Mechanism of the Inactivation of the Bacteriophage T₁ in Aerosols

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The mechanism of inactivation of bacteriophage T₁ in aerosols was studied by using 32P-labeled phage. During inactivation, viability decreased in parallel with the adsorption of 32P to the host, showing either that inactivated phage does not adsorb or that the deoxyribonucleic acid (DNA) has left the coat. A 32P band at the density of free DNA was found when inactivated phage was analyzed in a CsCl gradient.

Aerosols of many lipid-free ribonucleic acid (RNA) viruses rapidly lose infectivity at certain relative humidities. Recent evidence suggests that the inactivation is due to a damaged protein coat, whereas the nucleic acid remains functionally intact. Akers and Hatch (3) showed that the free infectious RNA of mengovirus 37A (single-stranded) survives aerosolization at humidities deleterious to the complete virus. De Jong (7), could recover a full complement of infectious RNA from murine encephalomyocarditis virus, which had lost all infectivity and hemagglutinating power in the aerosol state. Again, Dubovi (9) was able to extract infectious nucleic acid of the small lipid-free bacteriophages MS₂ (single-stranded RNA) and φ X 174 [single-stranded deoxyribonucleic acid (DNA)] from aerosol-inactivated virus.

In this paper, we describe experiments performed with phage T₁, a double-stranded DNA coliphage. This phage was chosen for this work because it has been shown to be a good model for aerosol inactivation studies of viruses due to its stability against osmotic shock (T. Trouwborst, Ph.D. thesis, 1971 Rijksuniversiteit, Utrecht, The Netherlands; T. Trouwborst, J. C. de Jong, and K. C. Winkler, J. Gen. Virol., in press). We present evidence that, during inactivation in aerosols of phage T₁, the nucleic acid is separated from the protein coat.

**Materials and Methods**

Bacteriophage T₁ was propagated on *Escherichia coli* B growing in the synthetic medium of Adams (1). This contained 0.1 m glucose, 0.1 m NaCl, 0.02 m NH₄Cl, 1 m m CaCl₂, 1 m m MgSO₄, 0.05 m NaH₂PO₄, and 0.02 m KH₂PO₄, pH 6.8. For labeling experiments the phosphate concentration was ten times lower.

**Labeling of Phage with 32P.** From an overnight culture of *E. coli* B, 0.3 ml was inoculated into 15 ml of the growth medium with reduced phosphate. After shaking at 37°C for 5 hr, the pH was adjusted with NaOH to 6.9, and 0.3 ml of NaH₂¹³PO₄ (carrier-free) was added per ml of culture. After further incubation for 15 min, 0.1 ml of a suspension of bacteriophage T₁ [10¹⁴ plaque-forming units (PFU)/ml] was added. After shaking at 37°C for 2 hr, the bacteria were removed by centrifugation and filtration. The lysate contained 10¹⁴ PFU/ml.

**Purification of the Phage.** To remove the non-phage-bound 32P, the lysate was dialyzed against 0.1 m NaCl at 4°C and further purified and concentrated by centrifugation in a CsCl gradient in a rotor-type SW39L (Spinco, Beckman Corp.) centrifuge at 115,000 × g. The gradient was made according to the two-layer method of Brunk and Leick (6). The lower layer contained 1.8 ml of phage suspension to which 2.7 g of solid CsCl was added. The upper layer consisted of 2.5 ml of phage suspension and 0.8 g of CsCl. After centrifugation, the phage-containing fractions were collected and dialyzed against 0.1 m NaCl.

**Aerosol Equipment.** The aerosols were generated with a spray gun-type FK-8 apparatus, which nebulizes 1 ml of virus suspension in about 4 sec, and kept in a double-walled static system of 2,000 liters, previously described by de Jong and Winkler (8). The aerosol was sampled with three simultaneously operating "raised Porton" impingers (11) filled with 10 ml of adsorption medium (AM: synthetic medium without glucose). Sampling time was 1 min, and sampling volume 11.5 liters.

**Measurement of Radioactivity.** The virus suspension before spraying and the impinger fluid after collection were tested for radioactivity in a liquid scintillation counter (Tracer-Lab.), using Cerenkov radiation. Corrections for background radiation were made.

**Adsorption Experiments and Calculations.** Cells of *E. coli* B were prepared for adsorption by inoculating 100 ml of nutrient broth with 10 ml of a 20-hr-old culture of *E. coli* broth. After 4 hr of incuba-
tion, the cells were centrifuged and washed with adsorption medium. Ten milliliters of the phage suspension to be adsorbed, viz., impinger fluid or a dilution of the spray suspension (control), was added to the sediment. The homogenized mixture was kept at 37°C for 10 min and centrifuged, leaving less than 1% of the bacteria in the supernatant fluid. The supernatant fluid was immediately plated for nonadsorbed, viable phage with Adams’ double agar layer technique (2).

The sediment was washed with AM and tested for adsorbed radioactivity by liquid scintillation counting.

Phage recovery was calculated from the ratio between phage titer and radioactivity. This method implicitly includes correction for losses by physical fall-out.

Percentage recovery =

phage titer, aerosol sample
radioactivity, aerosol sample

phage titer, control sample
radioactivity, control sample × 100.

Adsorption of 32P after aerosolization to the bacteria was expressed relative to the adsorption of 32P of the control (nonaerosolized) suspension.

Percentage adsorption =

radioactivity in sediment of
adsorbed aerosol sample

radioactivity in control sample

Analysis of phage in a CsCl gradient. The aerosol samples for the gradient analyses were taken with a single-stage May impinger (10) filled with 10 ml of synthetic medium. Sampling time was 2 min. An aerosol sample of 110 liters was collected.

The gradient was made by preparing two layers in the centrifuge tube. The lower layer was made by mixing 1.8 ml of impinger fluid with 2.7 g of CsCl; the upper by mixing 2.5 ml of impinger fluid with 1.1 g of CsCl. The tube was centrifuged at 115,000 × g at 14°C for 20 hr. Thereafter, fractions of the gradient were measured for radioactivity. The density of each fraction was determined by measuring the index of refraction.

RESULTS

Adsorption properties of phage after aerosolization. Phage particles with intact coat but inactivated DNA would still adsorb to bacteria. By measuring the adsorption of the 32P label and the viable phage titer, such phage particles could be detected.

One milliliter of phage suspended in 0.1 M NaCl and 5 × 10⁻⁴ M phenylalanine was aerosolized at a relative humidity of 88%. The phenylalanine was added to slow down the inactivation rate (T. Trouwborst, Ph.D. thesis). Aerosol samples were taken at 10 sec, 5, 10, and 30 min and tested for viable phage and adsorbable 32P. The results are presented in Table 1 and Fig. 1. The phage suspension before aerosolization (Table 1, control) adsorbs well (cf. columns 5 and 8); it still contains a high percentage of non-phage-associated 32P label (presumably low-molecular-weight 32P as concluded from gradient analysis), but this does not disturb the results of the adsorption experiments because of the method of calculating. The rate of aerosol inactivation of phage can be seen from column 3. Viable phage is adsorbed to the extent of more than 90% (columns 2 and 4). It appears that the values for the amounts of 32P adsorbed (column 8) are broadly the same as the values calculated with the assumption that aerosol-inactivated particles will not adsorb to bacteria (column 7). The results are graphically represented in Fig. 1, together with viability of the phage. The decrease in adsorption parallels exactly the inactivation of the phage. This applies to the inactivation observed for the first sample as well as to the inactivation thereafter. The values for the samples at 10 and 30 min are less accurate because of low radioactivity counts.

The experiment was repeated at a relative humidity of 64.6%. The results (Table 2) again show that 32P adsorption and viability decrease at the same rate (columns 3 and 9). In another experiment at a relative humidity of 20% without protection by phenylalanine, over 95% of the phage is inactivated within the first 10 sec, and the remainder inactivates slowly with time. No accurate 32P adsorption values could be obtained at this level. However, it was also observed in this experiment that 32P adsorption had decreased more than 95% in the first sample, paralleling phage inactivation.

Behavior of inactivated phage in CsCl gradient. The adsorption experiments show that 32P of inactivated phage does not adsorb to the host cell. This could mean that the adsorbing site of the phage is inactivated or, alternatively, that the labeled DNA is separated from the virus coat which could still adsorb. We tried to differentiate between these possibilities by analyzing an aerosol sample in a CsCl gradient. The sample was taken 12 min after aerosolization at a relative humidity of 20.2% with 99% of the phage in the inactivated form. The gradient analysis of the phage before aerosolization is shown in Fig. 2; the analysis after aerosolization (impinger fluid) is shown in Fig. 3. The 32P of aerosolized phage banded at a density of about 1.66, that is, very near to the band at d = 1.67 observed for phage DNA ob-
TABLE 1. Decrease of infectivity and capacity of adsorption onto Escherichia coli cells of bacteriophage T₁ after aerosolization at 88% relative humidity from a phage suspension in 0.1 M NaCl with 5 × 10⁻⁴ M D,L-phenylalanine

<table>
<thead>
<tr>
<th>Sample</th>
<th>Phage titer in control suspension or impinger fluid (PFU/ml)</th>
<th>Phage recovery (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Phage titer of supernatant fluid&lt;sup&gt;b&lt;/sup&gt; (PFU/ml)</th>
<th>Radioactivity in control suspension or impinger fluid (counts/min)</th>
<th>Radioactivity (counts/min) expected in sediment&lt;sup&gt;c&lt;/sup&gt; if:</th>
<th>Aerosol-inactivated phage will adsorb&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Only aerosol-active phage will adsorb&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Radioactivity found in sediment&lt;sup&gt;c&lt;/sup&gt; (counts/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Sample taken at t = 10 sec</td>
<td>4.3 × 10⁷</td>
<td></td>
<td>2.1 × 10⁸</td>
<td>2,354</td>
<td>117</td>
<td>15.2</td>
<td>19.0</td>
<td>344</td>
</tr>
<tr>
<td></td>
<td>6.0 × 10⁴</td>
<td>5.3</td>
<td>1.5 × 10⁸</td>
<td>621</td>
<td>90</td>
<td>4.8</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.5 × 10⁴</td>
<td>1.6</td>
<td>5.0 × 10⁸</td>
<td>501</td>
<td>72.6</td>
<td>1.0</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.0 × 10⁴</td>
<td>0.5</td>
<td>4.0 × 10⁸</td>
<td>335</td>
<td>48.8</td>
<td>0.2</td>
<td>0.3</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Plaque-forming units.
<sup>b</sup> Percentage of surviving phage; see text for calculations.
<sup>c</sup> After adsorption onto the E. coli cells.

TABLE 2. Decrease of infectivity and capacity of adsorption onto Escherichia coli cells of bacteriophage T₁ after aerosolization at 64.8% relative humidity from a suspension in 0.1 M NaCl with 10⁻³ M phenylalanine

<table>
<thead>
<tr>
<th>Sample</th>
<th>Phage titer in control suspension or impinger fluid (PFU/ml)</th>
<th>Phage recovery (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Phage titer of supernatant fluid&lt;sup&gt;b&lt;/sup&gt; (PFU/ml)</th>
<th>Radioactivity in control suspension or impinger fluid (counts/min)</th>
<th>Radioactivity (counts/min) expected in sediment&lt;sup&gt;c&lt;/sup&gt; if:</th>
<th>Radioactivity found in sediment&lt;sup&gt;c&lt;/sup&gt; (counts/min)</th>
<th>Percentage&lt;sup&gt;d&lt;/sup&gt; &lt;sup&gt;e&lt;/sup&gt; &lt;sup&gt;f&lt;/sup&gt; of adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (nonaerosolized)</td>
<td>4.0 × 10⁷</td>
<td></td>
<td>3.8 × 10⁸</td>
<td>3,155</td>
<td>161</td>
<td>62.5</td>
<td>38.7</td>
</tr>
<tr>
<td>Sample taken at t = 30 sec</td>
<td>4.7 × 10⁴</td>
<td>34.9</td>
<td>4.9 × 10⁴</td>
<td>1,061</td>
<td>142</td>
<td>50.8</td>
<td>35.6</td>
</tr>
<tr>
<td></td>
<td>3.6 × 10⁴</td>
<td>30.2</td>
<td>3.2 × 10⁴</td>
<td>938</td>
<td>43.0</td>
<td>40.4</td>
<td>23.4</td>
</tr>
<tr>
<td></td>
<td>2.4 × 10⁴</td>
<td>23.9</td>
<td>2.2 × 10⁴</td>
<td>792</td>
<td>28.7</td>
<td>28.1</td>
<td>23.4</td>
</tr>
<tr>
<td></td>
<td>1.3 × 10⁴</td>
<td>19.3</td>
<td>1.0 × 10⁴</td>
<td>531</td>
<td>16.0</td>
<td>16.1</td>
<td>20.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Plaque-forming units.
<sup>b</sup> Percentage of surviving phage; see text for calculations.
<sup>c</sup> After adsorption onto the E. coli cells.

pHtained by heating (Fig. 2) and at a much higher density than the band of active phage at d = 1.52 (Fig. 2). Analysis of samples from aerosols at higher relative humidity yielded the same results.

**DISCUSSION**

The adsorption experiments show that the ³²P-labeled DNA from inactivated phage does not adsorb to the host. The gradient analysis shows a ³²P band at the site of free DNA. This suggests that the coat protein becomes separated from the DNA during inactivation. The separation could be stimulated by shear forces during collection or by the high salt concentration in the CaCl₂ gradient. Bresler et al. (5), studying inactivation of phage by gentle heating, observed that the DNA of heat-inactivated phage T₁ was expelled from the coat but remained connected with it by a labile bond which could be broken by increasing temperature or salt concentration. That in our case separation only occurs in the gradient is not plausible because radioactivity of aerosol-inactivated phage was unadsorbable to bacteria before gradient analysis.

The rate of adsorption of viable phage is fast, over 90% being adsorbed within 10 min. That this rate does not markedly change with increasing inactivation (Tables 1 and 2) sug-
Fig. 1. Decrease of infectivity and capacity of adsorption of $^{32}$P onto E. coli cells of bacteriophage T, after aerosolization at 88% relative humidity. Sprayed from a $^{32}$P-labeled phage suspension in 0.1 M NaCl with $5 \times 10^{-4}$ M D. L-phenylalanine. For calculation of $^{32}$P adsorption, see text.

Fig. 2. Analysis of radioactive phage T, in a CsCl gradient before (O) and after (x) heating.

Fig. 3. Analysis of radioactive phage of Fig. 2 in a CsCl gradient after aerosol inactivation at 20.2% relative humidity. Sample taken at 12 min after aerosolization from 0.1 M NaCl. Active phage is found at d = 1.52, free DNA at d = 1.67.

Inactivation of phage T, in aerosols

The separation of nucleic acid from the virus coat may be a more general consequence of virus inactivation in aerosols. It has also been observed by de Jong (manuscript in preparation) for encephalomyocarditis virus after aerosol and heat inactivation. Detection of the biological activity of this “surviving” nucleic acid requires special methods. That it still could be of practical importance for epidemiology has been shown by Akers et al. (4), who could infect animals with aerosols of free nucleic acid of encephalomyocarditis virus.

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LITERATURE CITED