Biophysical Characterization of Rotavirus Particles Containing Rearranged Genomes

By M. McIntyre,1 V. Rosenbaum,2 W. Rappold,2 M. Desselberger,2 D. Wood3 and U. Desselberger1*

1Institute of Virology, University of Glasgow, Church Street, Glasgow G11 5JR, U.K.,
2Institut für Physikalische Biologie, Universität Düsseldorf, Universitätsstrasse 1, 4 Düsseldorf 1,
F.R.G. and 3North Manchester Regional Virus Laboratory, Booth Hall Children's Hospital,
Manchester M9 2AA, U.K.

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SUMMARY

Human rotaviruses containing rearranged genomes were found to package up to 1800 additional base pairs in virus particles. The viruses compared were indistinguishable in respect of their particle diameters and their apparent S values. Particles containing rearranged genomes were found to be of a higher density than rotavirus particles containing a standard genome as determined by equilibrium density ultracentrifugation. The increase in density was directly proportional to the number of additionally packaged base pairs.

At present, five groups (A to E) of rotaviruses can be distinguished by serology and genome analysis (Bridger et al., 1986; Pedley et al., 1986). The rotavirus genome consists of 11 segments of dsRNA. The segments of group A rotaviruses have between 660 and approximately 3400 bp, resulting in a total genome size of approximately 18600 bp (Holmes, 1983; Rixon et al., 1984).

Recently, group A rotaviruses have been described (Pedley et al., 1984), which were obtained from immunodeficient, chronically infected children and in which one or several of the RNA segments were rearranged. Normal RNA segments were missing from the electrophoretic profile or decreased in concentration, but more slowly migrating additional bands of dsRNA were observed in the gel and found to represent concatemeric forms of normal RNA segments (Pedley et al., 1984). Similar forms of genomic rearrangements were also observed in variants of bovine rotavirus (BRV) isolated after serial passage at a high multiplicity of infection (Hundley et al., 1985). Direct sequence analysis of one of the concatemeric forms of a BRV isolate (Pocock, 1987) revealed partial duplication of a normal RNA segment (Scott et al., 1986). Furthermore it has been found that rotaviruses possessing rearranged genomes reassort with normal (standard) rotaviruses and that rearranged bands of genomic RNA replace normal RNA segments structurally and functionally in reassortants (Allen & Desselberger, 1985; Graham et al., 1987). Recently, we have been able to grow human rotavirus isolates with genome rearrangements in vitro in BSC-1 cells. These isolates consisted of various subpopulations differing grossly in genotype and possessing different combinations of rearranged RNA bands (Hundley et al., 1987). We calculated that these viruses had between 450 and 1790 additionally packaged bp, amounting to 2.4 to 9.6% of the standard genome size. We therefore considered that the biophysical characteristics of such particles also might have changed. We now report electron microscopical observations and velocity and equilibrium sedimentation data for rotavirus particles possessing rearranged genomes and compare these to rotaviruses having a standard genome.

BRV (UK Compton strain) was grown in MA104 cells and semipurified by differential centrifugation as previously reported (Follett et al., 1984). Human rotaviruses (HRV), isolated
from faeces of a chronically infected immunodeficient child, were adapted to growth in BSC-1 cells. From the yields numerous subpopulations of rotaviruses possessing different forms of genome rearrangements were obtained after several rounds of plaque-to-plaque purification (Hundley et al., 1987). The RNA profiles of some of these viruses selected for biophysical study are shown in Fig. 1. Rearrangements of segment 11-, segment 10- and segment 8-specific sequences into concatemeric forms are indicated. The HRV of genotype 2 has RNA segment 11 replaced by band e; HRV of genotype 3 carries band c instead of segment 11 and band a instead of segment 8; HRV of genotype 7 carries band c instead of segment 11, band d instead of segment 10 and band a instead of segment 8; and HRV of genotype 9 carries band e instead of segment 11, band d instead of segment 10 and band f instead of segment 8 (Hundley et al., 1987).

As a result, rotaviruses of genotypes 2, 3, 7 and 9, respectively, have approximately 450, 1070, 1570 and 1790 additionally packaged bp. The HRVs were semipurified in the same way as described for BRV, and all viruses were Freon-extracted according to the procedure described by McCrae (1985). The viruses were then banded using CsCl equilibrium ultracentrifugation (density of CsCl–virus suspension before centrifugation, 1.36 to 1.37 g/ml; centrifugation at 150000 g for 18 h at 15 °C). Bands which consisted of single-shelled particles containing the RNA genome (density 1.37 to 1.38 g/ml; Rodger et al., 1975; Tam et al., 1976) were collected and diluted with phosphate-buffered saline (PBS). Virus was then pelleted (100000 g, 90 min, 4 °C) and resuspended in PBS. A second round of CsCl gradient centrifugation as described above was performed with mixtures of the different single-shelled virus particles and with the individual virus suspensions as controls. Whereas the controls yielded single bands again, mixtures of BRV and of HRVs of genotypes 3, 7 and 9 formed visible double bands (results not shown). The RNA of gradient fractions (120 ~tl) was extracted and analysed on polyacrylamide gels (Follett et al., 1984); it was found that the lower band contained the HRVs with rearranged genomes (data not shown).

Bands of single-shelled particles of HRV genotype 7 and of BRV obtained by ultracentrifugation were isolated, negatively stained with 3% phosphotungstic acid (pH 6) and examined by electron microscopy. The diameters of 100 particles of each virus stock were measured from the electron microscopical image using a calibrated MOP-Videoplan system for image analysis (Kontron Bildanalyse, Munich, F.R.G.). Single-shelled particles of BRV measured 54.8 ± 5.1 nm (arithmetic mean ± s.d.) compared with 55.4 ± 4.2 nm for particles of HRV genotype 7. The small difference is not significant (t-test, P < 0.05).

It had been observed that after density gradient ultracentrifugation a mixture of HRV genotype 3 (1070 additional bp) and of BRV could still be resolved as a narrow double band by the naked eye. This was less distinct than the resolution obtained with the HRV 7/BRV mixture. However, mixtures of HRV genotype 2 (450 additional bp) and BRV could no longer be resolved visually after ultracentrifugation nor by analysis of the resulting fractions. The resolution of double bands of HRV/BRV mixtures was increased and quantified in terms of density differences by analytical ultracentrifugation. The experiments were performed using a Spinco Model E ultracentrifuge equipped with a high intensity ultraviolet illumination system, photoelectric scanner and electronic multiplexer (Flossdorf, 1980) as well as with fluorescence detection optics (Rappold, 1986). Velocity sedimentation centrifugation was carried out using charcoal-filled Epon single-sector centrepieces of 3 mm optical pathlength, at a rotor speed of 10000 r.p.m. and a temperature of 22 °C. The composition of the sample solution was 0.15 M-NaCl (buffered at pH 7.2 with 0.01 M-phosphate), 6% (w/w) sucrose and 10⁻⁵ M-ethidium bromide. Sedimentation profiles were monitored with the fluorescence detection optics recording the movement of ethidium bromide bound to virus particles. For equilibrium density gradient centrifugation in CsCl, charcoal-filled Epon single-sector centrepieces of 12 mm optical pathlength were used. The rotor speed was maintained at 40000 r.p.m. throughout the experiment with the temperature at a constant 24 °C. The virus particles were suspended in TNE buffer (100 mM-Tris-HCl pH 7.2, 100 mM-NaCl, 10 mM-EDTA), and CsCl was added to give an overall density of 1.391 g/ml before centrifugation. Several scans were made in the period between 2 and 12 h after the start of the experiment using the u.v. absorption optics at 265 nm. On the scans the difference in density between the various virus particles is known to be
Fig. 1. RNA profiles of BRV and of HRV with rearranged genomes of genotypes 2, 3, 7 and 9. RNAs were extracted from semipurified virions, separated by electrophoresis on 2.8% polyacrylamide-6 m-urea slab gels and silver-stained as described (Follett et al., 1984). The segment numbers (1 to 11) are indicated to the left. Position and origin of rearranged bands of genomic RNA are identified on the right; bands a and f are derived from RNA segment 8, band d from segment 10, and bands c and e from segment 11 (see text). The numbers of additionally packaged bp (indicated below the profiles) were calculated from the relative migration rates of rearranged RNA bands and the RNA segments of BRV, the sizes of which are known (Rixon et al., 1984).

Fig. 2. (a) Scans after analytical equilibrium centrifugation in CsCl of mixtures of single-shelled particles containing RNA of BRV and of HRV of genotypes 2, 3 or 9. Scans of all mixtures were made in the same experiment after 2 h 15 min. The viruses contained in the peaks are denoted, and the numbers of additionally packaged bp are indicated below the profiles. (b) Plot of difference in density as determined from data shown in (a) against number of additionally packaged bp.
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proportional to the separation in distance of the corresponding peaks. This density difference was calculated from densities of individual bands according to the equation (Schachman, 1959):
\[ \rho_r = \rho_s + \left[ \left( r - r_e \right) \times \omega^2 \times r_e / \beta(\rho_s) \right] \]
where \( \rho_r \) is the density of band at radius \( r \) from the centre of rotation; \( \rho_s \) is the density of original mixture before ultracentrifugation and reached at radius \( r_e \) after ultracentrifugation; \( r_e = \sqrt{[(r_m^2 + r_b^2)/2]} \) where \( r_m \) denotes the radius at the meniscus and \( r_b \) the radius at the base of the cell; \( \omega \) is the angular velocity in radians/second, \( \beta(\rho_s) = 1.388 \times 10^9 \) cm\(^5\)/g.s\(^2\).

Results of equilibrium centrifugation experiments are shown in Fig. 2(a). HRV of genotypes 2, 3 and 9 differed in density from BRV by 0.0031, 0.0062 and 0.0096 g/ml, respectively. When the difference in density was plotted against the number of additionally packaged bp, an approximately linear relationship was obtained (Fig. 2b) for the three sample mixtures (HRV 2/BRV, HRV 3/BRV, HRV 9/BRV) analysed in the same run (Fig. 2a).

During ultracentrifugation the absolute particle density was found to increase at a constant rate in time (2 to 12 h); this rate differed from one virus preparation to another. Consequently, the density differences in BRV/HRV mixtures were also a function of time. This is illustrated by two examples in Fig. 3(a). The increase in absolute density of all particles must be due to a physical change, possibly the influx of CsCl or the loss of capsid protein. Therefore, in order to obtain data about the particles in their original state at the start of the experiment, the data shown in Fig. 3 (a) were extrapolated to time zero (Fig. 3a) and plotted against the differences in additionally packaged bp (Fig. 3b). A straight line by definition passing through the origin (\( \Delta \rho = 0, \Delta bp = 0 \)) was obtained. These results indicate that the earlier the recordings are taken, the more representative they are of the viruses in their original state. The data shown in Fig. 2 (t = 2 h 15 min) give a result for the differences in density per number of additional bp which is very close to that obtained from the extrapolated values (t = 0) in Fig. 3. Therefore the data of Fig. 2 correctly describe the density differences for the viruses in their original state.

In velocity sedimentation experiments, no differences in the apparent S value of BRV and the HRVs was observed; BRV and HRV of genotype 7 both sedimented with apparent S values of 390 ± 8. This corresponds to an S value under standard conditions \( s_{20,w}^0 \) (0% sucrose, 20 °C) of 440 Svedberg units (results not shown).

The biophysical data reported here further characterize rotaviruses with genome rearrangements. They show that up to 1800 additional bp, found as concatemeric forms in segment-specific, rearranged bands of genomic dsRNA, are packaged into virus particles without evident physical constraint. The apparent particle size does not change measurably. The value of approximately 55 nm for the diameter of single-shelled particles is in agreement with data reported by McNulty (1979). The apparent S value of 390 agrees well with that measured by Tam et al. (1976) for single-shelled particles containing RNA of density 1.38 g/ml. As the RNA content of rotavirus particles is approximately 15% by weight (for reovirus particles 14-6% RNA content was found; Gomatos & Tamm, 1963), an additional 2 to 10% of RNA packaged will amount to a differential RNA content in particles of 0-3 to 1-5% changing the apparent S value by that amount. The s.d. in the determination of the S value under the conditions used was 2%, explaining why the subtle change was not registered.

The change in density, however, could be resolved on CsCl gradients by equilibrium centrifugation and could be accounted for by the extra amount of RNA packaged. Tam et al. (1976) saw four different bands of rotaviruses in CsCl density gradients after equilibrium ultracentrifugation. These had specific densities of 1.28, 1.30, 1.36 and 1.38 g/ml corresponding to double-shelled empty, single-shelled empty, double-shelled and single-shelled particles both containing RNA, respectively, as determined by electron microscopy. Similar data were obtained by Rodger et al. (1975). Thus, packaging of the total standard rotavirus genome of 18600 bp increases the specific density of particles by 0.08 g/ml. By simple scaling, this predicts density increases of 0-0019 to 0-0077 g/ml (0-024 x 0-08 to 0-096 x 0-08) for packaging of 450 to 1800 additional bp. These values are in close agreement with the experimental data obtained.

The results show that rotaviruses have considerable capacity to package additional genomic RNA, and one wonders what the upper limit of this capacity might be. Whereas a total of 11 RNA segments/rearranged bands are invariably packaged, there seems to be much less
constraint on the length of individual RNA segments/bands assembled into the maturing virus particle.

As genome rearrangements have recently also been found in rotaviruses isolated from immunocompetent hosts, both human (Besselaar et al., 1986) and animal (calves: Pocock, 1987; rabbit: Thouless et al., 1986; pigs: Bellinzoni et al., 1987), and also in orbiviruses (Eaton & Gould, 1987), it is suggested that genome rearrangements are an important mechanism of the evolution of rotaviruses and possibly of other double-stranded RNA viruses.

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