendency of the coat proteins to unfold at that inter-
face. The step of adsorption is strongly dependent on the surface properties of the virus,
which determine the tendency of the particle to reach the surface and the equilibrium posi-
tion of the particle in that interface (see Fig. 6a). If the virus contains hydrophobic parts,
these parts will tend to be located in the air phase and this could result in inactivation by
irreversible unfolding and rearrangement of molecules. Such a process has been described
for inactivation of enzymes at interfaces (James & Augenstein, 1966). A lipid-containing
virus like SFV could well be inactivated by such a mechanism because the lipids might
contribute to the hydrophobic nature of the virion surface.

The rate of surface inactivation is strongly dependent on the salt concentration (Fig. 1a).
Surface inactivation could be influenced by the salt concentration in at least three ways.
(1) at a higher salt concentration the surface tension of the air/solution interface is raised
Fig. 6. (a) Spherical (virus) particle in the air/water interface. $\gamma_{v,a}$, $\gamma_{a,l}$, $\gamma_{v,1}$ are the surface tensions along the virus/air, air/liquid and virus/liquid interface, respectively. Stresses tangential to the surface of such a virus would balance at the air/liquid interface provided

$$\gamma_{v,a} = \gamma_{v,1} - \gamma_{a,1} \cos \theta,$$

leaving a net radial stress of $\gamma_{a,1} \sin \theta$. The effect of the latter would depend on the deformability of the virus. No adsorption is found, if

$$\gamma_{v,a} > \gamma_{v,1}$$  (particle entirely immersed in solution)

or

$$\gamma_{v,1} > \gamma_{v,a}$$  (particle unwetted, entirely in the air phase).

(b) Schematic representation of deformation of a sphere by surface forces. If $\gamma_{v,a} > \gamma_{v,1}$ and the particle is deformable, the radius of curvature ($r$) of the part of the sphere in the air phase must be larger than that in the liquid phase, because $\gamma/r$ must be equal in both phases.

and according to Fig. 6 this could result in a better adsorption of the virus on to the interface. (2) At a higher salt concentration the electrostatic repulsion of a double layer becomes less, and adsorption of the virus will be favoured. (3) The surface tension along a spherical particle will give an excess pressure inside the sphere given by

$$P = 2\gamma/r,$$

where $P$ denotes for the excess pressure inside the sphere, $\gamma$ is the surface tension along the surface of the sphere and $r$ is the radius of curvature of the sphere. If $\gamma_{v,1}$ differs from $\gamma_{v,a}$ (Fig. 6a) a pressure difference inside the particle will be established which will lead to deformation of the particle in order to equalize the quotient $\gamma/r$ in both parts (Fig. 6b). This deforming force, which is related to $\Delta P = (2/r)(\gamma_{v,a} - \gamma_{v,1})$, could be affected by the NaCl concentration to an extent increasing $\gamma_{v,a} - \gamma_{v,1}$ and consequently the deforming force.

Phage MS2 and EMC virus are both icosahedral-shaped RNA-containing viruses. It is then remarkable that phage MS2 is sensitive to surface inactivation, whereas EMC virus is not sensitive. A difference is found in the size of the virus: phage MS2 has a diam. of 246 Å (Hohn & Hohn, 1970) whereas EMC virus has a diam. of 380 Å (Weil et al. 1952). According to formula (1) a small particle will get a stronger deforming force at the same surface tensions than a large particle. Another difference is given by the presence of the A protein in the shell of phage MS2, which is responsible for adsorption of phage to the host and at which point the RNA is released from the coat protein after adsorption. It could be that surface adsorption has an analogy with adsorption to the host.

REFERENCES


Surface-inactivation of viruses


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