The Aerosol Stability of a Strain of Foot-and-Mouth Disease Virus and the Effects on Stability of Precipitation with Ammonium Sulphate, Methanol or Polyethylene Glycol

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SUMMARY

A strain of foot-and-mouth disease virus (O1 BFS 1860) was found to be comparatively stable in aerosols at relative humidities above 55%: the inactivation rate was greater at lower relative humidities.

Virus precipitated with ammonium sulphate or methanol was considerably more unstable than untreated virus at relative humidities below 55%: this instability was not due to residual precipitant not removed by dialysis. Virus precipitated by polyethylene glycol or centrifuged at high speed was as stable as untreated virus. Possible reasons for the instability of virus precipitated by ammonium sulphate or methanol are discussed.

INTRODUCTION

It has recently been shown that foot-and-mouth disease (FMD) virus is excreted as an aerosol by cattle, sheep and pigs infected with the disease (Sellers & Parker, 1969) and much of the spread of the disease in recent epidemics in Great Britain has been attributed to dispersal by airborne particles (Hurst, 1968; Hugh-Jones & Wright, 1970; Tinline, 1970). Airborne FMD virus has also been shown to be capable of infecting cattle (Hyslop, 1965). G. J. Harper, J. N. Wilson & R. F. Sellers (personal communication) have shown that FMD virus survives best at high relative humidities.

Previous studies on the aerosol stabilities of other picornaviruses (poliovirus (Harper, 1961), Columbia SK group viruses (Akers, Bond & Goldberg, 1966) and mengovirus) have shown that all survive best at high relative humidity.

The present paper reports on the infectivity stability in aerosol of the O1 BFS 1860 strain of FMD virus after spraying at various relative humidities. Since the chemical precipitation methods used to concentrate the virus affected its stability, the mechanism of this effect was investigated.

METHODS

Virus. The O1 BFS 1860 strain of FMD virus was used at the ninth passage in baby hamster kidney (BHK 21) cells and was grown in Eagle's medium. Tissue culture harvest virus was separated from cell debris by centrifugation at 4000 rev./min. and the supernatant fluid was either stored at −70° for use as untreated virus or was concentrated by precipitation.

Infectivity assay. Infectivity assays were performed using renal swine (IB-RS-2; de Castro, 1964) monolayers. A sample of 0.4 ml. of virus dilution was spread onto each monolayer and an overlay containing 1% (w/v) Special Agar Noble was added after incubation at 37°
for 30 min. Five replicate plates were used for each dilution and the plates were stained with neutral red after 24 hr.

Precipitation methods

Ammonium sulphate. An equal volume of saturated ammonium sulphate was added to tissue culture virus suspension at $4^\circ$ (Brown & Cartwright, 1963). After 30 min, the virus suspension was centrifuged at 4000 rev./min. for 20 min. The precipitate was usually resuspended in 0.04 M-phosphate buffer containing 0.5% bovine serum albumin (pH 7.6). Excess ammonium sulphate was removed by dialysis overnight.

Methanol. Tissue culture virus was precipitated by 20% methanol at $-6^\circ$ overnight (Bachrach & Breese, 1958). The virus suspension was centrifuged at 4000 rev./min. for 20 min. and then resuspended in 0.04 M-phosphate buffer containing 0.5% bovine serum albumin. Excess methanol was removed by dialysis overnight.

Polyethylene glycol. Tissue culture virus was precipitated by the addition of 7.5% (w/v) polyethylene glycol at $4^\circ$ (Fayet, 1969). After 2 hr, the virus suspension was centrifuged at 4000 rev./min. for 20 min. and resuspended in 0.04 M-phosphate buffer containing 0.5% bovine serum albumin.

High speed centrifugation. The virus suspension was centrifuged for 2 hr at 115,000g in a MSE Superspeed 25 centrifuge. The pellet was resuspended overnight at $4^\circ$ in 0.04 M-phosphate buffer containing 0.5% bovine serum albumin.

Aerosol generation, storage and collection. Aerosols containing virus were generated with a single jet Collison spray and stored in a 75 l. rotating drum containing air at controlled relative humidity (Henderson, 1952; Druett, 1969). The characteristics of the Collison spray are such that essentially monodisperse aerosols are produced. The mass median diameter of the particles produced was about 1.4 $\mu$m. Samples of the aerosol were collected by Porton raised impingers (May & Harper, 1957) containing 10 ml. of collecting fluid (phosphate buffered saline with 0.25% bovine serum albumin and silicone antifoam emulsion). Samples were taken about 1 sec. after generation and after storage in the drum. The drum and cloud were rotated at 4 rev./min. to reduce physical loss by sedimentation (Goldberg et al. 1958).

The experiments were performed at the ambient temperature which varied from 19 to 22$^\circ$.

Physical decay. The number of infective virus particles in an aerosol falls as a result of physical and biological losses (Anderson & Cox, 1967). To estimate the physical losses which occurred while generating the aerosol and holding in the drum, [3H]-uridine in distilled water was sprayed in the apparatus and collected in distilled water. The extent of physical loss in the drum over 24 hr was statistically insignificant. As the uridine was sprayed from distilled water, the physical decay was probably less than that for virus sprayed from tissue culture supernatant fluid. *Bacillus subtilis* var. niger (BG) spores were tested as a routine tracer. They showed a physical decay rate of $0.014 \pm 0.01$ log./hr over 24 hr. This exaggerated the physical loss between spray and drum since a higher proportion of virus than BG spores was often recovered from the drum after 5 min. This was presumably due to the larger size of BG spores than virus particles. BG spores were therefore not used routinely as a tracer. The error involved in neglecting this very small physical decay rate will be small, particularly at low relative humidities.

Calculation of results. The viable spray factor (VSF) was obtained as follows from the infectivity of collected samples.

\[
\text{VSF} = \frac{\text{p.f.u./litre of cloud in spray tube} \times \text{flow rate through apparatus (l./min)}}{\text{Flow rate through spray (l./min) \times p.f.u./ml. of original spray fluid} \times 10^3}
\]
Tracer experiments with [3H]-uridine gave a maximum VSF of around $6 \times 10^{-6}$ for samples taken at 1 sec. and after ageing in the drum. This value was taken as 100% and all other results expressed as a percentage viability.

\[
\text{Percentage viability} = \frac{\text{VSF of virus sample}}{6 \times 10^{-6}} \times 100\%.
\]

Some of the observed viabilities exceeded 100% due either to the impinger collecting virus more efficiently than [3H]-uridine or to the inaccuracies of the assay systems. At least four experiments were performed at each relative humidity and the percentage viabilities and log. decay rates were calculated as the arithmetic means of the values obtained.

**Decay rates.** After the initial 5 min., the logarithm of the percentage viability was found to decrease linearly with time. A least squares method was used to determine the aerosol decay rates. These decay rates are quoted as logarithms of the percentage viability per hr (log./hr) and the errors given are twice the standard error. Most experiments were of 2 hr duration with the exceptions of those for the faster decay rates which were shorter. A few experiments on slow decay rates were over 24 hr.

**Protein determinations.** Protein was assayed by the method of Lowry et al. (1951).

**Heat stabilities.** Samples of untreated or ammonium sulphate precipitated virus were held at 50° in a water-bath. At various times up to 2 hr, samples were removed and stored at 4° until titrated for infectivity immediately after the 2 hr period.

**RESULTS**

**Stability of untreated virus**

Untreated virus was very stable above 55% RH, and no loss of virus was detected after 1 sec. At relative humidities below 55%, considerable losses occurred during the first sec. After 5 min., considerably less infective virus was recovered (Table 1). The pattern of dependence on relative humidity was similar to that after 1 sec. (Fig. 1A).

<table>
<thead>
<tr>
<th>Relative humidity (%)</th>
<th>Viability (%) at 1 sec.</th>
<th>Viability (%) at 5 min.</th>
<th>Decay rate after 5 min. (log./hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>120</td>
<td>12</td>
<td>$0.063 \pm 0.006$</td>
</tr>
<tr>
<td>50</td>
<td>37</td>
<td>7.5</td>
<td>$0.73 \pm 0.24$</td>
</tr>
<tr>
<td>45</td>
<td>9.3</td>
<td>2.9</td>
<td>$0.85 \pm 0.28$</td>
</tr>
<tr>
<td>40</td>
<td>1.8</td>
<td>0.40</td>
<td>$4.2 \pm 0.8$</td>
</tr>
<tr>
<td>30</td>
<td>0.40</td>
<td>0.009</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.29</td>
<td>0.009</td>
<td></td>
</tr>
</tbody>
</table>

Decay rates were calculated from regression lines and the errors quoted are ± twice the standard error.

Once the initial loss had occurred during the first 5 min., virus was inactivated comparatively slowly at high relative humidities. Thus at 60% RH, the inactivation rate was $0.63 \pm 0.006$ log./hr and remained constant over 24 hr. At the same temperature, the corresponding rate of inactivation in the initial spray fluid was $0.03 \pm 0.01$ log./hr. This is significantly lower than the inactivation rate in the aerosols ($> 99.9\%$ significance, Student's $t$-test). The inactivation rate in the aerosols was greater at lower relative humidities (Fig. 2A and Table 1).
Fig. 1. A. Survival aerosols of infectivity of untreated virus at various relative humidities. B. Survival in aerosols of infectivity of ammonium sulphate precipitated virus at various relative humidities.

% viability at 1 sec. •——, 5 min. ▲——▲.

**Stability of ammonium sulphate precipitated virus**

Virus precipitated by ammonium sulphate was found to be considerably more unstable than untreated virus (Fig. 1B, 2B and Table 2). The survivals of infectivity at 1 sec. at various relative humidities were similar to those with untreated virus. By 5 min. only 0.22% of the virus survived at 50% RH and 0.032% at 45% RH, compared with 7.5% and 2.9% respectively with the untreated virus at these humidities.

It was calculated that the residual ammonium sulphate concentration would have been reduced to less than 0.0003 M by dialysis. Ammonium sulphate at 0.001 M had no detectable effect on the stability of untreated virus or on virus precipitated by polyethylene glycol. Thus the increased inactivation of virus was unlikely to be due to residual ammonium sulphate.

**Methanol precipitated virus**

The stability in aerosols of methanol precipitated virus was similar to that for ammonium sulphate precipitated virus (Table 3). The estimated initial concentration of methanol was about 1 to 2% and this would have been reduced to less than 0.0005% by dialysis. Untreated
Aerosol stability of FMDV after concentration

Fig. 2. A. Decay of infectivity of untreated virus in aerosols held for up to 60 min. B. Decay of infectivity of ammonium sulphate precipitated virus in aerosols held for up to 60 min. 60 % RH, ○—○, 50 % RH, △—△, 40 % RH •—•.

Table 2. Aerosol stability of infectivity of ammonium sulphate precipitated virus at various relative humidities

<table>
<thead>
<tr>
<th>Relative humidity (%)</th>
<th>Viability (%)</th>
<th>Decay rate after 5 min. (log./hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>92</td>
<td>15</td>
</tr>
<tr>
<td>55</td>
<td>110</td>
<td>1·7</td>
</tr>
<tr>
<td>50</td>
<td>37</td>
<td>0·22</td>
</tr>
<tr>
<td>45</td>
<td>12</td>
<td>0·032</td>
</tr>
<tr>
<td>40</td>
<td>5·7</td>
<td>&lt; 0·003</td>
</tr>
<tr>
<td>30</td>
<td>3·9</td>
<td>&lt; 0·003</td>
</tr>
<tr>
<td>20</td>
<td>2·2</td>
<td>&lt; 0·003</td>
</tr>
</tbody>
</table>

Virus was not affected by the addition of 5% methanol and so the increased inactivation was unlikely to be due to residual methanol.

Polyethylene glycol precipitated virus

Virus precipitated by polyethylene glycol was not significantly more sensitive to aerosol inactivation than untreated virus (Table 4).
Table 3. Aerosol stability of infectivity of methanol precipitated virus at various relative humidities

<table>
<thead>
<tr>
<th>Relative humidity (%)</th>
<th>Viability (%)</th>
<th>Decay rate after 5 min. (log./hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At 1 sec.</td>
<td>At 5 min.</td>
</tr>
<tr>
<td>60</td>
<td>99</td>
<td>11</td>
</tr>
<tr>
<td>55</td>
<td>28</td>
<td>0.65</td>
</tr>
<tr>
<td>50</td>
<td>17</td>
<td>0.20</td>
</tr>
<tr>
<td>45</td>
<td>8.6</td>
<td>&lt; 0.03</td>
</tr>
<tr>
<td>40</td>
<td>3.5</td>
<td>&lt; 0.03</td>
</tr>
</tbody>
</table>

Table 4. Aerosol stability of infectivity of virus after pelleting by centrifuging at high speed or precipitation by polyethylene glycol

<table>
<thead>
<tr>
<th>Relative humidity (%)</th>
<th>Viability (%)</th>
<th>Polyethylene glycol precipitated virus</th>
<th>Pelleted virus</th>
<th>Untreated virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At 1 sec.</td>
<td>At 5 min.</td>
<td>At 1 sec.</td>
<td>At 5 min.</td>
</tr>
<tr>
<td>60</td>
<td>51</td>
<td>9.2</td>
<td>117</td>
<td>10</td>
</tr>
<tr>
<td>55</td>
<td>28</td>
<td>5.0</td>
<td>27</td>
<td>8.1</td>
</tr>
<tr>
<td>45</td>
<td>7.1</td>
<td>1.9</td>
<td>16</td>
<td>6.2</td>
</tr>
<tr>
<td>40</td>
<td>4.3</td>
<td>0.53</td>
<td>6.8</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Sedimented virus

The stability of virus was unchanged after pelleting by centrifuging at high speed and re-suspension in the same buffer solution as the chemically precipitated virus (Table 4). Thus the distinct instability of the chemically precipitated virus was not due to the suspending medium.

Protein concentrations

As the precipitation concentrated other proteins with the virus protein and the precipitated virus was re-suspended in a buffer solution containing 5 mg./ml. of bovine serum albumin, the protein concentrations of the precipitated virus suspension were higher than those in the untreated virus suspension. Considerable variations were observed between batches of virus, the protein concentration before precipitation being from 0.5 to 0.9 mg./ml. and that after centrifugation from 7 to 10 mg./ml. Virus pelleted by high speed centrifugation showed a similar protein concentration to that for the chemically precipitated virus. The instability of the precipitated virus was not, therefore, simply related to its protein concentration.

DISCUSSION

Relevant studies on the aerosol stability of FMD virus are important in view of the likelihood of airborne spread during an epidemic. However, important reservations must be made before the results can be applied to natural conditions. The virus studied here was suspended in an artificial medium and its properties had almost certainly been modified by its nine passages in tissue culture. The conditions under which the aerosol was stored were very different from those of the normal atmosphere to which the virus is exposed in nature. In the open air, sunlight and the presence of extraneous factors contribute to the inactivation
of virus. Thus *Escherichia coli* is inactivated faster in the open air than in a closed drum (May, Druett & Packman, 1969).

The strain of FMD virus used was very stable in aerosols at above 55% RH. Virus infectivity was not depressed significantly by the initial stress of spraying. The decay rate of 0.063 log./hr at 60% RH was very little higher than the solution inactivation rate of 0.03 log./hr at the same temperature. At lower relative humidities, most of the virus infectivity was lost during the process of spraying and in the following 5 min. Thus, below 40% RH less than 0.01% of infectivity survived after 5 min. After this initial loss, the inactivation was much slower, but the virus was still inactivated faster at lower relative humidities.

The aerosol stabilities of other strains of FMD virus suspended in salivary fluid have been investigated (Donaldson, 1972). All the strains, including O1 BFS 1860, were considerably more unstable than virus suspended in tissue culture fluid. It is likely that this is due to the high pH (8.9 to 9.1) of the salivary fluid; this is being investigated.

Poliovirus (Harper, 1961; de Jong & Winkler, 1968) and the encephalomyocarditis groups of viruses (Akers *et al.* 1966; Akers & Hatch, 1968) also show a rapid decrease in recoveries below 55 to 60% RH. However, survival for these viruses is better below 40% than between 40 and 60% R.H., whereas this strain of FMD virus shows the reverse behaviour. Poliovirus is considerably more resistant to spraying than FMD virus, whereas the recoveries with the encephalomyocarditis viruses appear to be similar to those obtained with FMD virus, except at low relative humidities. However, many of these differences may be due to differences in composition of the spray fluids.

In aerosols at relative humidities below 55%, virus which had been precipitated by ammonium sulphate or methanol was considerably more unstable than untreated virus. This was not due to residual precipitant as the ammonium sulphate and methanol were removed almost completely by dialysis and both untreated and polyethylene glycol precipitated virus were unaffected by concentrations of ammonium sulphate higher than those remaining after dialysis.

It has been shown that suspending medium and protein concentration can influence considerably the stability of airborne viruses (Harper, 1963; Benbough, 1969, 1971). It is important therefore to show that the instability of the chemically precipitated virus was not related to differences in the spraying suspensions. Virus which had been pelleted by centrifuging at high speed and resuspended in the same medium as the chemically precipitated virus was as stable as untreated virus. Also, virus precipitated by ammonium sulphate and resuspended in medium harvested from infected cells behaved similarly to virus resuspended in buffer solution. Instability was not due to differences in protein concentration.

Virus precipitated by polyethylene glycol or centrifuging at high speed was not markedly more unstable than untreated virus. Thus the ammonium sulphate and methanol precipitations appear to induce some chemical change in the virus particles. Although this change was apparent after spraying, the ammonium sulphate precipitated virus was not more susceptible than untreated virus to heat inactivation in solution. Ammonium sulphate and methanol both act by competing with protein molecules for the water available for solvation. At high concentrations of the precipitant, insufficient water molecules are available for full solvation of the protein. Protein–protein interactions then become more important and precipitation takes place as a dehydration process. Polyethylene glycol precipitates by the formation of an aqueous two-phase system (Albertsson, 1960) and the process does not involve dehydration of the virus particle. Webb, Bather & Hodges (1963) have postulated that dehydration is an important factor in aerosol inactivation and the dehydration involved
in the precipitation by ammonium sulphate and methanol may account for the greater instability of the virus in aerosols.

Ammonium sulphate and methanol precipitation are regularly used for concentration of FMD virus with complete recovery of infectivity. However, the concentration procedure modifies the virus particle in some way so that its infectivity is more susceptible to inactivation in aerosols.

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REFERENCES


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