A High Density Component in Several Vertebrate Enteroviruses

By D. J. ROWLANDS, M. W. SHIRLEY, D. V. SANGAR AND F. BROWN

Animal Virus Research Institute, Pirbright, Surrey GU24 0NF

(Accepted 17 July 1975)

SUMMARY

In addition to the major infective component, which bands at a density of 1.34 g/ml in caesium chloride ('light component'), a component with a density of 1.44 g/ml ('heavy component') has been found in harvests of poliovirus (type 1), Coxsackie B5 virus, a bovine enterovirus (VG-5-27) and swine vesicular disease virus (SVDV). With SVDV about 98% of the infectivity equilibrated at 1.34 g/ml but approx. 2% was present as a peak at 1.44 g/ml. The morphology of the two forms was similar but the heavy component had a smaller diameter (28 nm) than the light component (30 nm). No inter-conversion of the two forms was observed on re-cycling in fresh caesium chloride gradients and the two components had the same proportions of RNA and protein and the same polypeptide composition. Each component gave a similar proportion of the light and heavy forms on replication, but the light component had a specific infectivity about fourfold higher than that of the heavy component and was also much more efficient in eliciting the formation of neutralizing antibodies in guinea pigs. Although these results suggest that the two particles are alternative stable configurations of the virus, iodination failed to reveal any differences in the extent or pattern of labelling of the polypeptides in the two forms.

INTRODUCTION

Buoyant density in caesium chloride has been used as one of the criteria for the classification of the picornaviruses (Newman, Rowlands & Brown, 1973). The range of densities within the family extends from 1.34 g/ml for the enteroviruses to 1.45 g/ml for equine rhinovirus but the range of values for different members within each group is narrow and characteristic of that group. Although the buoyant density of any member of the family is highly reproducible under standard conditions, minor alterations can be induced by varying certain experimental parameters such as the length of time of centrifuging (Rowlands, Sangar & Brown, 1971) or the pH of the caesium chloride (Wood, 1971; Rowlands et al. 1971).

In this paper we report the presence in several enteroviruses of a minor component with a much higher density than that of most of the virus. The properties of the two forms show that the high density of the minor component is not due to contamination by cellular material but is probably caused by a difference in the configuration of the capsid proteins.
METHODS

Viruses. Swine vesicular disease virus (SVDV It 1/66, Hong Kong/71 and England 27/72 isolates) was grown in monolayers of IB-RS-2 pig kidney cells, poliovirus (type 1, Mahoney strain) and Coxsackie B5 (Faulkner strain) in HeLa cell monolayers, and a bovine enterovirus (VG-5-27; McFerran, 1962) in BHK 21 cell monolayers, using Eagle's medium for each cell type. For the preparation of viruses labelled with [3H]-uridine, Eagle's medium containing 4 µc/ml of isotope was used. Viruses labelled with [14C]- or [3H]-amino acids were grown in Earle's saline containing 1 or 4 µc/ml of [14C]-protein hydrolysate or [3H]-amino acids. Viruses labelled with [3H]- or [35S]-methionine were grown in methionine-free Eagle's medium containing 4 µc isotope/ml. [82P]-virus was grown in phosphate-free Earle's saline containing 50 µc carrier-free [32P]-phosphate/ml. All the viruses were purified by the method described by Brown & Cartwright (1963) for foot-and-mouth disease virus, except that SDS replaced sodium deoxycholate in the final purification step.

Virus infectivity. The only assays were made with SVDV (It 1/66) in IB-RS-2 monolayers, using the plaque method.

Antisera. These were prepared by inoculation of virus fractions which had been inactivated at 37 °C for 6 h with 0.05 % acetylthyleneimine or at 20 °C for 72 h with 0.05 % formaldehyde. Guinea pigs were inoculated subcutaneously with mixtures of the inactivated antigens and aluminium hydroxide gel and serum was collected 21 days later.

Neutralizing activity of antisera. This was measured by mixing serial fivefold dilutions of serum with 50 p.f.u. of virus and estimating the amount of residual infectivity by plaque assay on IB-RS-2 monolayers.

Buoyant density determinations. Virus samples were layered on to 5 ml linear gradients of caesium chloride ranging from 1.3 to 1.5 g/ml and centrifuged for 6 h at 24,000 rev/min in the six-bucket rotor, No. 59108, in an MSE 65 ultracentrifuge. The gradients were fractionated by puncturing the bottom of the tube and collecting 0.3 ml volumes. The density of the fractions was estimated from refractive index measurements.

Sucrose gradient sedimentation. Linear 15 to 45 % (w/v) gradients in 0.04 M-phosphate, pH 7.6, were used for centrifuging virus samples. For the analysis of RNA, 5 to 25 % (w/v) gradients in 0.1 M-acetate/0.1 % SDS, pH 5.0, were used and the samples centrifuged for 16 h at 18,000 rev/min in the 3 × 23 ml rotor, No. 59590, of the MSE ultracentrifuge.

Extraction of RNA. Virus samples in caesium chloride were diluted tenfold with distilled water and extracted with water-saturated phenol. BHK 21 cell RNA was added as carrier to the aqueous phase and the mixture of virus and cell RNA precipitated with 2 vol. ethanol at −20 °C overnight. The RNA precipitate was collected and dissolved in 0.1 M-acetate/0.1 % SDS for analysis.

SDS-polyacrylamide gel electrophoresis of virus polypeptides. The method used has been described in full previously (Talbot et al. 1973).

Iodination of virus. The virus was iodinated using chloramine-T to oxidize the [125I]-Na. The method is given in Talbot et al. (1973).

Electron microscopy. Each sample was mixed with an equal vol. of 3 % phosphotungstic acid, applied to carbon-Formvar grids and examined in a Siemens Elmiskop I. For the comparison of the diameter of the heavy and light components, catalase crystals were included as an internal standard (Wrigley, 1968).
A high density component of enteroviruses

**RESULTS**

*Presence of a high-density component in several enteroviruses*

In experiments to characterize the virus causing swine vesicular disease, the virus isolated in Hong Kong in 1971 (Mowat, Darbyshire & Huntley, 1972) was grown in IB-RS-2 cell monolayers and a portion of the harvested fluid centrifuged in a 5 ml preformed caesium chloride gradient (1.3 to 1.5 g/ml) for 6 h at 24,000 rev/min, using the 59108 rotor of the MSE 65 ultracentrifuge. Fractions (0.3 ml) collected from the tube were titrated in IB-RS-2 cells by the plaque method. Although most of the infectivity was located as a sharp band at a density of 1.34 g/ml, a small but significant amount of infectivity was also found at 1.44 g/ml (Fig. 1a). The same distribution of infectivity was also found when virus grown in the presence of [35S]-methionine or [14C]-amino acid mixture was first purified by treating...
with 1% SDS and centrifuging in a sucrose gradient prior to centrifuging to equilibrium in a caesium chloride gradient (Fig. 1 b).

The occurrence of a heavy component was not confined to virus grown in 1B-RS-2 cells but was also found in extracts of epithelium removed from a pig infected with the virus. This demonstrated that the presence of the heavy component was not a peculiarity of virus grown in tissue culture cells.

A heavy infective component was also found in the serologically distinct Italian (It 1/66) and English (UK 27/72) isolates of the same virus and in a bovine enterovirus (VG-5-27; McFerran, 1962). A heavy component with the same density was found in poliovirus (type 1, Mahoney strain) and Coxsackie B5 virus (Faulkner strain), but the infectivity was not tested (Fig. 2). The proportion of radioactivity in the heavy component of Coxsackie B5
A high density component of enteroviruses

Fig. 3. Re-cycling in caesium chloride gradients of previously separated heavy and light components of SVDV (It 1/66): (a) initial separation; (b) re-cycled heavy component; (c) re-cycled light component.

virus was rather higher than in the other viruses examined (Fig. 2). The work discussed below was done with the It 1/66 isolate of SVDV.

Properties of the heavy and light components

Buoyant density

Each component banded at its own individual density when re-cycled through a second caesium chloride gradient (Fig. 3), suggesting that the initial sedimentation step was separating two components which were present in the virus sample, rather than producing the heavy from the light component. If such a conversion of light to heavy component were occurring during the initial separation, a heavy band would be expected each time the light component was re-cycled in caesium chloride.

When the separated light and heavy components were centrifuged through sucrose gradients, all the light component sedimented at about 160 S (Fig. 4a). This component
banded at 1.34 g/ml in a subsequent cesium chloride gradient. However 10 to 50% of the heavy component sedimented at about 75S and the remainder at 160S (Fig. 4b). The portion of the heavy component that sedimented at 75S banded at a density of 1.34 g/ml when centrifuged in a second cesium chloride gradient, whereas the portion sedimenting at 160S re-cycled at 1.44 g/ml. Since direct re-cycling of the heavy component in a cesium chloride gradient without an intervening cycle in a sucrose gradient gave a single peak at 1.44 g/ml, it would seem that the conversion to a component sedimenting at 75S and banding at 1.34 g/ml occurred only when the particles were transferred from cesium chloride to sucrose.

The different densities of the two components could be due to configurational differences leading to greater interaction of the RNA of the heavy component with cesium ions. Unlike foot-and-mouth disease virus, which bands at 1.43 g/ml in cesium chloride at pH 7.6 and has an increased density at pH 9 or after prolonged centrifuging at pH 7.6, the heavy component of SVDV did not increase in density at the higher pH or after prolonged
A high density component of enteroviruses

centrifuging. However, considerable losses of the component occurred after prolonged runs, presumably owing to adsorption on to the walls of the tube.

Morphology

The separated heavy and light components were usually similar in their morphology when stained with phosphotungstic acid, although there was a rather higher proportion of penetrated particles (about 5 to 10%) in the heavy component preparations than in the light component, where the proportion did not exceed 5%. The heavy particles also had a smaller diam. than the light particles. Based on measurements of 160 heavy particles, the average diam. was 28.2 nm ± 0.76 whereas the average diam. of 157 light particles was 30.1 nm ± 0.74.

Specific infectivity

The specific infectivity of the two particles was compared by measuring the infectivity: radioactivity ratio in samples prepared from purified virus labelled with [14C]-amino acids. The ratio was about 4 times greater for the light component so that, provided the two particles were labelled to the same extent during their growth, the light component must be 4 times more efficient than the heavy component in initiating infection. The conversion of the heavy component to a particle sedimenting at 75S in sucrose, referred to above (Fig. 4), did not entirely account for its lower specific infectivity since that part of the heavy component which sedimented at 160S also had a fourfold lower specific infectivity than the 160S fraction of the light component. Despite this difference in specific infectivity, the RNA in the two particles contained the same information, since the virus produced by infection of IB-RS-2 cells with the separated and plaque-purified samples of the two contained the same proportions of heavy and light components.

Chemical composition of the two components

Sedimentation in a caesium chloride gradient of virus labelled with [3H]-uridine and [14C]-amino acids gave two peaks of radioactivity with similar ratios of the two isotopes (Fig. 5), indicating a similar RNA:protein ratio.

Polyacrylamide gel electrophoresis in SDS gels of the polypeptides of the two components gave similar patterns. One minor peak, mol. wt. 35 × 10^3, seen as a shoulder, and three major peaks, mol. wt. 30, 26 and 7 × 10^3, were obtained. If it is assumed that the peak with mol. wt. 26 × 10^3 contains two proteins (as appears likely from its radioactivity relative to the other peaks), these would correspond to VP1, VP2 + VP3 and VP4 respectively of other enteroviruses. This pattern for the Italian isolate of SVDV differs from that obtained by Delagneau, Bernard & Lenoir (1975) for the French isolate of SVDV but is similar to that obtained for many picornaviruses. Double label experiments in which heavy component labelled with [3H]-amino acids was mixed with light component labelled with [14C]-amino acids and the proteins compared in SDS-polyacrylamide gels showed that the mol. wt. and proportions of the polypeptides of the two components were similar (Fig. 6). The low counts in the heavy component preparation make it difficult to decide whether this component had the same or a reduced amount of the protein mol. wt. 35 × 10^3 compared with the light component.

The RNAs from the two components were also similar. A mixture of the RNAs extracted from [32P]-heavy and [3H]-uridine light components was centrifuged in a 5 to 25% sucrose gradient prepared in 0.1 M-acetate/0.1% SDS, pH 5.0. The RNAs from the two components sedimented together as a sharp homogeneous peak at 35S (Fig. 7).
It seemed likely from the experiments described above that the higher density of the heavy component was due to the greater degree of interaction of its RNA with the caesium ions which in turn would be a reflection of the arrangement of the polypeptides in the capsid structure. However, no difference was found between the SDS-polyacrylamide gel profiles of the polypeptides following iodination of the intact components (Fig. 8). This indicates that the tyrosine residues of the different polypeptides are equally accessible in the two components. As with bovine enterovirus (Carthew & Martin, 1974) and FMDV (Talbot et al. 1973) VP1 is labelled to a much greater extent with radioactive iodine than the other polypeptides of the virus.

Similarly, the two components were equally resistant to low pH environments. Neither component lost infectivity at pH 3 to 7 in 15 min when 100-fold dilutions of the appropriate caesium chloride fractions were incubated in 0.1 M-acetate buffer. This contrasts with the acid lability of the high-density foot-and-mouth disease virus.
A high density component of enteroviruses

Fig. 6. SDS-polyacrylamide gel co-electrophoresis in 12.5% gels of the polypeptides prepared from [14C]- and [3H]-amino acid labelled components of SVDV. The polypeptides were mixed and co-run on the same gel: •, [14C]-light component; ○, [3H]-heavy component.

Fig. 7. Sucrose gradient co-sedimentation of the RNAs extracted from [32P]-heavy and [3H]-uridine light components of SVDV (It 1/66): •, [32P]; ○, [3H]. The positions of the 28S and 18S BHK cell ribosomal markers were determined by measuring the extinction at 260 nm.
Fig. 8. Polyacrylamide gel electrophoresis of the polypeptides of $^{[125]}$I-labelled (a) light and (b) heavy components of SVDV (Lt 1/66).

Antigenic properties of the two components

Sera were prepared in groups of 4 guinea pigs by inoculating equal amounts (as judged by radioactive counts) of re-cycled heavy or light component inactivated with acetyleneimine (AEI) or formaldehyde. The sera collected 21 days after inoculation were titrated by the plaque reduction method, using either separated heavy or light component as challenge. The antisera produced by inoculating the AEI- or formaldehyde-inactivated light component contained a high level of antibody that neutralized either component equally well (Table 1). However, the antibody level produced by the heavy component was very low when AEI-inactivated antigen was used and was about fivefold lower than that produced by the light component when formaldehyde-inactivated antigen was used. The antibody produced by the formaldehyde-inactivated heavy particles neutralized either particle equally well (Table 1).
A high density component of enteroviruses

Table 1. Neutralizing activity of guinea pig sera 21 days after inoculation of inactivated heavy and light components

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Inactivating agent</th>
<th>Heavy component</th>
<th>Light component</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heavy component</td>
<td>Acetylationeimine</td>
<td>16 (0-64)</td>
<td>19 (0-64)</td>
</tr>
<tr>
<td>Light component</td>
<td>Acetylationeimine</td>
<td>320 (256-512)</td>
<td>512 (256-1024)</td>
</tr>
<tr>
<td>Heavy component</td>
<td>Formaldehyde</td>
<td>100 (25-625)</td>
<td>50 (25-125)</td>
</tr>
<tr>
<td>Light component</td>
<td>Formaldehyde</td>
<td>625</td>
<td>460 (125-625)</td>
</tr>
</tbody>
</table>

* Each value is the mean of titres obtained with sera from 4 separate guinea pigs. The figures in parentheses indicate the range of titres obtained.

DISCUSSION

The vertebrate picornaviruses have densities in caesium chloride which range from 1.34 g/ml for the enteroviruses to 1.45 g/ml for an equine rhinovirus (Newman et al. 1973). Since the picornaviruses contain the same proportions of RNA and protein, this wide range of buoyant density must be a reflection of the structural arrangement of the capsid components. It has been postulated that differing degrees of interaction between the RNA and protein lead to varying availability of the RNA for interaction with solute cations such as caesium (Rowlands et al. 1971; Ruecker, 1971; K. Lonberg-Holm, personal communication).

The bacteriophage MS2 has been shown to exist in two forms with different buoyant density (Rohrmann & Krueger, 1970). The two forms are structurally and antigenically similar but differ in specific infectivity, the light form being less infective.

The results described in this paper show the presence in several enteroviruses of a small proportion of particles with a buoyant density of 1.44 g/ml within a population of particles, the majority of which have a density of 1.34 g/ml. The progeny virus obtained by cultivating either of the two density forms contained the same proportion of heavy and light components as the original virus, showing that the two density forms are genetically identical. This differs from the results of Goodheart (1965), who found that density variants present in harvests of encephalomyocarditis virus were genetically determined and stable on replication.

The higher density of the heavy component does not appear to be due to the presence of adventitious contaminating material, as was described for a high-density component of Talfan virus (Warlinton, 1967), since extensive purification of SVDV did not alter the proportion of heavy component and attempts to inter-convert the two forms were unsuccessful. Additionally, the heavy component was somewhat less stable than the light component.

The difference in density is not due to any difference in the RNA protein ratio of the two components. Similarly, the RNA and protein moieties of the two particles appeared to be identical in physical properties.

Whereas the particles of FMDV, which have a density almost as high as those of the heavy component of SVDV, increase in density on prolonged centrifuging in caesium chloride gradients or if the pH of the gradients is increased to pH 9, the heavy component of SVDV retains its original buoyant density under these conditions. Similarly, the heavy component of SVDV is unaffected at pH values as low as 3, whereas the higher-density picornaviruses such as FMDV and human rhinovirus are disrupted at pH 5. In fact, the light and heavy components of SVDV were equally stable in an acid environment, both being unaffected at pH 3.
The heavy component was somewhat less efficient than the light component in its ability to initiate infection and raise neutralizing antibody in guinea pigs. No neutralizing activity was detectable in the sera of guinea pigs which had been inoculated with the heavy component unless this was first fixed with formaldehyde. Similar amounts of the light component produced a good response irrespective of the method of inactivation (Table 1).

The similarity in the composition and biological properties of the two density forms led us to examine whether the difference in density was due to a configurational difference between the two components. To test this idea, the separated components were iodinated and the proteins separated by polyacrylamide gel electrophoresis. No difference between the profiles was observed, showing that the tyrosine residues in each component were equally accessible to iodination. This observation, however, does not preclude the possibility that the two components differ in the structural arrangement of the capsid proteins and it is clearly desirable to test whether compounds reacting specifically with other amino acids will reveal differences between the two components. It seems unlikely that the two density forms are related to the two forms found in electrophoresis experiments with poliovirus (Mandel, 1971) since the two density forms have similar specific infectivity and are not readily interconvertible.

Since this work was completed and prepared for publication Yamaguchi-Koll, Wiegers & Drzeniek (1975) have published the results of a similar study with poliovirus.

We wish to thank Mr C. J. Smale for the electron microscopy.

REFERENCES


(Received 30 April 1975)