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Biophysical and biochemical properties of an unusual birnavirus pathogenic for rotifers

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A cytoplasmic dsRNA virus, rotifer birnavirus (RBV), has recently been isolated from the rotifer *Brachionus plicatilis* and is associated with a high mortality rate. Histologically, the viral lesions consist of characteristic inclusions, particularly amorphous dense bodies containing occluded particles. Purified virions are about 59 nm in diameter, single-shelled and display four capsomers per edge. The purified virions have a buoyant density of 1·290 (full particles) and 1·250 (empty particles) in CsCl gradients. Four major structural polypeptides of M_rs 60K, 52K, 33K and 27K were detected by SDS–PAGE. The genome is composed of two linear segments of dsRNA with M_rs of $2{\cdot}45 \times 10^6$ and $2{\cdot}31 \times 10^6$; additionally, small circular ssRNA molecules were detected by electrophoresis in overloaded agarose gels, but their significance is currently unknown. Except for this last feature and the structural instability of purified virions under freeze storage, all the other biochemical and biophysical characters indicate that RBV is a member of the *Birnaviridae* family with, for the moment, a unique position in this group.

Introduction

Rotifers are minute aquatic multicellular invertebrates of the phylum *Rotifera*. The species *Brachionus plicatilis* is cultivated in marine fish hatcheries for first-feeding of the fry. Problems have occurred in its production for several years but the causes have never been established clearly; recently, however, a virus was detected by electron microscopy and subsequently isolated from dying rotifer populations (Comps *et al.*, 1990, 1991). Some similarities with the birnavirus group, as defined by Dobos *et al.* (1979), were reported previously, i.e. its major morphological features and the probable presence of two pieces of RNA forming the genome. We report here further studies on rotifer birnavirus (RBV) which reveal some unusual biochemical and biological properties which have not been described so far for other birnaviruses.

Methods

Source of rotifers. For all experiments, frozen *B. plicatilis* from Station Ifremer, Palavas, France from dying populations reared in 2200 l culture tanks using the semi-continuous culture method (Lubzens, 1987) were used.

Virus purification. Depending on the experiment, 30 to 100 g of rotifer was homogenized in TN buffer (0·02 M-Tris–HCl, 0·4 M-NaCl, pH 7·4) using a glass tissue blender. After clarification by centrifugation for 10 min at 4500 r.p.m. in a JS 13.1 rotor on a J2-21 Beckman centrifuge, the supernatant was pelleted for 1 h at 28000 r.p.m. in an SW28.1 rotor on a L7 Beckman ultracentrifuge and resuspended in TN buffer. The viral suspension was then treated with active charcoal and filtered on Celite 535. This clear extract was pelleted and resuspended once more prior to being centrifuged through a 25 to 45% (w/w) sucrose gradient for 1 h at 28000 r.p.m. in an SW28.1 rotor. In addition, some preparations were extracted three times with Freon 113 (1,1,2-trichlorotrifluoroethane) before centrifugation through the sucrose gradient. Fractions of 1 ml were recovered from the gradient using a Büchler Auto-densiflow equipped with a u.v. absorbance monitor (ISCO UA5) which recorded the absorbance at 254 nm. Virus-containing fractions were pooled and diluted in TN buffer, then pelleted for 1 h at 28000 r.p.m. in an SW28.1 rotor. Final purification was achieved by isopycnic centrifugation (15 h at 28000 r.p.m. in an SW28.1 rotor) in a preformed 25 to 37% (w/w) CsCl gradient in 0·1 × PBS. As for sucrose gradients, 1 ml fractions were collected and the absorbance was measured. For density determination, the refractive index was measured by sampling the first three drops from each of two tubes. For further analysis, fractions containing virions were pooled, diluted and centrifuged for 1 h at 28000 r.p.m. in a SW28.1 rotor. The final pellets were resuspended in 0·5 ml 0·1 × PBS.

Effect on purified virions of freezing. Aliquots of purified virus suspension were stored at 4 °C, −35 °C and in liquid nitrogen (−180 °C). Each day, a sample was removed and virus particles were observed by transmission electron microscopy on negatively stained grids.

Electron microscopy. Infected rotifers were pelleted, fixed in 4% glutaraldehyde, 2% osmium tetroxide and embedded in Epon. Ultrathin sections were stained by the method of Reynolds (1963).

Purified virus suspensions were negatively stained with 2% sodium phosphotungstate (PTA) pH 7. Suspensions of purified tobacco mosaic virus and negatively stained catalase crystals were used as standards for size calibration.

Extracted nucleic acid was spread on carbon-coated grids according to the method of Delain & Brack (1974) and rotationally shadowed with platinum–palladium. Molecule length was measured using a Numonics digitizer with a microcomputer using the Digikick version 1.0 (ArpAsoft) program. Circular molecules of ss and ds ΦX174 DNA [native and replicative (RF) forms] were used as the internal standard. Observations were made using a Hitachi HU11C electron microscope operating at 75 kV.

Cell lines. Fish cell lines (BF2 and RTG) were used for virus isolation. Cell cultures were inoculated either with suspensions of purified full virus particles or with dilutions of freshly extracted, infected rotifers.

Nucleic acid extraction. Suspensions of full virus particles were firstly digested with proteinase K (final concentration 50 μg/ml) at 37 °C for 2 h and then treated with Sarkosyl (final concentration 50 μg/ml) for 2 h at the same temperature. The nucleic acid was phenol–chloroform-extracted (Maniatis *et al.*, 1982) and precipitated overnight with cold ethanol. The pellet was dried and resuspended in 50 μl 0·1 × TE buffer (Maniatis *et al.*, 1982).

Spectrophotometry. The absorbance of the purified nucleic acid was recorded between the wavelengths 200 and 340 nm using a Kontron K8000 spectrophotometer. To characterize the RNA, neutral formaldehyde was added to a final concentration of 1·8% and spectra were recorded over 24 h. The final measurement was made after heating the sample at 100 °C for 20 min and then quickly cooling on ice.

Protein and nucleic acid dosages. The amount of viral protein was determined by the colorimetric method of Lowry *et al.* (1951), modified by Hartree (1972). The amount of viral RNA was determined by the orcinol method (Mejbaum, 1939).

SDS–PAGE of viral proteins. Full and empty viral particles were treated with dissociating medium (0·1% SDS, 1% 2-mercaptoethanol, 5 M-urea), heated to 100 °C for 3 min and electrophoresed on a 10% polyacrylamide gel using the buffer 0·1 M-Na_2HPO_4, NaH_2PO_4 pH 7 containing 0·1% SDS. Polypeptides were separated by PAGE using an electrophoresis tank (GE 2/4 LS; Pharmacia) and revealed by staining the gel with Coomassie blue, or by silver staining (Daiichi silver stain kit). For M_r determination, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and α-lactalbumin (M_rs 94K, 67K, 43K, 30K, 20·1K and 14·4K, respectively) were used as markers. The M_r of the viral polypeptides was estimated by the method of Weber & Osborn (1969).

Agarose gel electrophoresis. Electrophoresis was performed on 1·5% agarose gels containing 0·5 μg/ml ethidium bromide using the method of Maniatis *et al.* (1982). Lambda *Hin*dIII, and Lambda *Eco*RI and *Hin*dIII DNA digests were used as M_r markers. For determination of the M_r of the RBV genome pieces, we used P virus genome segments as a reference; this reo-type virus, isolated from the marine crab *Macropipus depurator* (Bonami, 1973, 1980; Bonami *et al.*, 1976), is used in our laboratory as an M_r marker for dsRNA after being standardized by co-electrophoresis with RNA from human reovirus 3 (H. Montanié, unpublished data). The P virus genome consists of 12 pieces of dsRNA, ranging in M_r from $2{\cdot}60 \times 10^6$ to $0{\cdot}51 \times 10^6$; the fragments are seen on a 10% polyacrylamide gel, but bands 2 and 3 cannot be separated on an agarose gel.

Some viral suspensions and nucleic acid samples were digested with RNase A, DNase I and nuclease S1 (Maniatis *et al.*, 1982) prior to analysis by agarose gel electrophoresis.

Results

Ultrastructure of viral lesions

In thin sections of infected rotifer cells, virions appeared icosahedral or spherical in shape, unenveloped and single-shelled, with a diameter between 52 and 56 nm. In the cytoplasm of infected cells, beside a spread of individual virions, three types of inclusions were seen. The first (I1) consisted of a large, irregular, finely granular and electron-dense area; the second (I2) was large rod-shaped, amorphous and highly electron dense bodies; the third (I3) was formed by the association of viral particles in paracrystalline arrays (Fig. 1*a* and *b*).

RBV morphogenesis occurs in the cytoplasm from I1 inclusions which we thought to be viroplasms; virions develop at the periphery and also in the inner part (Fig. 1*b*) of these virogenic areas. In heavily infected cells, the particles were spread throughout the whole cytoplasm. In some areas, complete virus particles were associated to form paracrystalline arrays (I3), 1·5 to 2·0 μm in size. Virions often were occluded into I2 bodies which were detected easily by light microscopy on fresh squashes of infected rotifers because of their size (about 2 to 7 μm) and their refringence. However, because of the lack of crystalline structure and despite containing occluded virions with a polygonal profile, it was not possible to put the I2 inclusions in the same category as occlusion bodies of insect cytoplasmic polyhedrosis viruses (Payne & Harrap, 1977; Hukuhara & Bonami, 1991).

Purification and buoyant density of the virions

After centrifugation in a sucrose density gradient, virions were located in the upper part of the tube and two bands of empty and full particles could be distinguished. In the CsCl isopycnic gradient (Fig. 2), the virions formed two distinct bands: empty particles in an upper band with a density of $1{\cdot}250 \pm 0{\cdot}005$ and the lower band containing full virions with a density of $1{\cdot}290 \pm 0{\cdot}005$. After negative staining, electron microscopy revealed a good level of purification and separation of both types of particles.

Purified virions are hexagonal in shape and exhibit a mean diameter of 59 nm, with an average of 56 ± 1 and 61 ± 1 nm ($n = 50$) side-to-side and point-to-point respectively (Fig. 3*a*); these values are a little higher than those measured on sections, a fact which is commonly attributed to shrinkage in sections caused by the fixative used, i.e. glutaraldehyde (Dawes, 1980). Empty particles exhibited an electron-dense centre surrounded by a single layered capsid composed of substructural elements consisting of spherical capsomers about 7 nm in diameter; four capsomers (Fig. 3*b*) were distinguished

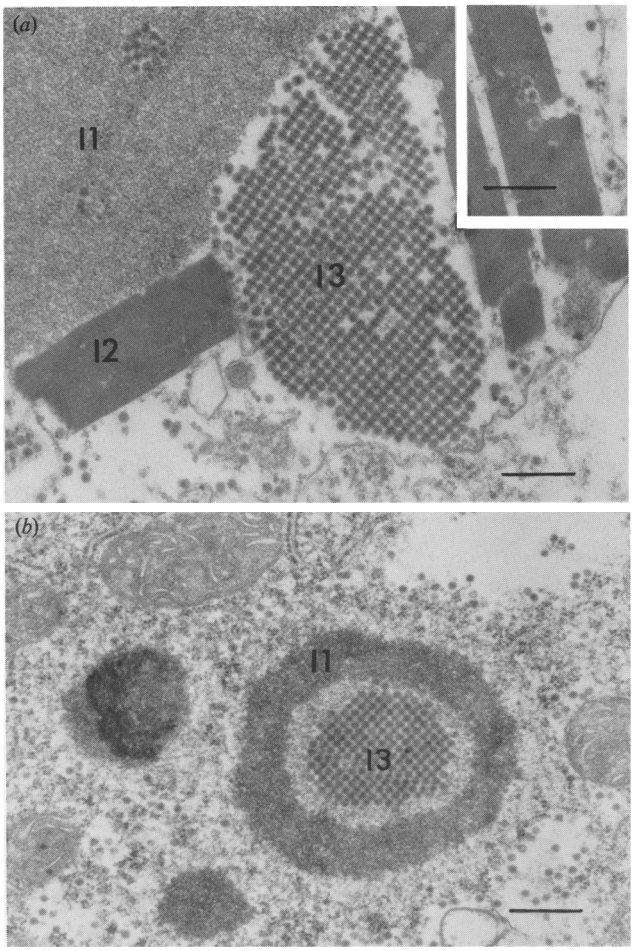

Fig. 1. (*a*) Ultrathin section of a typical RBV lesion in the cytoplasm of a stomach gland cell. All three types of inclusions are present. I1, granular virogenic stroma; I2, amorphous inclusion showing occluded particles (inset); I3, paracrystalline array of virions. (*b*) Different aspects of RBV morphogenesis; I3 (paracrystalline array of virions) is directly formed in the I1 inclusion (virogenic) area. Bar markers represent 0·5 μm.

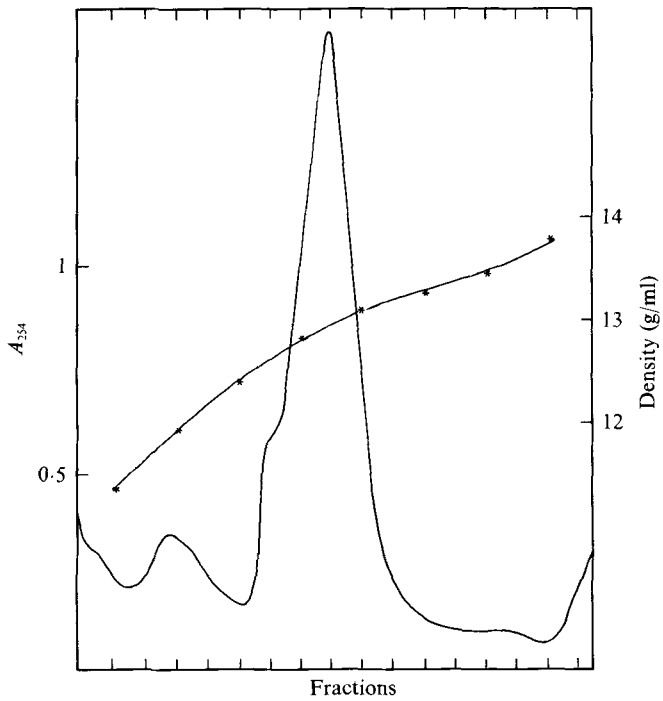

Fig. 2. CsCl equilibrium centrifugation of RBV virions. Solid line, A_{254}; *, density.

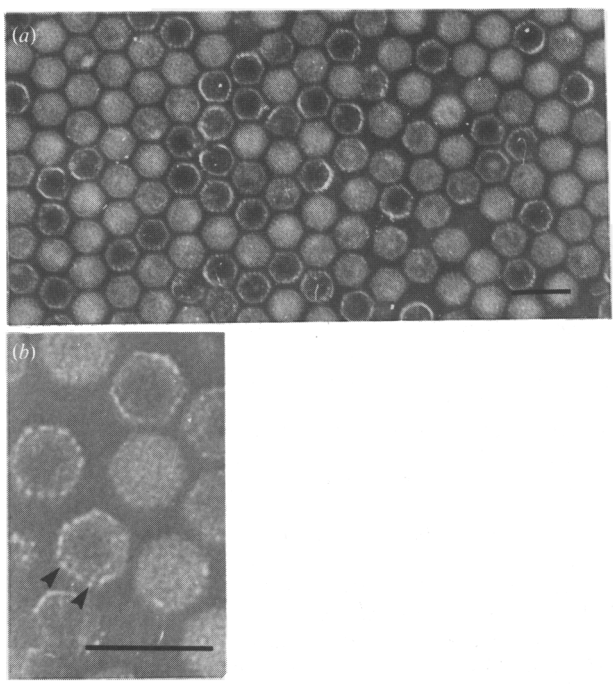

Fig. 3. (*a*) Purified suspension of RBV. (*b*) Detail showing the four capsomers per edge (arrows). Negative staining with PTA. Bar markers represent 100 nm.

per edge. Moreover, tubular elements about 60 nm in diameter and 0·3 to 1·0 μm long also were present in suspensions of empty particles (Comps *et al.*, 1991) which consisted of capsomer-like structures 7 nm wide organized in parallel lines at an angle of 60 ° to the axis of the tubule.

Infection of cell lines

To date, all attempts to cultivate RBV in BF2 and RTG cells have been unsuccessful, despite two blind passages; no c.p.e. was observed nor was any viral replication detected by electron microscopy. This negative result emphasizes the unusual aspect of this agent in comparison with all other aquatic birnaviruses (Hill, 1976*a*; Hill *et al.*, 1982; Underwood *et al.*, 1977; Dobos *et al.*, 1979; Nicholson *et al.*, 1979; Bovo *et al.*, 1984; Clotilde, 1984), which are known to infect such cell lines and produce well defined c.p.e.

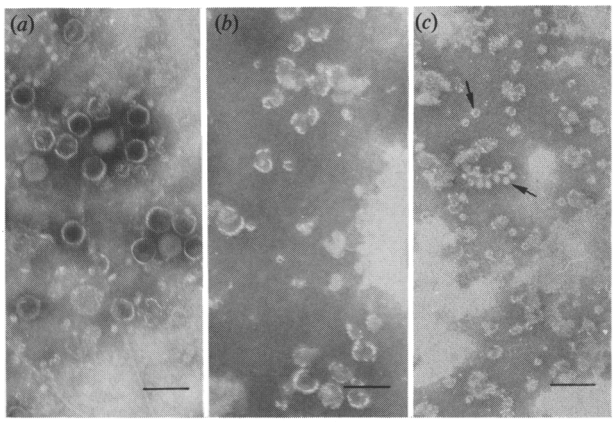

Fig. 4. Stages of degradation of RBV virions at −180 °C after 1 h (*a*), 1 day (*b*) and 4 days (*c*). Arrows in (*c*) point at reassociated capsomers. Bar markers represent 100 nm.

Sensitivity of purified particles to freezing and thawing

Our early results showed that purified virus was quickly damaged by freezing and thawing; freezing at −35 °C and −180 °C confirmed the high sensitivity of purified virions to freezing at low temperatures (Fig. 4). After 1 h at −180 °C the particles appeared empty and irregular in shape; after 1 day the capsids began to be disrupted and after 4 days, subunits of the capsid were separated in the preparation and some had re-associated to form spherical structures, 21 to 25 nm in diameter. However, this degradation of virions was not noticeable when virus-infected rotifers were deep-frozen, even after several months.

Chemical properties of RBV

The RNA nature of the viral nucleic acid had been suggested already by the flame red cytoplasmic fluorescence of virus-infected cells stained with acridine orange (Comps *et al.*, 1991); the positive reaction with orcinol confirms that the RBV genome consists of RNA.

After quantitative analysis of the nucleic acid and proteins from full particles from three different preparations, the percentage of nucleic acid contained in the virions was estimated to be about 6·6%, close to the value of 5·75% obtained by spectrophotometric measurements on purified virions (Layne, 1957).

Virus polypeptides

By SDS–PAGE, four polypeptides (VP1 to VP4) were revealed after Coomassie blue staining; their M_rs were 60K, 52K, 33K and 27K, respectively (Fig. 5). Identical results were obtained using either Freon-extracted or non-Freon-extracted particles. After silver staining,

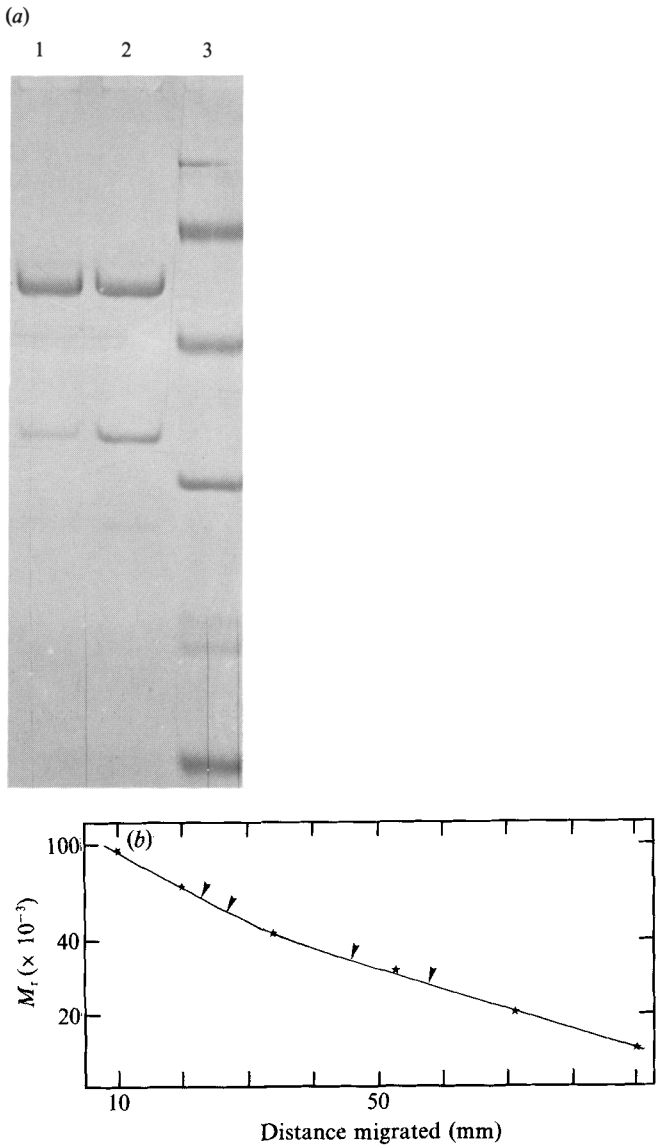

Fig. 5. (*a*) SDS–PAGE of RBV (Coomassie blue staining). Lane 1, RBV without Freon treatment; lane 2, RBV extracted with Freon; lane 3, protein markers. (*b*) Determination of the M_r of RBV polypeptides (arrows); protein markers (stars).

minor bands were seen with M_rs ranging between 29K and 48K.

Nucleic acid

Extracted RNA exhibited a typical spectrum with a maximum absorbance at 258 nm and a minimum at 230 nm; the A_{260}/A_{280} was close to 2. When treated with formaldehyde, the absorbance values of the RNA sample were only slightly modified, but after heating at 100 °C a 22% increase in hyperchromicity was recorded, indicating that the nucleic acid has a double-stranded structure (Fig. 6).

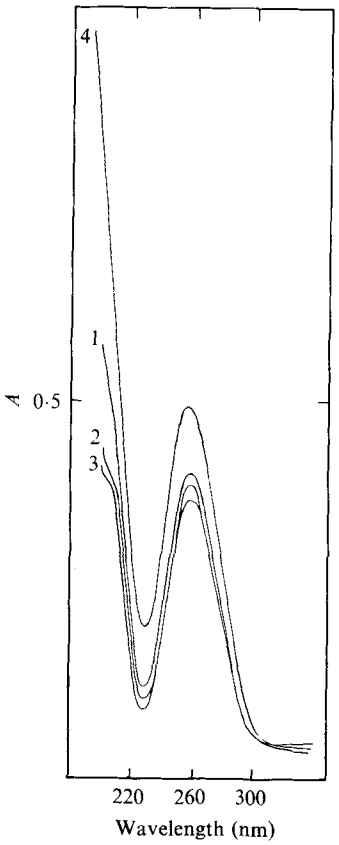

Fig. 6. Formaldehyde treatment of RBV RNA purified after 1, no heating; 2, 3 and 4, heating at 100 °C for 30 min, 16 h and 24 h.

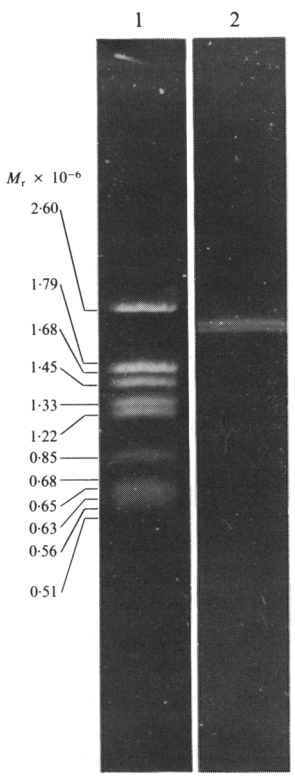

Fig. 7. Agarose gel electrophoresis of extracted RBV genomic RNA. Lane 1, P virus RNA (marker); lane 2, RBV RNA.

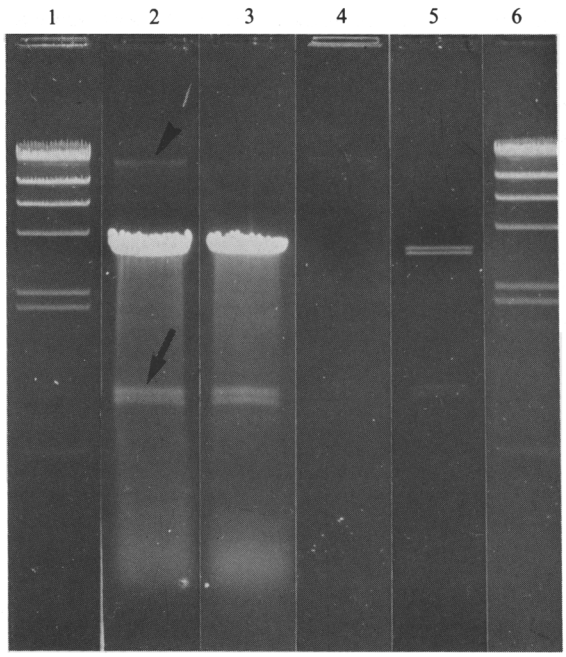

Fig. 8. Agarose gel electrophoresis of extracted RBV nucleic acid after various treatments. Lanes 1 and 6, Lambda DNA digested with *Hin*dIII. Lane 2, extracted RBV nucleic acid (overloaded well); both genomic segments are indistinguishable in the same band; two light bands are clearly seen (arrow); a heavy band (bacterial DNA contaminant) is used as a control for nuclease treatment (arrowhead). Lane 3, DNase treatment of RBV RNA; only the DNA contaminant is digested. Lane 4, RNase treatment of RBV RNA; only the DNA contaminant is still present. Lane 5, nucleic acid extracted from Freon-treated RBV; light bands are also present.

By agarose gel electrophoresis, two bands were observed (Fig. 7). Using P virus dsRNA pieces as markers, the M_rs of these bands were $2{\cdot}45 \times 10^6$ and $2{\cdot}31 \times 10^6$, respectively. When wells were overloaded with extracted nucleic acid, unusual additional bands of small size (between 1 and 0·5 kbp) were seen, suggesting a 'plasmid-like' pattern (Fig. 8). These bands were obtained always from either purified virions without or with Freon treatment, or from virions treated with DNase I and RNase A (Fig. 9). RNase A digested these bands, but DNase I was ineffective, indicating that this component was RNA (Fig. 8).

After treatment of the total extracted nucleic acid with nuclease S1, the additional bands were digested, but the genomic segments were not (Fig. 9); this result indicates that the two genomic pieces are made up of dsRNA, confirming the result obtained previously from formaldehyde treatment, whereas the small bands are single-stranded.

Observation of total extracted nucleic acid by electron microscopy after rotational shadowing revealed two types of molecules (Fig. 10): linear molecules (two genomic segments, about 1·3 and 1·4 μm in length, respectively) and small circular molecules (0·34 μm in

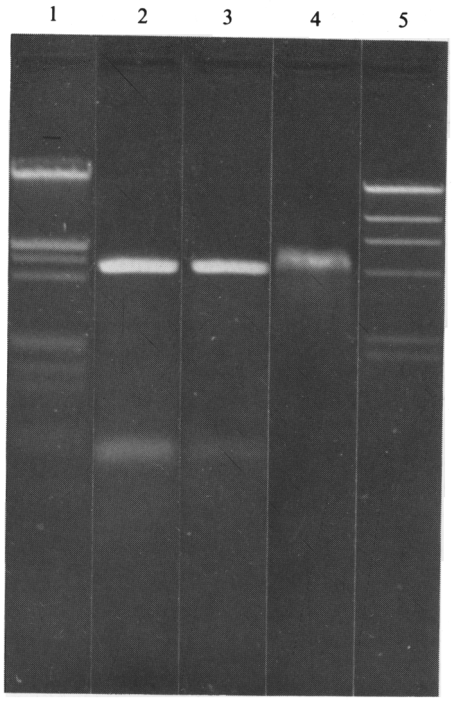

Fig. 9. Agarose gel electrophoresis of RBV dsRNA. Lane 1, Lambda DNA digested with *Hin*dIII and *Eco*RI; lane 2, RBV RNA extracted from purified virions without treatment; lane 3, RBV RNA extracted from purified virions previously treated with RNase A and DNase I; lane 4, RBV RNA treated with nuclease S1; lane 5, Lambda DNA digested with *Hin*dIII.

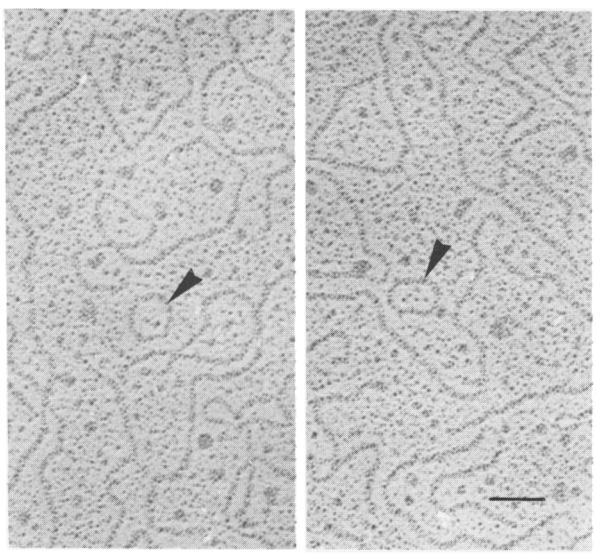

Fig. 10. RBV RNA molecules in extracted nucleic acid. Note the presence of small circular dsRNA molecules (arrows) among the linear dsRNA molecules of the genome. Bar marker represents 1 mm.

length). Compared with the size of the RF ϕX174 DNA which contains 5386 bp, the size of the two segments of the RBV genome can be estimated at about 3150 and 2950 bp, or an M_r of $2{\cdot}09 \times 10^6$ and $1{\cdot}95 \times 10^6$, respectively. But, according to Rixon *et al.* (1984) and Taylor *et al.* (1985), a correction factor of 1·16 to 1·17 must be applied; this gives values for the genomic pieces of about 3670 and 3430 bp, i.e. M_rs of $2{\cdot}43 \times 10^6$ and $2{\cdot}27 \times 10^6$ respectively, and for the small circular molecules about 1180 bases and an M_r of $0{\cdot}4 \times 10^6$. These M_r values for the two genomic segments are very close to those obtained from gel electrophoresis using the P virus as a marker.

Discussion

In the first reports on the viral infection of the rotifer *B. plicatilis*, considering essentially the morphological character of the virions, we suggested that this virus was closely related to the *Birnaviridae* (Comps *et al.*, 1990, 1991). This is confirmed by the present study on the physicochemical properties of this virus. Indeed, the genome was established as being one or two segments of linear dsRNA, one of the fundamental characteristics of birnaviruses as defined by Dobos *et al.* (1979).

From the size, shape and structure of the capsid and from buoyant density values, purified RBV particles look like all other described birnaviruses, i.e. drosphila X virus, infectious bursal disease virus, oyster virus, Tellina virus (TV), crab virus and infectious pancreatic necrosis virus (IPNV) (Dobos *et al.*, 1979; Bonami & Adams, 1991).

We consider the values obtained for the M_r of the RBV genome by comparison with the dsRNA marker (P virus) to be the most accurate, i.e. $2{\cdot}45 \times 10^6$ and $2{\cdot}31 \times 10^6$ for the two segments. These values are similar to those obtained by length measurement of the molecules by electron microscopy, are close to the values determined for IPNV and TV ($2{\cdot}4 \times 10^6$ and $2{\cdot}2 \times 10^6$; Dobos *et al.*, 1979), and are within the range of M_r values for this virus group.

Like the majority of birnaviruses, RBV contains four viral polypeptides, with VP2 and VP3 being the major components; in contrast, no polypeptide with high M_r (between 90K and 100K) was detected in RBV.

By its size, its icosahedral symmetry, the presence of four capsomers per edge, its single-layered capsid and its bisegmented linear dsRNA genome, RBV is clearly related to the birnaviruses as defined by Dobos *et al.* (1979). Nevertheless, there are some unique aspects of this new virus. In contrast to the birnaviruses of aquatic invertebrates (Hill, 1976*a*, *b*; Underwood *et al.*, 1977; Hill & Alderman, 1977; Hill *et al.*, 1982; Clotilde, 1984; Bovo *et al.*, 1984), RBV does not replicate in the fish cell lines BF2 and RTG2, and we have no evidence that this virus can replicate in fish fed with infected rotifers. However, the high rate of diseased rotifers in some hatchery cultures and the large cytoplasmic amounts of

virus particles allow isolation of the virus and employment of the usual purification procedures used for most other non-cultivatable viruses of marine invertebrates (Bonami, 1980; Mari, 1987).

The most unusual feature of RBV, compared with other marine invertebrate birnaviruses, is the occurrence of lesions characterized by the constant presence of virus particles in the cytoplasm of epithelial cells during the course of infection. Developed from virogenic areas interpreted as viroplasms, the virions form paracrystalline arrays frequently associated with large rod-shaped inclusions of electron-dense material. Some viral particles are occluded in such inclusions; the chemical composition of these inclusions has not been defined and their significance is uncertain. The absence of a clearly defined crystalline structure does not allow us to interpret these inclusions as occlusion bodies similar to those described in insects infected with cytoplasmic polyhedrosis virus (Matthews, 1982).

The high sensitivity of the RBV virions to freezing is not characteristic of the aquatic birnaviruses except, but to a lesser extent, TV1 (serogroup B1) isolated from *Tellina tenuis* (B. J. Hill, personal communication).

At the molecular level, an unusual feature is the constant presence of small RNA elements (as demonstrated by RNase A digestion) associated with the particles and co-extracted with the genomic dsRNA. Because they are present after treatment of purified virus with RNase A and DNase I prior to nucleic acid extraction, we hypothesize that they have an internal location, or at least are protected by capsid proteins. Their digestion by nuclease S1 indicates that they are single-stranded and electron microscopy reveals that they are circular. Their significance is unknown but the presence of several bands, as observed in gel electrophoresis, suggests that these circular molecules could occur in different forms (from supercoiled to circular and perhaps linear molecules). When observed by electron microscopy, the number of small circular molecules is relatively low compared with that of linear genomic strands, but it is difficult to identify whether they are coiled or supercoiled elements from our micrographs. To our knowledge, circular RNA structures closely associated with the genome have never been observed in the *Birnaviridae* family (Brown, 1986).

Some of the properties of RBV correspond to the essential characters of the *Birnaviridae* family as defined by Dobos *et al.* (1979), i.e. cytoplasmic particles, icosahedral shape, diameter of about 59 nm, the single-shelled capsid, four capsomers per edge and the bisegmented dsRNA genome (M_r 2·45 × 10^6 and 2·31 × 10^6). This family appears to be homogeneous, except perhaps for a recently described virus characterized by a bisegmented dsRNA genome, which has been proposed to be a 'picobirnavirus' (Pereira *et al.*, 1988). We propose that RBV be included in the *Birnaviridae* family. However, the presence of occlusion-like bodies in the cytoplasm of infected cells, the apparent absence of infectivity for the BF2 and RTG-2 cell lines, the high structural sensitivity to freezing, and the presence of small circular RNA molecules shared by the virions and co-extracted with the RNA genome, suggest that RBV could have a unique status within this group.

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