Buoyant Density of Picornaviruses in Caesium Salts

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SUMMARY

Factors affecting the determination of buoyant density in caesium salts of several animal picornaviruses have been studied. The values obtained for the acid-labile foot-and-mouth disease virus increased with the duration of the centrifugation and were higher at elevated pH values. In contrast, the acid-stable enteroviruses had the same values irrespective of the time of centrifugation or the pH of the caesium solution. Viruses of intermediate stability, such as vesicular exanthema virus and the rhinoviruses, were affected in the same way as foot-and-mouth disease virus but to a smaller extent. The implications of these observations in respect of the structure of the picornavirus group are discussed.

INTRODUCTION

Despite their similar size and composition, the members of the picornavirus group exhibit a wide range of buoyant densities in caesium chloride. At one end of the range, poliovirus and the enteroviruses have densities of 1.34 g./ml., while at the other foot-and-mouth disease virus has a density of 1.43 g./ml. Between these extremes, values of 1.36 to 1.38 g./ml. for vesicular exanthema virus and the feline picornaviruses and 1.38 to 1.41 g./ml. for the human rhinoviruses have been reported (Table I).

Table 1. Buoyant density of picornaviruses in caesium chloride

<table>
<thead>
<tr>
<th>Virus</th>
<th>Buoyant density (g./ml.)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polio</td>
<td>1.34</td>
<td>Mattern (1962), Schaffer &amp; Frommhagen (1965)</td>
</tr>
<tr>
<td>Bovine entero</td>
<td>1.34</td>
<td>Martin et al. (1970)</td>
</tr>
<tr>
<td>Vesicular exanthema</td>
<td>1.36 to 1.38</td>
<td>Oglesby (1965), Wawrzkiewicz, Smale &amp; Brown (1968)</td>
</tr>
<tr>
<td>Feline picorna</td>
<td>1.37 to 1.39</td>
<td>Studdert, Martin &amp; Peterson (1970), Prydie (personal communication)</td>
</tr>
<tr>
<td>Human rhino</td>
<td>1.38 to 1.41</td>
<td>Chapple &amp; Harris (1966),Dans, Forsyth &amp; Channock (1966), McGregor, Phillips &amp; Mayor (1966), Gerin et al. (1968)</td>
</tr>
<tr>
<td>Foot-and-mouth disease</td>
<td>1.43 to 1.51</td>
<td>Trautman &amp; Breese (1962), Liebermann &amp; Grahiger (1968)</td>
</tr>
</tbody>
</table>

The reasons for this range of densities within the picornavirus group are not fully understood, but several possibilities have been suggested: (a) the dense viruses have a greater proportion of RNA; (b) the reaction of caesium ions with the RNA is greater in the denser viruses because of the greater accessibility of the RNA; (c) the viruses have different degrees of hydration.

It is unlikely that (a) is the factor involved since most of the picornaviruses contain about
25 to 30% RNA and the molecular weights of the RNAs fall within a fairly narrow range (2.4 to 2.8 x 10^6). The evidence provided in this paper does not allow us to distinguish between (b) and (c) but the results provide an explanation for the rather large range of values which have been published for individual members of the group. For example, values ranging from 1.38 to 1.41 for the human rhinoviruses have appeared in the literature. Although it is possible that these variations occur because the experiments were performed in different laboratories, the width of the range of values for this group of viruses suggested to us that there was a crucial difference in methodology between the various laboratories. It is known, for example, that determinations are made either by centrifugation for a short time in pre-formed gradients or by prolonged centrifugation using conditions in which the gradients are produced during centrifugation. While this difference in technique may not be important for some viruses, preliminary results obtained during the investigation of the physical properties of several strains of foot-and-mouth disease virus suggested that the duration of centrifugation was an important factor in determining the buoyant density of this virus.

Apart from the slight variations in technique which exist between different laboratories, however, it seems that other factors which cannot be due to this are also involved in the discrepancies which appear in the literature. For example, Strauss & Sinsheimer (1963) reported that the density of the RNA phage MS 2 was 1.38 to 1.46 g./ml., depending on the concentration of the virus. Further, Oglesby (1965) reported values ranging from 1.36 to 1.38 for vesicular exanthema virus and Studdert, Martin & Peterson (1970) gave values of 1.37 to 1.39 for the morphologically similar feline picornaviruses.

The aim of the work described in this paper has been to study the factors which appear to be important in the determination of the buoyant density of picornaviruses in caesium salts and to provide a standardized method for the determination.

**METHODS**

*Viruses.* The foot-and-mouth disease virus strains were grown at 37°C in monolayers of BHK 21 cells. The pig enterovirus (ITALIAN 1/66, Nardelli et al., 1968) and vesicular exanthema virus (type E) were grown in the IB-RS-2 pig kidney cell line (de Castro, 1964) and the equine rhinovirus (strain NM 11) was grown in RK 13 cells. For the preparation of viruses labelled with [3H]- or [14C]uridine, the usual growth medium containing 5 µg [3H]-uridine or 1 µc [14C]uridine and 1 µg. actinomycin D/ml. was used. Virus labelled with [14C]- or [3H]amino acids was grown in the presence of Earle's saline containing 1 µc [14C]-protein hydrolysate or [3H]amino acids/ml. Virus labelled with [32P] was grown in the presence of Earle's saline in which the phosphate was replaced with 0.01 M-tris buffer, pH 7.6, and contained 50 µc carrier-free [32P]phosphate and 1 µg. actinomycin D/ml. All the viruses were purified by the method of Brown & Cartwright (1963) with the exception of vesicular exanthema virus, where 0.1% sodium deoxycholate was used instead of sodium dodecyl sulphate in the final step (Wawrzkiewicz, Smale & Brown, 1968).

Poliovirus (labelled with [3H]uridine) was a gift from Dr M. L. Fenwick, University of Oxford, and human rhinovirus (Type 4 labelled with [32P]phosphate) was kindly provided by Dr E. J. Stott, Clinical Research Centre, Harrow, Middlesex. The bovine enterovirus (VG-5-27, labelled with [3H]uridine) was a gift from Dr S. J. Martin, University of Belfast.

The titrations of foot-and-mouth disease virus and the bovine enterovirus VG-5-27 were made by plaque assay on BHK 21 cell monolayers (Mowat & Chapman, 1962; Martin, Johnston & Clements, 1970).
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**Isopycnic centrifugation method.** Caesium chloride or sulphate solutions were prepared in 0.04 M-phosphate, pH 7.6, or in 0.1 M-tris buffer, pH 7.1, 8.1 or 9.0. Linear gradients containing 5.0 ml. were made with the aid of a gradient-making machine and 0.05 ml. samples of the virus were then added. The tubes were centrifuged at 20° in the six-bucket rotor no. 59108 in an MSE 65 ultracentrifuge. The gradients were fractionated by puncturing a hole in the bottom of the tube and collecting 0.25 ml. fractions. After every third fraction one drop was collected for determination of refractive index. The density of the caesium solutions was calculated from the refractive index measurements after correction for the refractive index of the buffer solution. The accuracy of these determinations was checked occasionally by direct weighing.

**Extraction and centrifugation of virus RNA.** The appropriate fractions from the caesium chloride gradients were diluted with 0.04 M-phosphate, pH 7.6, and extracted twice with water-saturated phenol. Ribosomal RNA prepared from BHK 21 cells was added to the aqueous layer and the mixture of RNAs precipitated with two vol. of cold ethanol. After overnight storage at −20°, the precipitate was dissolved in 0.1 M-acetate, 0.1% sodium dodecyl sulphate, pH 5.0, and centrifuged at 20,000 rev./min. for 15 hr in 5 to 25% (w/v) sucrose gradients prepared in 0.1 M-acetate, 0.1% sodium dodecyl sulphate. One ml. fractions were collected for radioactive analysis and determination of extinction at 260 nm.

**Counting of radioactivity.** Samples were mixed with bovine serum albumin solution and precipitated with a final concentration of 10% trichloroacetic acid. The precipitates were collected on glass fibre discs and counted in PPO-POPOP-toluene scintillant in a Packard scintillation counter.

**RESULTS**

Preliminary observations on the density of foot-and-mouth disease virus

Although the buoyant density of foot-and-mouth disease virus is usually given as 1.43 g./ml. (Trautman & Breese, 1962), we have frequently obtained, over a number of years, values as divergent as 1.41 and 1.47 g./ml. with different strains of virus. These observations indicated that it was necessary to standardize the conditions of centrifugation much more rigorously. For example, it had been our practice to use caesium chloride in 0.04 M-phosphate, pH 7.6. The virus sample was then mixed with this solution to a density of 1.43 g./ml. and the mixture centrifuged for 24 hr at 35,000 rev./min. in the SW 39 rotor of the Spinco Ultracentrifuge to establish the gradient. Alternatively, the virus sample was added to the top of a pre-formed gradient prepared from solutions with densities of 1.38 and 1.48 g./ml. and centrifugation continued for 4 hr at 35,000 rev./min. On most occasions with individual virus strains the two methods gave similar values. With some strains, however, values as high as 1.46 g./ml. were obtained with the 24 hr centrifugation method compared with 1.43 g./ml. with short centrifugation through a pre-formed gradient. Furthermore, in an experiment to determine the density of a bovine rhinovirus (P. Ide & F. Brown, unpublished results) a sample of radioactive foot-and-mouth disease virus was included as an internal marker and the caesium chloride was prepared in a buffer solution at pH 6.95. Centrifuging through a pre-formed gradient for 4 hr, the foot-and-mouth disease virus gave a peak at a density of 1.41 g./ml.

In view of these widely different values obtained for the density of foot-and-mouth disease virus, several factors which might influence the determinations were studied. The effects of length of time of centrifugation, pH and nature of the anion on the density of the virus were examined. The implications from the results led us to study examples of
picornaviruses which are stable at low pH and those of stability intermediate between the enteroviruses and foot-and-mouth disease virus.

Establishment of standard conditions for density determination

In all the experiments, the temperature of the rotor was maintained at 20°C and the refractive index readings on the fractions were made at the same temperature. All the determinations were made with the virus samples layered on to pre-formed gradients.

![Graph](image)

**Fig. 1.** Demonstration of minimum time required for foot-and-mouth disease virus to reach equilibrium in a pre-formed caesium chloride gradient. The samples of [32P] virus were added at the top and bottom of each gradient (1.4 to 1.5 g./ml.) and centrifuged at 24,000 rev./min. for (a) 1 hr; (b) 2 hr; (c) 4 hr; (d) 6 hr. ●●●● [32P]; ○---○, density.

The minimum time to reach equilibrium was determined by adding samples of [32P]-labelled virus (type O, strain 1) to the top and bottom of pre-formed gradients (1.4 to 1.5 g./ml.) and then centrifuging for 1, 2, 4, 6 or 22 hr. The sample added to the bottom of the gradient was made to a density of 1.55 g./ml. and inserted through the pre-formed gradient by means of a fine capillary. The results in Fig. 1 show that the samples of virus merged at 6 hr (but not before), and at this time the peak of radioactivity was at 1.43 g./ml. After centrifuging for 22 hr, the peak was found at 1.44 g./ml.

The standard procedure used for density determinations was as follows: centrifugation at 24,000 rev./min. (average 90,000 g) for 6 hr in 5 ml. pre-formed gradients, using the six-bucket rotor of the MSE 65 and maintenance at 20°C throughout the run.
Effect of prolonged centrifugation on the density of foot-and-mouth disease virus and poliovirus

The increase in density of type O (strain 1) virus produced by prolonging the centrifugation run was confirmed in the following experiment. Virus labelled with \( ^{3} \text{H} \)uridine was layered on to two pre-formed gradients (1.4 to 1.5 g./ml.) in 0.04 M-phosphate, pH 7.6, and centrifuged at 24,000 rev./min. for 24 or 94 hr. A sample of \( ^{14} \text{C} \)uridine-labelled virus was then layered on to each of the gradients and centrifugation continued for a further 6 hr. In a third tube a mixture of the \( ^{3} \text{H} \) and \( ^{14} \text{C} \) viruses was centrifuged through a similar gradient for 6 hr. The results in Fig. 2 show that the density of the virus particles becomes greater with increasing centrifugation times. The effect of prolonged centrifugation on the distribution of infectivity in the gradients was also studied. The profiles in Fig. 3 show that the distribution of infectivity in the gradients coincides with the radioactive peaks but the recovery of infectivity was lower after prolonged centrifugation. A similar increase in
density was observed when another strain of foot-and-mouth disease virus (type A, ARG 61) was centrifuged for a prolonged period but the density of poliovirus was the same after 6, 30 or 100 hr centrifugation. A bovine enterovirus (VG-5-27: Martin et al. 1970) behaved similarly to poliovirus and the recovery of infectivity was the same after the three periods of centrifugation.

Fig. 3. Distribution of infectivity and radioactivity in caesium chloride gradients of 14C-labelled virus which had been centrifuged at 24,000 rev./min. for different times. (a) 6 hr; (b) 30 hr; (c) 100 hr.

Effect of age of virus on buoyant density

The infectivity of foot-and-mouth disease virus is reasonably stable at 2 to 4°C, especially in the unfractionated state. We had found with purified samples of the virus, however, that more than 99% of the infectivity could be lost without apparent breakdown of the particles, i.e. they still sedimented as a sharp peak at 140S in sucrose gradients. The particles were more fragile, however, in that preparation of the specimens for electron microscopy often led to their disruption. Such preparations had higher buoyant densities than the freshly purified viruses. Table 2 shows the increase in buoyant density of virus of type O, strain 1, after storage for different periods at 2 to 4°C. In contrast, the purified specimen of poliovirus we used in these experiments had the same density after many months of storage at 2 to 4°C.
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Effect of anion on the density of the picornaviruses

The density of single-stranded RNA in caesium sulphate is approximately 1.65 g./ml., compared with 1.92 g./ml. in caesium chloride. This difference is reflected in the density of some of the members of the picornavirus group. We have found, for example, that the densities of poliovirus and the pig enterovirus ITALIAN 1/66 are similar in the two salts—a result in accordance with Zolotor & Engler (1967), who found that there was little change in the density of poliovirus in a variety of caesium salts. In contrast, the densities of the other members of the group which we examined were much lower in caesium sulphate compared with caesium chloride (Table 3). These results suggest that the RNAs of the higher density viruses are affected by the medium to a much greater extent than the enterovirus RNAs.

Table 2. Effect of storage at 2°C on buoyant density of foot-and-mouth disease virus

<table>
<thead>
<tr>
<th>Storage period (weeks)</th>
<th>6 hr centrifugation</th>
<th>24 hr centrifugation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1.43</td>
<td>1.44</td>
</tr>
<tr>
<td>12</td>
<td>1.43</td>
<td>1.46</td>
</tr>
<tr>
<td>25</td>
<td>1.46</td>
<td>1.46</td>
</tr>
</tbody>
</table>

Table 3. Comparison of buoyant densities of picornaviruses in caesium chloride and caesium sulphate at pH 7.1

<table>
<thead>
<tr>
<th>Virus</th>
<th>Caesium chloride</th>
<th>Caesium sulphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polio</td>
<td>1.34</td>
<td>1.33</td>
</tr>
<tr>
<td>Porcine entero</td>
<td>1.34</td>
<td>1.34</td>
</tr>
<tr>
<td>Vesicular exanthema</td>
<td>1.36</td>
<td>1.32</td>
</tr>
<tr>
<td>Human rhino</td>
<td>1.40</td>
<td>1.36</td>
</tr>
<tr>
<td>Foot-and-mouth</td>
<td>1.42</td>
<td>1.39</td>
</tr>
<tr>
<td>Equine rhino</td>
<td>1.45</td>
<td>1.41</td>
</tr>
</tbody>
</table>

Effect of pH on the density of several picornaviruses

Foot-and-mouth disease virus (type O, strain 1) labelled with $^{32}$P was centrifuged in three separate pre-formed gradients of caesium chloride, prepared in 0.1 M-tris buffer, pH 7.1, 8.1 or 9.0. Using the standard conditions of centrifugation (24,000 rev./min. for 6 hr at 20°C), the density of the virus was 1.42 at pH 7.1, 1.45 at pH 8.1 and 1.46 at pH 9.0. The increase in density at the higher pH values could be accounted for by (a) partial breakdown of the virus with preferential loss of protein, or (b) changes in density of the individual components of the virus, or (c) configurational alterations so that the degree of binding between the RNA and protein was weakened.

To decide between these possibilities, preparations of virus labelled with $[^3]$H uridine and $[^1]$C amino acids were centrifuged for 6 hr at 24,000 rev./min. in gradients of caesium chloride prepared in 0.1 M-tris buffer, pH 7.1, 8.1 and 9.0. The distribution of the two isotopes in the gradients shows that there was no preferential loss of protein from the virus particles and the ratio of $[^3]$H to $[^1]$C was similar at the different pH values (Fig. 4). If the density change at pH 9.0 had been caused by loss of protein, the ratio of $[^3]$H to $[^1]$C would have increased from 0.70 to 1.03.
The density of foot-and-mouth disease virus protein (either the 12S component produced by mild acid disruption of the virus or the 75S empty particle (Graves, Cowan & Trautman, 1968)) was unaltered by increasing the pH of the gradients from 7·1 to 9·0. Owing to difficulties in measuring the density of RNA in cesium chloride, we have no data on the effect of pH on the density of RNA in this salt. However, the density of RNA in cesium sulphate decreased slightly over the same pH range, indicating that the increase in density of the viruses at higher pH values is not due to an increase in the densities of the individual components.

Fig. 4. Effect of pH on the density of foot-and-mouth disease virus in cesium chloride gradients. Virus labelled with [14C]amino acids and [3H]uridine was used to determine whether the increase in density at higher pH values was due to differential loss of protein. (a) pH 7·1; (b) pH 8·1; (c) pH 9·0. • O, 14C; ○ --- O, 3H; ■ --- ■ density.

The peaks of radioactivity obtained by centrifuging the foot-and-mouth disease virus preparation at the three different pH values coincided with the positions of the infectivity, although it is necessary to emphasize that considerable losses of infectivity were encountered at pH 8·1 and 9·0. These losses appear to be due to breaks in the RNA chains, since the RNA extracted from the radioactive peak fractions in the cesium chloride gradients gave heterogeneous profiles in sucrose density gradients (Fig. 5). These profiles are typical of those obtained with the RNA extracted from virus which has been inactivated by heating at 37° (Brown & Wild, 1966). The extent of the degradation was greater at pH 9·0. In contrast
the RNA extracted from virus which had been centrifuged in caesium chloride at pH 7.1 gave a profile similar to that of freshly prepared virus RNA. It appears, therefore, that the increased density values obtained at the higher pH values are caused by configurational changes in the virus particles which lead to breaks in the RNA chains.

The density of the bovine enterovirus VG-5-27 was unaltered at higher pH values, and the RNA extracted from the radioactive peaks in the caesium chloride gradients gave similar profiles in sucrose gradients (Fig. 6). The density of poliovirus also was unaltered at pH 9.0 but the densities of the viruses with values intermediate between those of poliovirus and foot-and-mouth disease virus were increased (Table 4). The effect was more marked with the higher density viruses. Thus, the value for the human rhinovirus increased...
from 1·40 at pH 7·1 to 1·42 at pH 9·0, compared with an increase from 1·36 to 1·37 with vesicular exanthema virus.

Recently, Wood (1971) has shown that slight changes of pH greatly affect the density of the bottom and middle components of cowpea mosaic virus. While the changes in density with picornaviruses are smaller than those which Wood describes, it seems likely that similar factors are involved.

**Table 4. Effect of pH on the buoyant density of picornaviruses in caesium chloride**

<table>
<thead>
<tr>
<th>Virus</th>
<th>pH 5·0</th>
<th>pH 7·1</th>
<th>pH 8·1</th>
<th>pH 9·0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polio</td>
<td>1·34</td>
<td>—</td>
<td>1·34</td>
<td>1·34</td>
</tr>
<tr>
<td>Porcine entero</td>
<td>1·34</td>
<td>—</td>
<td>1·34</td>
<td>1·34</td>
</tr>
<tr>
<td>Vesicular exanthema</td>
<td>—</td>
<td>1·36</td>
<td>1·36</td>
<td>1·37</td>
</tr>
<tr>
<td>Human rhino</td>
<td>—</td>
<td>1·40</td>
<td>1·40</td>
<td>1·42</td>
</tr>
<tr>
<td>Foot-and-mouth</td>
<td>—</td>
<td>1·42</td>
<td>1·45</td>
<td>1·46</td>
</tr>
<tr>
<td>Equine rhino</td>
<td>—</td>
<td>1·45</td>
<td>1·46</td>
<td>1·47</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The results described in this paper show that the density of the acid-labile picornaviruses depends on the exact method used for their determination. Factors such as pH, the anion present and the duration of centrifugation affect the value obtained. Although the density of the acid-stable picornaviruses (e.g. polio) is not affected, these factors have a considerable influence on the density of the other members of the group. For example, the density of foot-and-mouth disease virus increases from 1·42 to 1·46 g./ml. by altering the pH from 7·1 to 9·0 and a similar change is observed when the centrifugation time is increased from 6 hr to 100 hr. The anion also affects the density, the value in caesium chloride at pH 7·1 being 1·42 compared with 1·39 in caesium sulphate at the same pH. Other members of the group are similarly affected.

It is worthy of comment that the equine rhinovirus has a buoyant density in caesium chloride and caesium sulphate which clearly distinguishes it from the human rhinovirus group. Further evidence that the equine rhinovirus is, in fact, not closely related to the human rhinoviruses is found in the results of a base composition analysis of the virus RNA (J. F. E. Newman, personal communication) compared with the analysis on three strains of human rhinovirus (Brown, Newman & Stott, 1970; McGregor & Mayor, 1971; J. F. E. Newman, unpublished data).

The correlation between increasing density and acid lability in the animal picornavirus group has an interesting analogy with the DNA phages T 1 and T 5 (Lark, 1962; Hertel, Marchi & Muller, 1962; Ritchie & Malcolm, 1970). These groups of workers found that stability to heat of variants of the phages was correlated with a decreased density in caesium chloride which was not related to loss of DNA. Nishihara & Watanabe (1969) have demonstrated differences between the densities of the RNA phages in caesium chloride. Although they considered that the differences might be due primarily to variations in the RNA content of the phage particles, they concluded that the extent of the interaction between the caesium chloride and the phage particles might account for some of their observations.

It would appear, therefore, that there is a correlation between thermal or acid stability in both RNA and DNA viruses. In many instances the density differences have been shown not to be due to different contents of nucleic acid. Within the picornavirus group,
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the RNA of the different members appears to exhibit a density which is related to the extent of its reaction with caesium ions or to its degree of hydration. These in turn will depend on the degree of binding of the RNA to the virus protein. There appears to be a considerably greater degree of binding between the RNA and protein of the enteroviruses compared with the acid-labile rhino- and foot-and-mouth disease viruses. An understanding of these reactions would be useful in studies of the physical structure of the viruses.

We are grateful to Mr D. Goodridge for considerable assistance in growing the equine rhinovirus and vesicular exanthema virus and to Drs M. L. Fenwick, E. J. Stott and S. J. Martin for purified samples of poliovirus, human rhinovirus and bovine enterovirus, respectively.

REFERENCES


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