The Action of Protease on Influenza A2 Virus

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(Accepted 11 July 1967)

SUMMARY

The main effects of the proteolytic digestion of the Asian (A2) strain of influenza virus were a loss of the characteristic surface morphology and a change in density. The untreated virus was found to have a density of 1.204 g./cm.³ on a sucrose gradient, but after protease action the density was 1.168 g./cm.³. The density change was correlated with a change in particle mass as indicated by a fall in sedimentation coefficient (Sₘ₀) from the normal 788 Svedbergs to 562 Svedbergs.

From the data obtained with the protease-treated and control virus preparations it was calculated that the changes observed could be accounted for by a loss of 35 to 40% of the particle mass. The action of the protease appeared to be confined to the surface of the virus. Concomitantly with the destruction of the haemagglutinating activity there was a release of neuraminidase activity, and on a density gradient this could be separated from the residual virus and was located at the top of the gradient tube. In addition to the changes described, there appeared to be a destruction of the virus V antigen.

INTRODUCTION

Various authors have shown that certain constituents can be released from influenza viruses by proteolytic enzymes. Thus, Mayron et al. (1961) treated an A2 strain with trypsin and showed an apparent separation of the neuraminidase and haemagglutination activities. Trypsin, however, appeared to be irregular in its action, and Cleeland & Sugg (1963) suggested that the reason for this irregularity was the presence of sodium ions and that the removal of these appeared to be essential for optimal trypsic activity. Using radioactive-isotope labelling, Noll, Aoyagi & Orlando (1962) showed that trypsin caused a very slight decrease in density of A2 influenza virus. More recently other proteases have been investigated, for example Reginster (1965) allowed caseinase C (from streptomyces) to act on the PR8 (type A) strain of influenza virus and was able to recover the neuraminidase activity unchanged, although the haemagglutination activity and infectivity were greatly reduced. Seto, Drzeniek & Rott (1966) were able to remove neuraminidase from influenza virus with pronase.

In general, the work reported above was more concerned with the release products of proteolysis and little attention was paid to the residual virus. The present work was undertaken to try to identify the released products of virus digestion, and in particular to study the properties of the virus particle after certain protein constituents had been removed from it.
METHODS

Virus preparations. The A2/SINGAPORE/1/57/W variant of the classical A2 strain was used exclusively. Crude allantoic fluids were obtained by inoculating allantoically in 10- or 11-day-old chick embryo virus seeds stored at -60°. Seed virus was diluted 10⁻³ or 10⁻⁴ in nutrient broth containing penicillin 2500 units/ml. and streptomycin 3000 μg./ml., and 0.1 ml. inoculated into each egg. After 48 to 72 hr the eggs were chilled and the infected allantoic fluids harvested, pooled and stored at 4° until required. Crude allantoic fluid virus was purified by calcium phosphate chromatography of centrifuge-concentrated allantoic fluid. The calcium phosphate fraction was further concentrated by high-speed centrifugation and resuspended in tris+HCl buffer.

Haemagglutination tests were done by the ‘pattern’ method in WHO plastic trays (WHO, 1959). Serial twofold dilutions of virus were made in 0.5 ml. volumes of Palitzch's borate buffer saline pH 7.6, and equal volumes of 0.5% fowl red cell suspensions were added. Readings were made after 1 hr and end points determined by a standard method of interpolation.

Density gradient centrifugation. Gradients were preformed in 5 ml. ‘lusteroid’ tubes. Most of the experiments were made using sucrose as the gradient medium. One ml. quantities of 60, 48, 30 and 24% sucrose in borate-buffered saline and 0.5 ml. of 12% sucrose in borate buffer pH 7.6 were layered on top of one another. After the gradients had formed by diffusion at 4° overnight, 0.5 ml. of the sample was layered on top and the gradients run in the Spinco Model L preparative ultracentrifuge at 39,000 rev./min. in the SW39 swing-out rotor for 5 hr. At the end of the run the tubes were punctured at the bottom and usually 12 to 14 5-drop fractions were collected. For reasons to be discussed later, some experiments were in gradients of sodium glutamate (50 to 10%).

Virus purification and concentration. Three hundred ml. of infected allantoic fluid were centrifuged in the Spinco Model L preparative ultracentrifuge at 25,000 rev./min. for 1 hr at 54,000 g (no. 30 rotor). The supernatant fluid was removed and the virus pellet taken up in 20 ml. of 0.01 M-phosphate buffer, pH 7. The centrifuged concentrate was then chromatographed on freshly prepared calcium phosphate columns (Pepper, 1967). After adsorption of the virus by running in the crude allantoic fluid the column was washed with 0.2 M-phosphate buffer pH 7 and the virus eluted with 0.8 M-phosphate buffer at pH 7. The effluent was collected in 5 ml. fractions and those fractions containing virus were pooled and concentrated by centrifuging in the Spinco Model L for 1 hr at 25,000 rev./min. (no. 30 rotor). The virus pellet was taken up in 4 or 5 ml. of 0.1 M-tris+HCl buffer, pH 8 to give a final titre of 10,000 to 20,000 haemagglutinating (HA) units per ml. Overall recoveries by this method were generally from 60 to 80%, although this was critically dependent upon the properties of the calcium phosphate batch used (Pepper, 1967).

Refractometry. The refractive indices of fractions from the sucrose gradients were measured in an Abbe refractometer using the sodium D line at a wavelength of 589 mμ. Values for refractive indices were converted to density by use of the International Critical Tables (1927).

Analytical ultracentrifugation. Sedimentation coefficients were calculated from the data of runs in an MSE analytical ultracentrifuge equipped with the schlieren optical system. The sedimentation coefficient was extrapolated to zero concentration from
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values calculated for different concentrations. Further details are given later in the experimental section.

Complement fixation tests were performed on WHO plastic trays according to the method of Bradstreet & Taylor (1962). Dilutions were made in veronal saline buffer and commercial guinea-pig complement (Burroughs Wellcome and Co.) was used. The V antisera were prepared by immunizing rabbits with concentrated red cell eluates of the A2/SINGAPORE variant described above. Before using them, the sera were absorbed with suspensions of chorioallantoic membrane to remove antibodies against the host component of the virus. Human convalescent sera were used as S-antigen antisera.

Neuraminidase assay. The neuraminidase activity was measured by incubating the samples with a soluble substrate and assaying the amount of free sialic acid released after incubation at 37°. The substrate was a mucoprotein (collocalia mucoid) prepared from edible bird’s nest by repeated extraction with water at 62° (Howe, Lee & Rose, 1960; Biddle & Belyavin, 1963). Previous washing with cold distilled water was necessary to remove any free sialic acid. The final product containing about 10% of sialic acid (N-acetyl neuraminic acid) was freeze-dried and readily soluble in water. For the neuraminidase estimation 0.05 ml. of the sample was mixed with 0.1 ml. of 0.1% aqueous solution of collocalia mucoid and held at 37° for 1 hr, when the released sialic acid was determined by the Warren procedure (1959) using the extraction method of Aminoff (1961).

Electron microscopy. Materials were dialysed against 0.15 M-ammonium acetate solutions and then examined in a Philips EM 200 electron microscope using the negative-staining technique (Brenner & Horne, 1959). The negative staining material was 2% phosphotungstic acid.

RESULTS

Effects of protease on haemagglutination activity

The protease used in these experiments was a highly purified preparation from Bacillus subtilis (kindly donated by Seravac Laboratories, Maidenhead, Berkshire). The final experimental conditions chosen were to incubate 0.5 ml. of virus concentrate containing 10,000 to 20,000 HA units per ml. with 2 mg. of enzyme at 37°. The virus was suspended in 0.2 M-tris+HCl buffer, pH 8. For optimum proteolytic activity it was important to remove all traces of phosphate buffer from the medium, and before digestion with the protease the virus concentrate was dialysed against 0.1 M-tris+HCl buffer, pH 7. After 5 hr protease treatment at 37°, about 66% of HA activity was destroyed and after a total of 18 hr incubation 95 to 98% of the HA activity was removed consistently (Fig. 1).

Gradient centrifugation. Half ml. quantities of A2 virus concentrate treated with Bacillus subtilis protease for 18 hr and 0.5 ml. of untreated control virus were layered on to sucrose gradients. After centrifugation at 39,000 rev./min. for 5 hr the tubes were examined by scattered light and in 22 experiments it was found that the treated virus had banded at a higher position in the gradient than the untreated control (Pl. 1, fig. 1). This suggested that proteolytic digestion left a residue of virus material which was less dense or had a considerably reduced sedimentation coefficient. For a particle the size of influenza virus a period of 5 hr centrifugation in the gradient described should be sufficient for the virus to reach its isopycnic point. In fact, even
after 2 hr centrifugation the control and treated virus preparations occupied the same position as they did after 5 hr centrifugation. This suggested that the difference in banding observed was more likely due to a change in density than to a change in sedimentation coefficient, although both factors may have operated. In each gradient run a third gradient tube was included without a sample overlay, buffer being used instead.

Immediately at the end of the run a thermometer was inserted into this gradient and the temperature recorded. When the refractive index of the fractions was measured the corresponding densities were corrected for the difference in temperature between that at which the refractive index measurements were made and that of the gradients. Altogether 22 experiments of this type were performed and in 23 of these there was some slowly sedimenting haemagglutinin at the top of the control gradient, due presumably to spontaneous breakdown of the virus. In addition a major peak of HA activity was located at the lower part of the gradient in a position corresponding to the band observed by light scattering (Fig. 2). Usually no HA activity was demonstrated with the protease-treated virus on the gradient although occasionally some activity was detected in a position corresponding to the centre of the light scattering band.
Release of neuraminidase activity

Preliminary experiments showed that the amount of neuraminidase activity was undiminished after protease treatment; indeed, activity was increased up to 120%. The treated and control preparations were then run on density gradients. Material from sucrose gradients could not be used directly in the assay system for N-acetyl neuraminic acid since the sucrose interfered strongly with the test. At first, each sample from a gradient was dialysed before assay. Later, it was found that sodium glutamate could be used to form a satisfactory gradient and did not interfere with the assay. Gradients comparable to sucrose gradients could be prepared by overlayering aqueous solutions of sodium glutamate from 50% (w/v) to 10% (w/v).

![Graph showing distribution of haemagglutinating activity](image)

Fig. 2. Distribution of haemagglutinating activity of protease-treated (● - - - ●) and virus control (○ - - - ○) preparations on a sucrose density gradient. Density, ●—●.

After protease treatment the neuraminidase was released from the virus and on a sodium glutamate gradient occupied a position at the top of the tube. In the experiment shown (Fig. 3) some residual neuraminidase was left at a position corresponding to the light-scattering band and this is shown here to illustrate the difference between the treated and untreated preparations. However, all of the neuraminidase was usually released by protease and was found at the top of the gradient tube.

Refractive index measurements and densities

The densities of the control and treated preparations differed significantly. In three experiments the mean density of the treated preparations was 1.167 g./cm.³ (individual values 1.168, 1.167 and 1.167 g./cm.³) and of the control virus 1.204 g./cm.³ (individual values 1.208, 1.205 and 1.199 g./cm.³). The results are given for both the peak fraction of the haemagglutinating activity and the fraction corresponding to the centre of the light-scattering band.
Complement-fixation tests and antigenic structure

Each of the fractions from the density gradient experiments was tested by complement fixation using the appropriate controls. S antigen was never detected, suggesting that only the surface of the virus was attacked or that any released S antigen was destroyed by the protease. Again, after protease treatment no V antigen was present, although occasionally a small amount of activity was detected in samples with detectable residual haemagglutinin. On the other hand, in the virus control preparations there was marked complement-fixing activity against V antisera and this corresponded in distribution to the haemagglutinin. There was always anticomplementary activity in the top two fractions of the protease-treated virus. On eight occasions the gradient fractions were tested by double diffusion in gel using rabbit antiserum shown by the haemagglutination-inhibition and complement-fixation tests to contain a high concentration of V antibody against A2 virus. No precipitation lines were obtained in any test, suggesting that no small antigenic component had been released and that complete destruction had occurred. Since the fractions from the top of the gradient tube in which the protease-treated virus had been run were anticomplementary, low-molecular-weight antigenic material could not have been detected even if it

![Graph](image-url)
had been released. The negative findings in the gel-diffusion tests suggest that such material was not released.

Analytical ultracentrifugation

The observed change in density may have resulted from a change in the composition of the virus after proteolytic digestion with a corresponding change in particle mass. Ideally, the best way to determine this would be to calculate the macromolecular weights of the treated and untreated preparations. This is very difficult but since the sedimentation coefficient of a particle is a function of the molecular weight, a determination of the sedimentation coefficients seemed to be a satisfactory way of assessing any possible change in the mass of the virus particle.

Table 1. Sedimentation data for protease-treated (5 mg./ml. protease for 18 hr) and control preparations of influenza A2 virus

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Virus</th>
<th>S10 (Svedbergs) at relative concentrations</th>
<th>S20 (Svedbergs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>1</td>
<td>Treated</td>
<td>451</td>
<td>463</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>759</td>
<td>758</td>
</tr>
<tr>
<td>2</td>
<td>Treated</td>
<td>387</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>715</td>
<td>---</td>
</tr>
<tr>
<td>3</td>
<td>Treated</td>
<td>494</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>761</td>
<td>---</td>
</tr>
</tbody>
</table>

Quantities of virus with an initial HA titre of 20,000 to 40,000 units/ml. were treated with protease at a concentration of 5 mg./ml. Control untreated preparations of virus were held in parallel. After incubation the materials were run in the analytical ultracentrifuge without further treatment. In these initial experiments dilutions of the preparations were made in 0.1 M-tris + HCl buffer, pH 8. Three concentrations of each control and treated preparation were analysed, thus allowing the sedimentation coefficient to be extrapolated to zero concentration. In two experiments the values obtained for the extrapolated sedimentation coefficient (S20) of the protease-treated A2 virus were 505 S and 490 S. The corresponding values for the virus control were 758 S and 744 S. In the next stage protease was removed since its presence may have affected the determination of the virus sedimentation coefficient. The digested preparations were centrifuged at 40,000 rev./min. (no. 40 rotor) in the Spinco Model L centrifuge for 1 hr. The supernatant fluids were removed (during the period of centrifugation used the protease was unlikely to have sedimented) and the pellets taken up in 0.15 M-NaCl. Again, different concentrations were run in the analytical ultracentrifuge using 0.15 M-NaCl as diluent. Four concentrations were obtained for each preparation. The extrapolated sedimentation coefficient for the treated preparation was 562 S and that of the corresponding virus control 788 S (Table 1). Sedimentation diagrams for the two preparations are shown in Pl. 1, fig. 2. Experiments 1 and 2 are those in which the protease was not removed and Expt. 3 the experiment in which protease was removed before the estimation of S20.
Electron microscopy

In the electron microscope (Pl. 2, fig. 3) the treated virus was seen to have undergone a change in the characteristic surface pattern. In some preparations residual surface structure was present (arrows).

DISCUSSION

The findings reported here confirm those of Mayron et al. (1961) and Seto et al. (1966) that neuraminidase is easily released from influenza virus by proteolysis. However, it is strange that the released neuraminidase is not itself digested by the proteolytic enzymes since surface antigenic configurations appear to be destroyed. The neuraminidase appears to be protected, although the link between this and the virus surface must be easily accessible. The isolation and characterization of the released neuraminidase is under investigation in this laboratory.

Protease-treated virus either loses some surface constituents or the whole virus particle could have been broken down. The evidence presented here of the presence of a distinct gradient band of virus-like particles suggests that only part of the virus has been released.

The change in density after proteolytic digestion may be explained in the following way. Influenza virus contains about 24% lipid, 6% carbohydrate, 69% protein and 1% RNA (Ada & Perry, 1954; Frommhagen, Knight & Freeman, 1959). Taking the values for the individual partial specific volumes to be 1.0 for lipid, 0.61 for carbohydrate, 0.55 for RNA and 0.734 for protein (values as cm.³/g.), the partial specific volume of the unhydrated virus can be calculated to be 1.27 cm.³/g. However, in solution the virus is hydrated and hence, although the amount of associated water is difficult to measure, less dense than the non-hydrated form. Assuming a hydration of 0.25 g. water per g. of virus the density of the hydrated virus would be 1.203 g./cm.³, a value in agreement with that found in the density gradient experiments. Although absolute equilibrium is not attained in a sucrose gradient, a large particle like influenza virus should approach very closely to its isopycnic point.

It is reasonable to assume that the change in density observed after treatment with Bacillus subtilis protease is principally the result of protein loss and therefore a relative increase in the amount of lipid and carbohydrate present. It can be calculated that the composition of treated virus to give the observed density of 1.167 g./cm.³ might be lipid 37.5%, protein 51.5%, carbohydrate 9.4% and RNA 1.6%. The change in mass required to give these final figures would be about 36% on the assumption that no change in hydration occurs. Since for otherwise similar molecules the molecular weights are proportional to the 3/2 power of the sedimentation coefficient, it follows that the ratio of the molecular weights of the protease-treated and control virus is about 0.6. Thus on the basis of changes in molecular size about 40% of the virus has been destroyed by protease. This figure is in reasonably good agreement with the figure obtained from the change in density. It seems therefore that the changes observed can be interpreted as a loss of about 40% of the mass of the virus particle and that the only material lost is protein. The electron microscope findings are consistent with a change occurring only at the virus surface with no apparent effect on the internal component. The surface projections are probably predominantly protein...
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structures embedded in a lipid membrane which is left behind after proteolytic
digestion.

Hitherto attempts in this laboratory to attack the influenza viruses by means of
lipolytic enzymes have been unsuccessful but this might have been due to the protection
of the lipid by the overlying protein. An attempt will now be made to release the internal
components from virus particles already digested with protease.

Thanks are due to Dr J. P. Stevenson for the electron microscopy, to the Science
Research Council for the purchase of the MSE analytical ultracentrifuge and to the
National Fund for Research into Poliomyelitis for financial assistance.

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(Received 20 April 1967)
EXPLANATION OF PLATES

PLATE I

Fig. 1. Position of banded materials on a sucrose density gradient; on left is protease-treated material, band devoid of HA activity—on right is virus control, peak of HA activity localized in this band.

Fig. 2. (Top) Sedimentation pattern of A2 influenza virus treated with protease. Speed 16,390 rev./min., interval between pictures, 2 min., schlieren angle varying between 50° and 70°. (Bottom) Corresponding virus control. Speed 12,370 rev./min., interval between pictures 2 min., schlieren angle 50° to 70°. Sedimentation to left in both cases.

PLATE 2

Fig. 3. Electron micrographs of protease-treated influenza A2 virus (a, b) and untreated control (c). Arrows: residual surface projections.
Fig. 1

$\bar{S}_{20} = 556$

Fig. 2

$\bar{S}_{20} = 788$

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(Facing p. 28)
Fig. 3

a  b  c

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