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## Biochemical and Biophysical Characteristics of Rio Bravo Virus (*Flaviviridae*)

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### SUMMARY

Rio Bravo (RB) virus has been assigned to the family *Flaviviridae* on the basis of its antigenic relatedness to other members of this family. RB virus, unlike most members of the *Flaviviridae*, is believed not to have an arthropod vector. We examined biochemical and biophysical characteristics of RB virus to determine whether it should be assigned to the *Flaviviridae* and to compare it with arthropod-borne flaviviruses. Purified RB virus banded at a density of 1.18 g/ml in sucrose and had a sedimentation coefficient of about 200 S. Virions, negatively stained with ammonium molybdate, were spherical, had diameters of 42 nm, and appeared to be surrounded by envelopes bearing surface projections. The loss of infectivity after infectious virus was incubated with diethyl ether or sodium deoxycholate confirmed the presence of envelopes. Partially purified RB virions contained single-stranded RNA, lacking 3' poly(A) tracts, that sedimented in a 15% to 30% sucrose gradient as one discrete band with a sedimentation coefficient of about 40 S. Most of the viral proteins in preparations of purified virus and in immunoprecipitates had similar electrophoretic mobilities and glycosylation patterns to known flavivirus proteins. Therefore, they were assigned the following tentative designations using the nomenclature for flavivirus proteins: gp52 and gp47, envelope proteins; gp46, non-structural protein 1; p25, gp20(prM), precursor to membrane protein; gp < 18K. Putative core and membrane proteins were not identified. These physical and biochemical characteristics of RB virus are remarkably similar to those of the arthropod-borne members of the *Flaviviridae* and they confirm the classification of RB virus in this family. This is the first report of biochemical and physical properties of a non-arthropod-borne member of the *Flaviviridae*.

### INTRODUCTION

Rio Bravo (RB) virus (bat salivary gland virus; Burns & Farinacci, 1956; Burns *et al.*, 1957; Johnson, 1962) was shown to be antigenically related to viruses which were earlier known as group B arboviruses, but are now classified in the family *Flaviviridae* (de Madrid & Porterfield, 1974; Berge, 1975; Westaway *et al.*, 1985). This family contains 64 members that include 42 viruses with known arthropod vectors (Brinton, 1986). Although the type species, yellow fever (YF) virus, and several mosquito-borne and tick-borne members have been well characterized (Brinton, 1986; Rice *et al.*, 1986), no detailed reports have been published on non-arthropod-borne flaviviruses.

RB virus is probably not arthropod-borne. It has not been isolated from field-collected (Johnson, 1962) or experimentally infected mosquitoes (Whitman, 1959) and it does not replicate in *Culex tarsalis* or *Aedes dorsalis* mosquito cell cultures (Main *et al.*, 1977; Cahoon *et al.*, 1978; Hendricks *et al.*, 1983). The possibility remains that a putative arthropod vector for

RB virus has not been discovered and for this reason it is included in the group of flaviviruses with unknown vectors. RB virus has been isolated repeatedly from Mexican freetail bats (*Tadarida brasiliensis mexicana*; Burns & Farinacci, 1956; Johnson, 1962; Sulkin, 1962; Constantine & Woodall, 1964; Allen *et al.*, 1970) and is believed to infect bats persistently (Constantine & Woodall, 1964; Baer & Woodall, 1966; Sulkin & Allen, 1974) and to be transmitted by aerogenic transmission (Constantine & Woodall, 1964). If it becomes clear from biochemical, physical and biological characteristics that RB virus is a flavivirus, then identification of the presumed block in its replication cycle in mosquito cell cultures may help to focus on the properties of arthropod-borne flaviviruses which allow them to be permissive in arthropods.

Biochemical and physical characteristics of members of the *Flaviviridae* are now well-defined (Westaway *et al.*, 1985) and they should be considered to be important criteria for inclusion of RB virus in this family. Flaviviruses are small (45 nm diameter), enveloped viruses which contain peplomers consisting of the envelope (E) glycoprotein and a nucleocapsid (diameter 20 to 30 nm) containing the core (C) protein. The third structural protein is the membrane-like (M) protein which is also associated with the envelope. The single-stranded RNA has a  $M_r$  of  $4.0 \times 10^6$ , a sedimentation coefficient of 44 S and a type 1 cap at the 5' terminus, but it does not contain a poly(A) tract at its 3' end.

We compared biochemical and physical properties of RB virus and flaviviruses and from these studies we confirm the assignment of RB virus to the *Flaviviridae*. We present here the first detailed report of biochemical and biophysical characteristics of a flavivirus that is considered not to be arthropod-borne.

#### METHODS

*Virus.* The prototype strain, M-64, of RB virus was obtained at the third intracerebral suckling mouse passage and a large plaque variant which grew to titres of  $10^7$  to  $10^8$  p.f.u./ml was derived by two cycles of plaque purification in cell culture.

*Cells and viral assays.* Clone 13 of BHK-21 cells was obtained from the American Type Culture Collection and grown in MEM (Gibco) supplemented with 10% foetal bovine serum (FBS), 10% tryptose phosphate broth and antibiotics. Plaque assays were carried out on Vero cells in six-well, cell culture plates (Falcon) as described previously (Hendricks *et al.*, 1983). Quantification of haemagglutination (HA) units was accomplished using a microtitre HA assay (Shope & Sather, 1979).

*Purification of RB virus.* Virus was concentrated and purified following a protocol that was derived from procedures described by several groups (Westaway & Reedman, 1969; Della-Porta & Westaway, 1972; Qureshi & Trent, 1973; Kitano *et al.*, 1974; Trent, 1977). Confluent monolayers of BHK-21 cells in 75 cm<sup>2</sup> cell culture flasks (Falcon) were infected at an m.o.i. of 1 and following viral adsorption for 1.5 h at 37 °C, MEM plus 0.2% bovine serum albumin (BSA) and antibiotics were added to each flask. Sixty to 72 h later, culture fluids were clarified and made 8% with respect to polyethylene glycol 6000 to precipitate virus which was then concentrated by centrifugation at 10000 g for 30 min. The viral pellet was resuspended in 1/30 of the original volume using TEN (0.01 M-Tris-HCl pH 7.8, 0.001 M-EDTA, 0.1 M-NaCl) buffer and this suspension was centrifuged at 120000 g for 1.5 h through a 10% to 30% sucrose gradient. Fractions containing the highest concentrations of virus as determined by radioactivity, infectivity or HA concentration, were pooled, diluted in TEN buffer and virus was centrifuged at 85000 g for 2.5 h into a 20% to 55% sucrose gradient. To determine the density of virus, fractions with the highest virus concentrations from the second centrifugation step were pooled, diluted and virus was centrifuged at 85000 g for 18 h into a 20% to 55% sucrose gradient. Densities of the fractions were determined from the refractive indices which were measured in a refractometer (American Optical). Sedimentation coefficients were determined by the method of McEwen (1967).

*Electron microscopic observation of purified virus.* A formvar-coated grid (300-mesh) was inverted on a drop of purified virus and after 1.5 h at room temperature, the grid was rinsed three times in phosphate-buffered saline. Preparations were stained with 1% aqueous ammonium molybdate (pH 6.8) for 15 s and virions were observed with a Zeiss EM 9S-2 electron microscope.

*Sensitivity of viral infectivity to lipid solvents.* Sensitivity of RB viral infectivity to diethyl ether or sodium deoxycholate was determined following the method of Shope & Sather (1979).

*Radiolabelling virus proteins or nucleic acid.* [<sup>3</sup>H]Leucine-labelled virus was grown in BHK-21 cells in 75 cm<sup>2</sup> flasks. Two days after infection, culture fluids were replaced with leucine-free MEM (Gibco) that was supplemented with 1/10 of the normal concentration of leucine and 2% dialysed FBS. Four h later, 5  $\mu$ Ci/ml of

[<sup>3</sup>H]leucine (70 to 100 Ci/mmol; New England Nuclear) was added to media and 24 h later virus was concentrated and purified from culture fluids as described in the previous section. Radiolabelled proteins that were used in radioimmunoprecipitation (RIP) assays were obtained from culture fluids and lysates of infected BHK-21 cells. Eighteen h after infection culture fluids were replaced with MEM supplemented with 2% FBS and either 50 µCi/ml of D-[1,6-<sup>3</sup>H(N)]glucosamine hydrochloride (43.2 Ci/mmol; New England Nuclear) or 100 µCi/ml of L-[<sup>3</sup>H(G)]serine (14.4 Ci/mmol; New England Nuclear). Two days after infection, culture fluids were removed from monolayers, adjusted to a concentration of 1 mM-PMSF, clarified by centrifugation at 12000 g for 5 min and stored at -70 °C. Monolayers were lysed in RIPA buffer (0.01 M-Tris-HCl pH 7.2, 0.15 M-NaCl, 1.0% Triton X-100, 1.0% sodium deoxycholate, 0.1% SDS, 1 mM-PMSF) and processed as for culture fluids. For preparation of [<sup>3</sup>H]uridine-labelled virus, medium from infected BHK-21 cells was replaced 4 h after infection with MEM that was supplemented with 0.2% BSA and 0.25 µg/ml actinomycin D (Sigma), a concentration which inhibited more than 90% of host transcription (unpublished data). Two h later, 10 µCi/ml [5,6-<sup>3</sup>H]uridine (36.8 Ci/mmol; New England Nuclear) was added to flasks. Two to 3 days after infection, virus was concentrated and purified from clarified culture fluids as described previously.

*Extraction of virion RNA.* [<sup>3</sup>H]Uridine-labelled RB viral RNA was extracted from viral preparations by an SDS-phenol method (Brawerman, 1974; Brawerman *et al.*, 1972). Viral preparations which contained 0.1 M-Tris-HCl pH 7.6 and 0.5% SDS were incubated with proteinase K (Boehringer Mannheim) at a final concentration of 50 µg/ml and then extracted with phenol. The phenol phase was re-extracted with 0.1 M-Tris-HCl pH 7.6 and then with 0.1 M-Tris-HCl pH 9.0 and RNA was concentrated by precipitation with ethanol using tRNA from *Escherichia coli* (Boehringer Mannheim) as carrier.

*Detection of poly(A)-containing viral RNA.* An oligo(dT)-cellulose (Collaborative Research, Waltham, Mass., U.S.A.) column was used to detect any viral RNA that contained poly(A) tracts, following the method of Aviv & Leder (1972).

*RIP assay.* The procedure for the RIP assay was described previously (Hendricks *et al.*, 1987). The normal mouse ascitic fluid and hyperimmune mouse ascitic fluid to RB virus which were used in these assays were research reference reagents, obtained from the National Institute of Allergy and Infectious Diseases, Bethesda, Md., U.S.A.

*SDS-PAGE.* Proteins were resolved by discontinuous SDS-PAGE (Laemmli, 1970). In brief, samples were boiled for 3 min in disruption buffer (final concentrations of 0.0625 M-Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol) and loaded on a vertical slab gel containing a 3% to 4% stacking gel and either a 10% or a 7.5% to 15% gradient separating gel. Following electrophoresis of non-radiolabelled preparations, gels were stained with Coomassie Brilliant Blue, photographed, destained with a solution containing 40% methanol and 10% acetic acid and then restained with a silver stain following the instructions of the manufacturer (Bio-Rad). After electrophoresis of radiolabelled preparations, gels were soaked for 30 min in ENLIGHTENING (New England Nuclear) and dried. Autoradiograms were prepared using Kodak Royal X-Omat film exposed at -70 °C.

## RESULTS

### *Sedimentation characteristics of RB virus*

RB virus that was purified from culture fluids of infected BHK-21 cells by two cycles of rate zonal centrifugation and one isopycnic centrifugation step produced one well-defined peak of infectivity coincidental with the peaks of radioactivity and HA units (Fig. 1). Most of the viral activity was concentrated in three fractions (7 to 9) which contained sucrose densities of 1.17 to 1.19 g/ml and 87% of viral infectivity in the gradient. Based on sedimentation of virus in the second rate zonal centrifugation step (data not shown), a Svedberg coefficient of 201.5S was calculated. A smaller and more diffuse band of slowly sedimenting material (fractions 11 to 14) could be clearly discerned only from the profile of radioactivity in the fractions. Although it is possible that this minor peak contained a slowly sedimenting (70S) haemagglutinin (SHA), described for flaviviruses (Russell *et al.*, 1980), it is likely that a putative SHA would have been resolved from virions in the two previous cycles of rate zonal centrifugation. The material in this band was not identified. Clearly, the density of RB virus and its sedimentation characteristics are consistent with its classification in the *Flaviviridae*.

### *Examination of RB virus by electron microscopy*

Observation of purified RB virus by electron microscopy revealed particles with spherical shapes and diameters of 42 nm (Fig. 2). Virions were homogeneous with regard to both shape and size. Although symmetry was indistinct, fivefold and sixfold axes of symmetry were

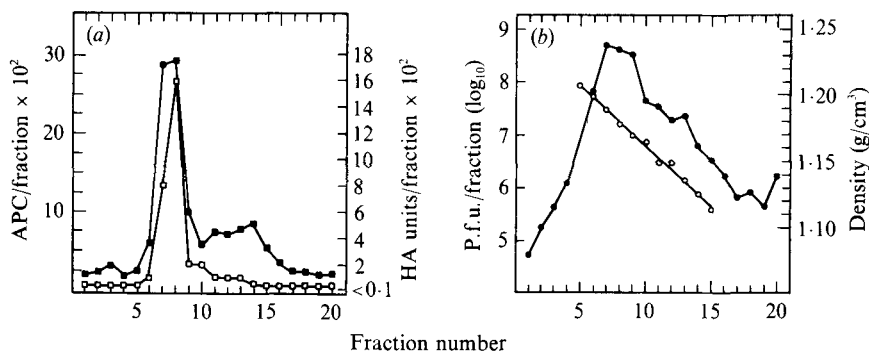


Fig. 1. Isopycnic centrifugation of RB virus. [<sup>3</sup>H]Leucine-labelled RB virus was concentrated from culture fluids and purified by two rate zonal centrifugation steps as described in Methods. Virus was then banded in a 20% to 55% sucrose gradient by centrifugation in an SW50-1 rotor at 85000 *g* for 18 h. The bottom of the gradient is fraction 1. (a) Acid-precipitable counts (APC) per minute, ■; HA units, □. (b) P.f.u., ●; sucrose density, ○.

Table 1. *Effects of lipid solvents on viral infectivity*

Lipid solvent	Solvent concentration* (%)	P.f.u./ml†
Diethyl ether	0	2.6 × 10 <sup>6</sup>
	50	<1.0 × 10 <sup>1</sup>
Sodium deoxycholate	0	3.1 × 10 <sup>5</sup>
	0.1	<1.0 × 10 <sup>1</sup>

\* Final concentration of lipid solvent in viral preparation.

† Residual infectivity after incubation for 16 h at 4 °C for the experiment with diethyl ether or for 1 h at 37 °C for the experiment with sodium deoxycholate.

suggested by pentagonal and hexagonal outlines of virions that were equally penetrated by negative stain (Fig. 2, insert). Virions appeared to be surrounded by envelopes close to the cores, and to be covered with peplomers. The presence of envelopes was confirmed by the loss of infectivity in viral preparations which were incubated with diethyl ether or sodium deoxycholate (Table 1). Some virions were penetrated by stain which occupied a space that was 29 nm in diameter; this may be the size of the viral core. The 'fibrous' material attached to numerous, apparently ruptured virions appeared similar to the 'osmotic blebs' observed by others (Murphy, 1980). The morphological characteristics of RB virus, a non-arthropod-borne flavivirus, are similar to those of the arthropod-borne members of the *Flaviviridae*.

#### *Partial characterization of virion-associated nucleic acid*

To determine whether RB virus contains a genome of RNA, virus was grown in BHK-21 cells that were incubated with [5,6-<sup>3</sup>H]uridine and virus, concentrated from culture fluids by precipitation with polyethylene glycol, was sedimented through a 10% to 30% sucrose gradient (Fig. 3). One peak of radioactivity (fractions 16 to 20) which coincided with viral infectivity and haemagglutination activity (data not shown) was obtained from fluids of virus-infected but not mock-infected cells. The smaller, less rapidly sedimenting peak of radioactivity (fractions 30 to 33) from the viral preparation may be due to intracellular, viral RNAs (e.g. 20 to 22S RNA; Brinton, 1986) or host RNAs which were released by infected cells. This hypothesis, however, is unlikely since viral cytopathogenic effects were not observed. These results demonstrate that RB virions contain RNA.

An effort was made to characterize further the virion-associated RNA by determining its Svedberg coefficient and whether it contains poly(A) tracts. [<sup>3</sup>H]Uridine-labelled virus that was

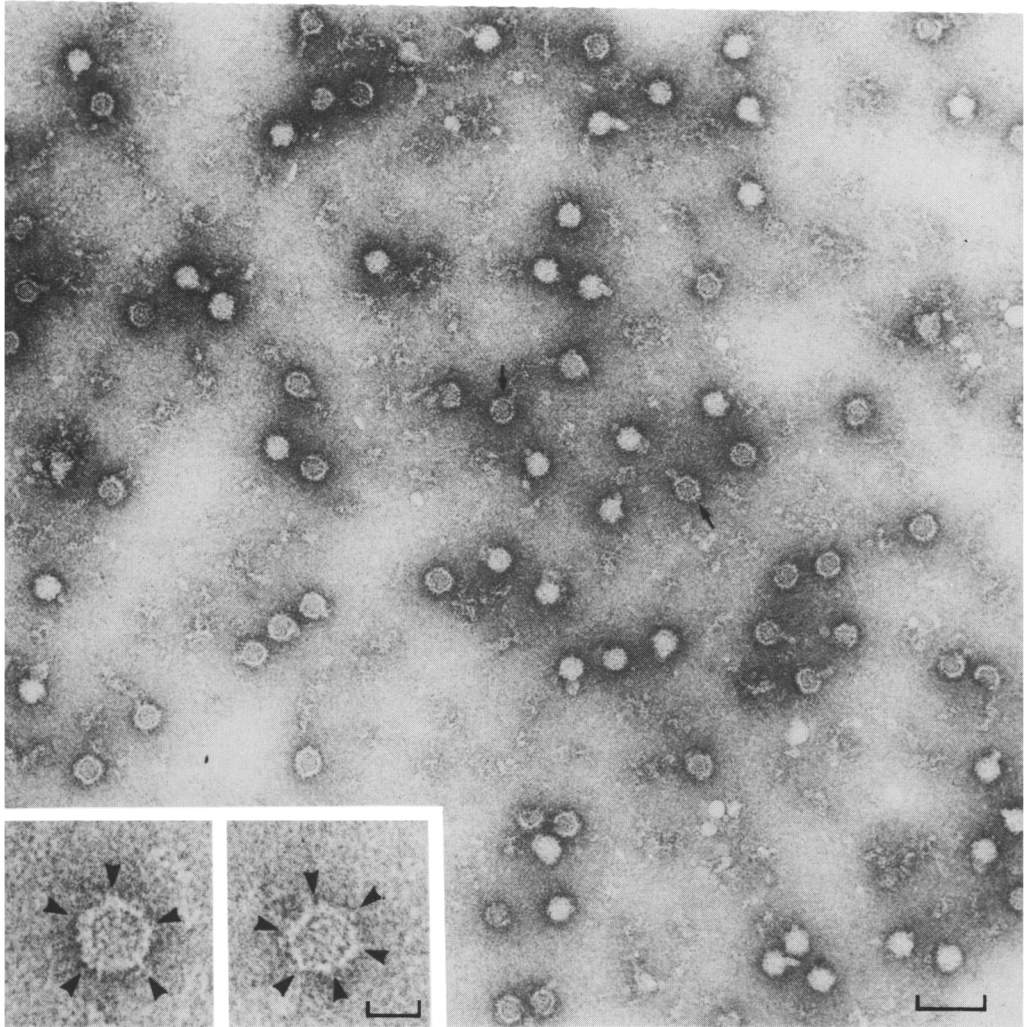


Fig. 2. Morphological characteristics of RB virus. RB virus was prepared for electron microscopy and stained with 1% ammonium molybdate as described in Methods. In main picture the arrows point to peplomers and the bar marker represents 100 nm. In the insets the arrows point to vertices of pentagonal and hexagonal outlines of virions and the bar marker represents 25 nm.

purified by one rate zonal centrifugation step (as shown in Fig. 3) was made 10% with respect to SDS and RNAs were resolved in a 15% to 30% sucrose gradient (Fig. 4); an  $s$  value was calculated from these data using the method of McEwen (1967). All of the radioactivity released from virions sedimented as one well-resolved band of about 40S. To determine whether virion RNA contained poly(A) tracts, [ $^3\text{H}$ ]uridine-labelled RNA was extracted from partially purified virus (as shown in Fig. 3) by the SDS-phenol method described in Methods and then passed over an oligo(dT)-cellulose column (Fig. 5). Most (87%) of the RB viral RNA did not anneal to the column, suggesting that it did not contain stretches of at least 10 adenine residues, the smallest number that will allow annealing. In contrast the oligo(dT)-cellulose column retained 97% of a commercial polyadenylate preparation which could be eluted by buffer containing no KCl. These results show that virion-associated RNAs did not contain poly(A) tracts at their 3' termini. The first three fractions from the oligo(dT)-cellulose column contained single-stranded

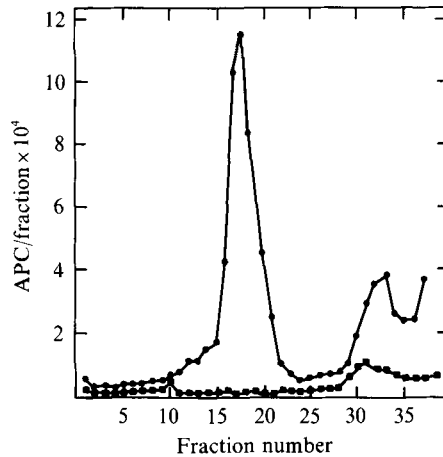


Fig. 3. Partial purification of [ $^3\text{H}$ ]uridine-labelled RB virus by rate zonal centrifugation. Material was concentrated from culture fluids as described in Results and resolved in a 10% to 30% sucrose gradient using an SW27 rotor and centrifugation at 120000  $g$  for 2.5 h. One ml fractions were collected and acid-precipitable counts (APC) per minute were determined. The bottom of the gradient is fraction 1. Virus infected,  $\bullet$ ; mock-infected,  $\blacksquare$ .

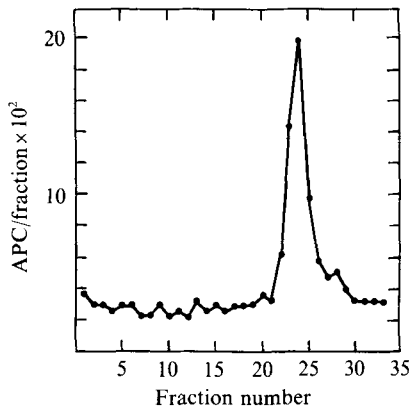


Fig. 4

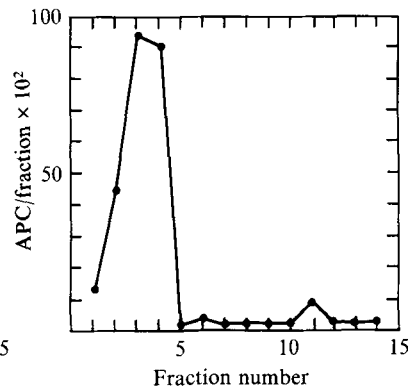


Fig. 5

Fig. 4. Resolution of virion-associated RNA in a 15% to 30% sucrose gradient. RNAs were resolved in a sucrose gradient by centrifugation at 195000  $g$  for 165 min. Fractions of 360  $\mu\text{l}$  were collected and acid-precipitable counts (APC) per minute were determined. The bottom of the gradient is fraction 1.

Fig. 5. Oligo(dT)-cellulose chromatography of RB virion-associated RNA. [ $^3\text{H}$ ]uridine-labelled RNA was loaded on the column in 0.01 M-Tris buffer (pH 7.6) containing 0.5 M-KCl and 1.0 ml fractions were collected until the absorbance at 254 nm was 0. After fraction 9, 0.01 M-Tris buffer (pH 7.6) without KCl was passed through the column and 1.0 ml fractions were collected. Acid-precipitable counts (APC) per minute were determined.

RNA since the radioactivity became acid-soluble following incubation of these fractions with ribonuclease T1 (10 units/ml) and bovine pancreatic ribonuclease A (0.05  $\mu\text{g}/\text{ml}$ ) at 37  $^{\circ}\text{C}$  for 15 min (data not shown).

#### *Identification of viral polypeptides*

Efforts were made to identify viral proteins by SDS-PAGE analysis of purified virus. Fig. 6 shows proteins in preparations of virus purified by two rate zonal centrifugation steps stained

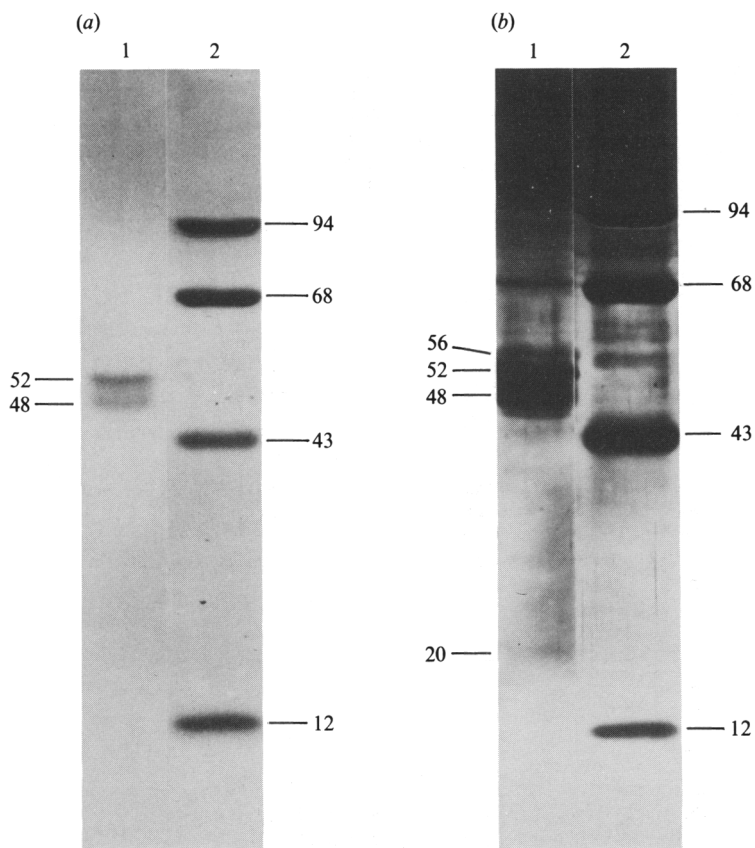


Fig. 6. SDS-PAGE analysis of virion-associated proteins. Virus that was purified in two rate zonal centrifugation steps was disrupted and proteins were resolved by SDS-PAGE in a 7.5% to 15% gradient gel. The gel was stained with Coomassie Brilliant Blue (a), destained, and then stained with a silver stain (b). Lane 1, purified virus; lane 2, standards whose molecular weights are shown in the right margins. Molecular weights of virion-associated proteins are shown in the left margins. All molecular weights are  $\times 10^{-3}$ .

first with Coomassie Brilliant Blue (Fig. 6a) and then destained and restained with a silver stain (Fig. 6b). Proteins with apparent  $M_r$  of 52K and 48K were the most prominent bands; they may be different forms of the E protein, an observation that has been reported for the E protein of YF virus (Schlesinger *et al.*, 1983). Two minor proteins of  $M_r$  56K and 20K only became apparent after the more sensitive silver stain was used. The smaller protein migrated with an electrophoretic mobility that was similar to the mobility of virion-associated prM (precursor to the membrane protein) proteins of other flaviviruses (Brinton, 1986; Westaway *et al.*, 1985). A small silver-stained protein, a putative M protein, migrated with the tracking dye in several gels (data not shown). Silver staining also revealed other minor bands that had  $M_r$  greater than 60K and that were likely to be protein contaminants in the electrophoresis buffer since they were also present in the gel between lanes.

To identify viral polypeptides more clearly, culture fluids or extracts of infected cells were assayed for [ $^3$ H]serine-labelled or [ $^3$ H]glucosamine-labelled viral antigens by immunoprecipitation (Fig. 7). Culture fluids contained an abundant or heavily labelled, virus-specific glycoprotein (putative E protein) whose heterogeneous electrophoretic mobility (apparent  $M_r$  51K to 63K) may have been due to molecules of different sizes or to different glycosylation patterns (Fig. 7a, lane 2). A similar, but less heavily labelled, heterogeneously migrating protein

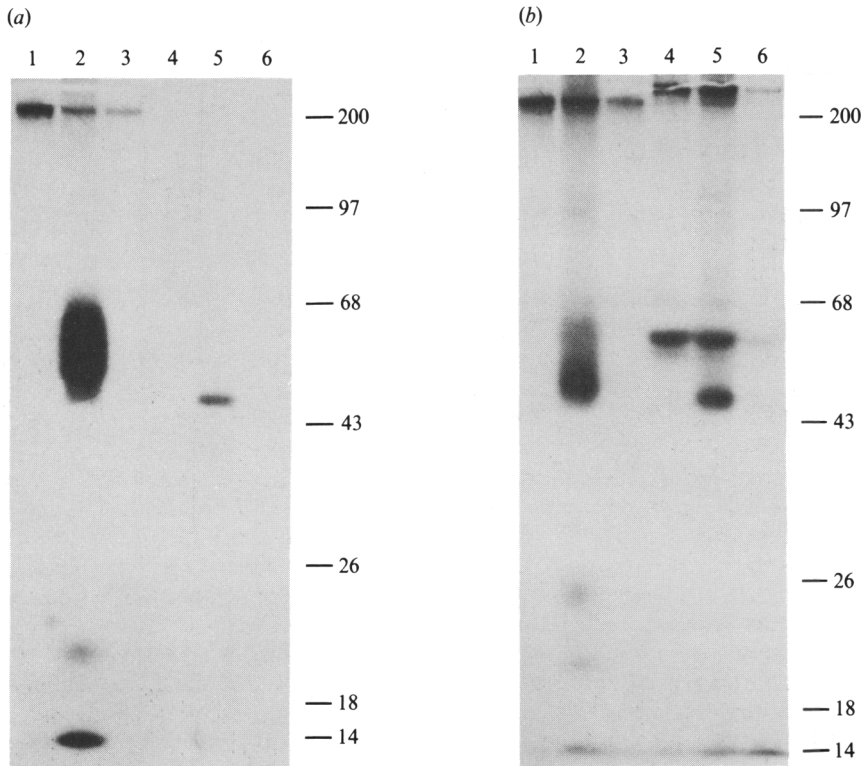


Fig. 7. SDS-PAGE analysis of immunoprecipitated viral proteins from culture fluids and cell extracts. RIPs were carried out as described in Methods using either [ $^3\text{H}$ ]glucosamine-labelled (a) or [ $^3\text{H}$ ]serine-labelled (b) proteins and either hyperimmune ascitic fluids to RB virus (lanes 1, 2, 4 and 5) or normal ascitic fluids (lanes 3 and 6). Precipitated proteins were resolved in 10% polyacrylamide gels. Lanes 1 to 3, culture fluid; lanes 4 to 6, cell lysate; lanes 1 and 4, mock-infected; lanes 2, 3, 5 and 6, RB virus-infected. Molecular weights of protein standards are shown in the right margins  $\times 10^{-3}$ .

was precipitated from the fluids of cells incubated with [ $^3\text{H}$ ]serine, and an additional, abundant species ( $M_r$  approx. 47K) was visible at the leading edge of this band (Fig. 7b, lane 2). The 47K protein, labelled with either [ $^3\text{H}$ ]serine or [ $^3\text{H}$ ]glucosamine, was resolved from the heterogeneous and larger protein in a 15% polyacrylamide gel (data not shown). The 47K protein appeared to have relatively few oligosaccharide moieties on the basis of its faint appearance after labelling with [ $^3\text{H}$ ]glucosamine. This protein was similar in size to the 48K protein noted in purified virions (Fig. 6).

Three additional virus-specific proteins in culture fluids were detected by RIP assays. Preparations of [ $^3\text{H}$ ]glucosamine-labelled antigens contained a 20K glycoprotein, perhaps the prM protein, and a heavily labelled or abundant glycoprotein which migrated with the tracking dye (Fig. 7a, lane 2). Because the small glycoprotein was not resolved from the tracking dye in a 15% polyacrylamide gel (data not shown), it was not assigned an  $M_r$ . Proteins with identical electrophoretic mobilities were detected in culture fluids that contained [ $^3\text{H}$ ]serine-labelled antigens (Fig. 7b, lane 2); these proteins are likely to be the 20K and very small glycoproteins. The [ $^3\text{H}$ ]serine-labelled preparation contained an additional protein with an  $M_r$  of 25K which is similar in size to the ns4a protein (formerly called NVX) of YF virus (Rice *et al.*, 1986). A putative C protein with an expected  $M_r$  of 13K to 16K was not identified.

Lysates of infected cells that were incubated with either [ $^3\text{H}$ ]glucosamine or [ $^3\text{H}$ ]serine contained only one virus-specific glycoprotein which had an apparent  $M_r$  of 46K (Fig. 7a and b, lanes 5), a size similar to the non-structural (NS1) glycoprotein of other flaviviruses (Rice *et al.*, 1986). The 60K, [ $^3\text{H}$ ]serine-labelled proteins that were precipitated from lysates of infected cells

were believed not to be virus-specific since they were also precipitated from mock-infected cells and from virus-infected cells by normal ascitic fluid.

#### DISCUSSION

The results in this report confirm the assignment of RB virus to the *Flaviviridae*. Non-arthropod-borne members of the *Togaviridae* (e.g. pestiviruses and rubella virus) have been partially characterized, in part because they cause important veterinary and human diseases; these studies have been summarized by Horzinek (1981), but this is the first study of a non-arthropod-borne flavivirus.

The sedimentation characteristics and appearance of RB virus by electron microscopy were similar to those features of arthropod-borne flaviviruses. The 200S sedimentation coefficient of RB virus is within the range of 175S to 218S reported for flaviviruses (Russell *et al.*, 1980) and somewhat smaller than the sedimentation coefficients of two togaviruses, Sindbis (270S) and rubella (240 to 350S) viruses (Horzinek, 1981). Likewise, the densities of RB virus, 1.18 g/ml and of flaviviruses, 1.19 to 1.20 g/ml in sucrose gradients are in agreement (Westaway *et al.*, 1985). RB viral particles are spherical and of uniform size with an average diameter of 42 nm, similar to the 45 nm diameter of Japanese encephalitis virus (Kitano *et al.*, 1974) and the 49 nm diameter of St Louis encephalitis (SLE) virus (Murphy, 1980), two arthropod-borne flaviviruses, and smaller than the average diameter (60 to 65 nm) of alphaviruses (Westaway *et al.*, 1985). Particles with diameters of 35 to 38 nm were observed in brains of newborn mice infected with Entebbe bat salivary gland virus (Peat & Bell, 1970) or Cowbone Ridge virus (Calisher *et al.*, 1969), two flaviviruses with unknown vectors. After penetration of some RB viral particles by negative stain, an internal area, perhaps occupied by the core, with a diameter of 29 nm, could be visualized. Similar sizes have been reported for putative cores of SLE and dengue viruses (Murphy, 1980). While viral symmetry was indistinct, particles that had pentagonal or hexagonal outlines, suggestive of fivefold and sixfold axes of icosahedral virions, could be visualized.

Most of the RB viral proteins identified in this study have electrophoretic mobilities and glycosylation patterns that were similar to flavivirus proteins. Therefore, we have assigned the following tentative designations [according to the convention of Westaway *et al.* (1980)] to these proteins: gp52 (E), gp47 (E), gp46 (NS1), p25, gp20 (prM), gp < 18K. Non-radiolabelled, purified virions contained two major proteins ( $M_r$  52K and 48K) whose electrophoretic mobilities were similar to flavivirus E proteins. A doublet of the E protein, one species containing mannose and the other not, has been described for purified [ $^{35}$ S]methionine-labelled YF virus (Schlesinger *et al.*, 1983). Kunjin (KUN), YF and tick-borne encephalitis viruses produce several intracellular forms of the glycosylated E protein and in the case of KUN virus a non-glycosylated form of the E protein is found in the intracellular compartment and in purified virions (Wright *et al.*, 1981; Wright, 1982; Heinz & Kunz, 1982; Schlesinger *et al.*, 1983). We suggest that the two glycoproteins ( $M_r$  51K to 63K and 47K) that were immunoprecipitated from culture fluids may be the two major proteins which we recovered from purified virions. The heterogeneity of gp51-63 (E) might be due to a mixture of virion-associated and non-virion-associated E proteins of different sizes in culture fluids. Non-virion-associated envelope glycoproteins that are smaller than virion-associated forms have been recovered from culture fluids of cells infected with herpes simplex, vesicular stomatitis, murine leukaemia and respiratory syncytial viruses (Chen *et al.*, 1978; Kang & Prevec, 1971; Bolognesi & Langlois, 1975; Little & Huang, 1977; Hendricks *et al.*, 1987). Since the faint, 20K protein in purified virions has the same  $M_r$  as the 20K glycoprotein in culture fluids, they may be the same protein, possibly the virion-associated glycoprotein, prM. Based on its  $M_r$ , p25 was tentatively identified as ns4a, a hydrophobic protein with unknown function (Rice *et al.*, 1986) which may aggregate or be membrane-associated in the culture fluids. The paucity of putative C and M proteins in purified virion is consistent with an observation for YF virus (Schlesinger *et al.*, 1983; Rice *et al.*, 1986).

Because the gp46 protein is glycosylated and apparently abundant in infected cells, it may be the NS1 protein, formerly called the soluble, complement-fixing antigen (Smith & Wright,

1985). Surprisingly, none of the viral proteins that were found in culture fluids was precipitated from cell lysates. This might be explained by the hypothesis that most of the intracellular radioactivity was incorporated in host proteins, a possible result of the long labelling period in BHK-21 cells which are apparently not adversely affected by viral replication (Wilhelm & Gerone, 1970; Hendricks *et al.*, 1983). It is likely that culture fluids which are expected to contain primarily exported proteins would have higher proportions of viral proteins, especially glycoproteins. If this hypothesis is correct, then results from immunoprecipitations suggest that the 46K protein is the most abundant intracellular viral protein.

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