Reaction of Glutaraldehyde with Foot-and-Mouth Disease Virus

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SUMMARY

Treatment of foot-and-mouth disease virus with 4% glutaraldehyde increases the diam. of the particles by 25% and makes them permeable to phosphotungstic acid so that they appear empty. The treated particles also resemble naturally-occurring empty particles in their low sedimentation coefficient (about 75S) but, in contrast to empty particles, they have a normal content of RNA and a higher than normal buoyant density in caesium chloride. The RNA can be removed from fixed particles by ribonuclease. Two models are suggested which account for these alterations in the structure of the virus particles. These results show that fixation with glutaraldehyde, far from maintaining the structural integrity of the virus particles, leads to considerable alterations in the arrangement of the RNA and protein subunits.

Foot-and-mouth disease virus is an acid-labile picornavirus. Below pH 7 the 140S particles are disrupted into virus RNA, a 125S protein subunit (comprising the three larger polypeptides of the virus VP1, VP2 and VP3) and an aggregate of the fourth protein VP4 (Burroughs et al. 1971). In the production of inactivated vaccines from this virus it is important to maintain the integrity of the 140S particles because the mixture obtained by disruption stimulates the production of only low levels of neutralizing antibody and induces little protection against challenge (Brown & Crick, 1959; Brown & Newman, 1963). Since glutaraldehyde is widely used for the fixation of biological materials prior to their examination in the electron microscope, and has also been used for preserving the integrity of ribonucleoprotein particles from poliovirus-infected cells (Baltimore & Huang, 1968), we investigated the action of the compound on foot-and-mouth disease virus in the expectation that it would be of value in the preparation of inactivated vaccines.

Foot-and-mouth disease virus (serotype O) which had been purified by the method described by Brown & Cartwright (1963) was reacted at 20 °C with concentrations of glutaraldehyde ranging from 0.0025 to 4%. The reaction was terminated at intervals by adding a twofold excess of sodium metabisulphite. Only low concentrations of glutaraldehyde gave satisfactory vaccines, but at these low levels it was often difficult to ensure that all infectivity was inactivated. In view of these observations, the use of glutaraldehyde for the preparation of vaccines was not pursued. However, we observed that virus particles which had been fixed with 4% glutaraldehyde appeared 'empty' and were rather larger, with a less distinct outline, than the unfixed particles (Fig. 1). These observations led us to examine the reaction in more detail.

For this investigation, virus labelled with [3H]-uridine and [14C]-amino acids (Rowlands, Sangar & Brown, 1971) was allowed to react with 4% glutaraldehyde in 0.04 M-phosphate, pH 7.6, for 1.5 h at 20 °C. The effect on the rate of sedimentation of the virus is shown in Fig. 2. Whereas untreated virus gave a peak at fraction 8, the fixed particles sedimented only to fraction 16. Accepting a value of 146S (Strohmaier, 1971) for the sedimentation coefficient of the virus is shown in Fig. 2. Whereas untreated virus gave a peak at fraction 8, the fixed particles sedimented only to fraction 16. Accepting a value of 146S (Strohmaier, 1971) for the sedimentation coefficient of the virus, the fixed virus particles had a sedimentation coefficient of about 75S. Natural empty particles in virus harvests also have a sedimentation coefficient of about 75S,
Fig. 1. Electron micrographs of foot-and-mouth disease virus (serotype O) after treating for 1.5 h with 4% glutaraldehyde and staining with 3% phosphotungstic acid (K salt, pH 7.0). (a) Untreated virus; (b) glutaraldehyde-fixed virus; (c) glutaraldehyde-fixed virus treated with 0.1 μg/ml ribonuclease.

Table 1. Effect of glutaraldehyde on the diam. of foot-and-mouth disease virus

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of particles measured</th>
<th>Mean estimated diam. (nm)</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated virus</td>
<td>99</td>
<td>28.0*</td>
<td>0.92</td>
</tr>
<tr>
<td>Glutaraldehyde-treated virus</td>
<td>100</td>
<td>31.9†</td>
<td>1.69</td>
</tr>
<tr>
<td>Glutaraldehyde-treated virus plus ribonuclease</td>
<td>101</td>
<td>29.7†</td>
<td>1.04</td>
</tr>
</tbody>
</table>

* Diam. estimated by comparison with crystal periodicity of catalase (C. J. Smale, unpublished data).
† Measurements were made only on intact particles with a roughly spherical outline (shown by arrows in Fig. 1). The measurements were made directly on the photographic plates with a travelling microscope. No allowance was made for any errors due to flattening. Photographs of the three specimens were taken in sequence and the sequence repeated six times without altering the microscope settings, with the exception of focus.

but these do not contain RNA (Cowan, Graves & Trautman, 1968; D. J. Rowlands & D. V. Sangar, unpublished observations). In contrast, the ratio of [³H] to [¹⁴C] in the fractions from the two gradients (Fig. 2a, b) showed that the particles fixed with glutaraldehyde contained the same proportion of RNA as the untreated virus.

In view of their altered appearance and sedimentation behaviour, the effect of ribonuclease on the fixed particles was examined. The virus was treated with 4% glutaraldehyde as described above and excess reagent was removed by filtration through Sephadex G-100.
The fixed particles were then treated with 0.1 μg ribonuclease/ml for 15 min at 37 °C before centrifuging in a 15 to 45% sucrose gradient. The distribution of radioactivity in the gradient showed that the rate of sedimentation of the particles was unaltered but that all the RNA was lost (Fig. 2c).

The fixed virus particles had a higher than normal buoyant density in caesium chloride.
Virus labelled with [3H]-uridine and [14C]-amino acids was centrifuged in preformed caesium chloride gradients for 6 h at 24,000 rev/min. The untreated virus equilibrated at a density of 1.43 g/ml, whereas the fixed virus particles had a density of 1.46 g/ml (Fig. 3a, b), suggesting that the RNA in the fixed particles was more accessible to caesium ions. After treatment with ribonuclease the fixed particles, which did not contain any RNA, equilibrated at 1.33 g/ml (Fig. 3c).
It was mentioned above that fixed virus particles had a greater diam. than untreated virus (Fig. 1). Subsequent treatment of fixed particles with ribonuclease reduced their diam. and sharpened their outline, although the enzyme-treated particles still had a diam. slightly greater than that of unfixed virus (Fig. 1c). Using catalase crystals as standard (Wrigley, 1968), the mean diam. obtained were 28 nm for unfixed virus, 32 nm for fixed particles and 30 nm for the enzyme-treated fixed particles (Table 1). The effect of fixation and subsequent treatment with ribonuclease on the size of the virus particles was also demonstrated by filtration through Sepharose 2B. Virus labelled with $^{14}$C-amino acids and fixed with 4% glutaraldehyde was freed from the aldehyde by filtration through Sephadex G-100 and then mixed with untreated $^3$H-amino acid-labelled virus.
before passing through a 30 x 1 cm column of Sepharose 2B in 0.04 M-phosphate-0.1 % SDS, pH 7.6. The fixed virus particles were eluted well ahead of the unfixed particles (Fig. 4a). After treatment with ribonuclease, the fixed particles eluted just ahead of unfixed particles (Fig. 4b). These observations confirmed that the fixed particles had a greater diam. than unfixed virus and that the diam. of the fixed particles was reduced by treatment with ribonuclease.

A model which is compatible with these observations is shown in Fig. 5a. In the intact virus, the RNA is inaccessible to ribonuclease. After fixation with glutaraldehyde the particles have a greater diam. (32 nm compared with 28 nm for unfixed virus, Fig. 1) and a smaller sedimentation coefficient and are eluted earlier from Sepharose 2B columns. The
RNA in the fixed particles is also more accessible to caesium ions, resulting in a higher buoyant density. Treatment with ribonuclease removes the RNA completely from the particles. This treatment also reduces the diam. slightly as shown by electron microscopy (Fig. 1c) and by its slower filtration rate through Sepharose 2B compared with fixed particles. The sedimentation rate is unaltered by ribonuclease treatment (Fig. 2c), presumably because the decrease in weight following the loss of the RNA is offset by the slight decrease in diam.

However, the outline of the fixed particles was less ragged after ribonuclease treatment. This observation led us to consider a second model in which part of the RNA is located outside the capsid after fixation (Fig. 5b). This model accounts for the ragged appearance of the fixed particles in the electron microscope (Fig. 1b) and also explains why they filter more slowly through Sepharose 2B columns after ribonuclease treatment. The lack of effect of ribonuclease on the sedimentation rate of the fixed particles may be explained if the decrease in frictional resistance, which occurs when the external RNA is removed, is exactly offset by the decrease in weight of the particles.

It should be possible to decide between these models by using insolubilized ribonuclease. This would not hydrolyse the RNA of the fixed particles in the first model but would do so in the second model. However, this approach was fruitless because free enzyme eluted continually from the carboxymethylcellulose-enzyme matrix, despite repeated washings with buffer solution prior to its use.

The observations described in this paper, which have also been made with other serotypes of the virus, show that glutaraldehyde changes both the appearance and physical properties of foot-and-mouth disease virus. This is in contrast to its effect on poliovirus, where there is no change in density or sedimentation coefficient after a short exposure to 4% glutaraldehyde (Baltimore & Huang, 1968). We have confirmed Baltimore & Huang’s observations with poliovirus and have found in addition that no phosphotungstic acid penetrable particles are produced. However, Wouters, Miller & Fenwick (1973) have shown that prolonged exposure of poliovirus to formaldehyde or glutaraldehyde increases the buoyant density of the virus and its permeability to phosphotungstic acid. Our observations and those of Wouters et al. (1973) show that caution must be exercised in the use of glutaraldehyde fixation in studies of the structures of biological particles.

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**REFERENCES**


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