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Purification and Buoyant Density of Infectious Bovine Rhinotracheitis Virus (39159)

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Infectious bovine rhinotracheitis virus (IBRV) is a member of the herpesvirus group. It is a DNA-containing virus possessing essential lipids (1). The mature virion is an enveloped nucleocapsid with icosahedral symmetry, measuring 150 nm in diameter (2).

The purification of herpes viruses as a whole has been a difficult task (3), and reliable procedures are needed to obtain sufficient viruses of relatively high purity for biochemical and immunological analysis. This paper describes optimal conditions for handling IRBV and presents a purification schedule that may be useful to other enveloped viruses. The buoyant density of IRBV in potassium tartrate is also determined.

Materials and methods. Cells. Monolayers of Madin–Darby bovine kidney (MDBK) cells were propagated in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum.

Virus. Infectious bovine rhinotracheitis virus was plaque-purified three times and propagated in MDBK cells.

Virus titration. Three-day-old monolayers of MDBK cells in 3-oz prescription bottles were used for virus assay. One-tenth milliliter of the virus inoculum was added into assay bottles in duplicate. Following adsorption for 2 hr at 37°, the bottles were overlaid with 0.8% tragacanth gum in MEM (4). Plaques were counted three days postinoculation.

Protein determination. Protein was determined as described by Lowry *et al.* (5) with bovine serum albumin as standard.

Concentration and purification. Virus was grown and harvested according to the method described previously (6). Crude virus was clarified by centrifuging at 5000*g* for 15 min.

Twenty-seven milliliters of clarified virus was layered onto 3 ml of a 40% sucrose solution (w/v) in 0.2 *M* phosphate buffer, pH 7.5, and centrifuged in a Spinco SW 25.1 rotor at 25,000 rpm for 1 hr at 4°. The virus pellet was suspended in 0.2 *M* phosphate buffer, pH 7.5 in 1/10 of its original volume and layered onto a preformed continuous 10–40% (w/v) potassium tartrate gradient prepared according to the method described by Brakke (7). The potassium tartrate gradients were centrifuged in a Spinco SW 25.1 rotor at 25,000 rpm for 1 hr, and 1-ml fractions were collected with an ISCO automatic fraction collector. Fractions containing the light scattering virus bands of each gradient were pooled, diluted 4-fold with distilled water, and centrifuged in a Spinco 30 rotor at 30,000 rpm for 4 hr. The concentrated and purified virus pellet was finally resuspended in 1 ml of 0.2 *M* phosphate buffer, pH 7.5. All virus solutions were stored at −70°.

Isopycnic centrifugation and buoyant density determination. Two milliliters of purified virus at 1×10^8 PFU/ml was applied onto a linear 10–40% potassium tartrate density gradient and centrifuged in a Spinco 25.1 rotor at 25,000 rpm for 12 hr. Infectivity assay and density profiles of each gradient were determined by plaque assay and by weighing 100-μl aliquots of consecutive fractions, respectively.

Electron microscopy. Purified virus preparations were examined by electron microscopy according to the negative staining technique described by Brenner and Horne (8). A drop of the virus solution containing 10^8–10^9 PFU/ml and 0.1% bovine serum albumin was placed onto a collodion-coated grid for 10 min. The excess fluid was blotted off with a filter paper, and the sample was stained with neutral 1.5% phospho-

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tungstate for 30 sec before being examined in an AEI EM-6B electron microscope.

Results. Purification and buoyant density of IBRV. Partial purification and concentration of IBRV was achieved by sedimenting clarified virus through a 3.0 ml cushion of 40% sucrose solution. Final purification of the pelleted virus was carried out by rate-zonal centrifugation in a 10–40% potassium tartrate gradient. The virus sedimented as a single band and over 90% of the virus layered onto the gradient can be recovered in the light-scattering band. Table I shows the recovery of virus at each step of the purification scheme. It was found that there was an 11-fold increase in virus concentration as well as a corresponding 300-fold increase in the specific infectivity (PFU/mg protein) of the purified virus preparation.

When purified virus was centrifuged isopycnically in a 10–40% potassium tartrate gradient at 25,000 rpm for 12 hr, a single light-scattering band appeared at the region where the density was approximately 1.220 g/cm^3 (Fig. 1). Infectivity assay of 1.0-ml fractions of the gradient demonstrated that maximal virus titer was detected in the light-scattering band.

Electron microscopy. In negatively-stained preparations of purified virus, both enveloped mature and unenveloped naked virions were evident (Fig. 2). There appears to be considerably more mature virions than naked ones. Mature virions were 180–200 nm in diameter. The envelopes of mature virions contained tiny surface projections about 8–10 nm in length (Fig. 2, inset), and they were distributed periodically around the surface of the virion. Ruptured virions and empty particles without distinct nucleocapsids were also present.

Discussion. This report describes a purification scheme for IBRV by rate-zonal centrifugation in a 10–40% potassium tartrate gradient. Clarified virus was partially purified and sedimented through a 3.0-ml cushion of 40% sucrose prior to layering onto the potassium tartrate gradient. This procedure is similar in principle to that described by Kaplan and Ben-Porat (9) for pseudorabies virus. The sucrose cushion holds cell components on top with densities of 1.176 g/cm^3 or less. Virus particles and other particulate molecules with heavier densities sedimented through the cushion and onto the bottom of the tube. In this study, both sucrose and potassium tartrate gradients

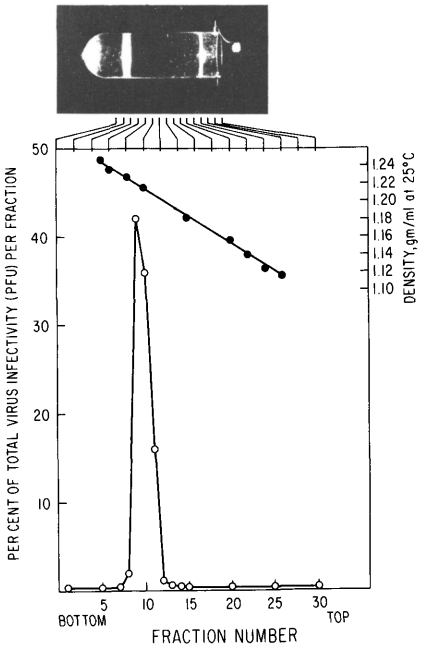

FIG. 1. Distribution of IBRV infectivity (PFU) and buoyant density of selected fractions in a 10–40% potassium tartrate gradient.

TABLE I. PURIFICATION STEPS FOR IBRV.

Purification steps	Volume (ml)	PFU/ml	Mg protein/ml	Specific infectivity (PFU/mg protein)	Total PFU	Recovery (%)
Crude virus	210	1.85×10^7	3.42	5.41×10^6	3.89×10^9	100
Clarified virus	210	1.27×10^7	2.17	5.85×10^6	2.68×10^9	69
Partially purified and concentrated virus	20	4.29×10^7	0.33	1.30×10^8	8.58×10^8	22
Purified virus 10–40% potassium tartrate	2.5	2.08×10^8	0.13	1.60×10^9	5.20×10^8	13

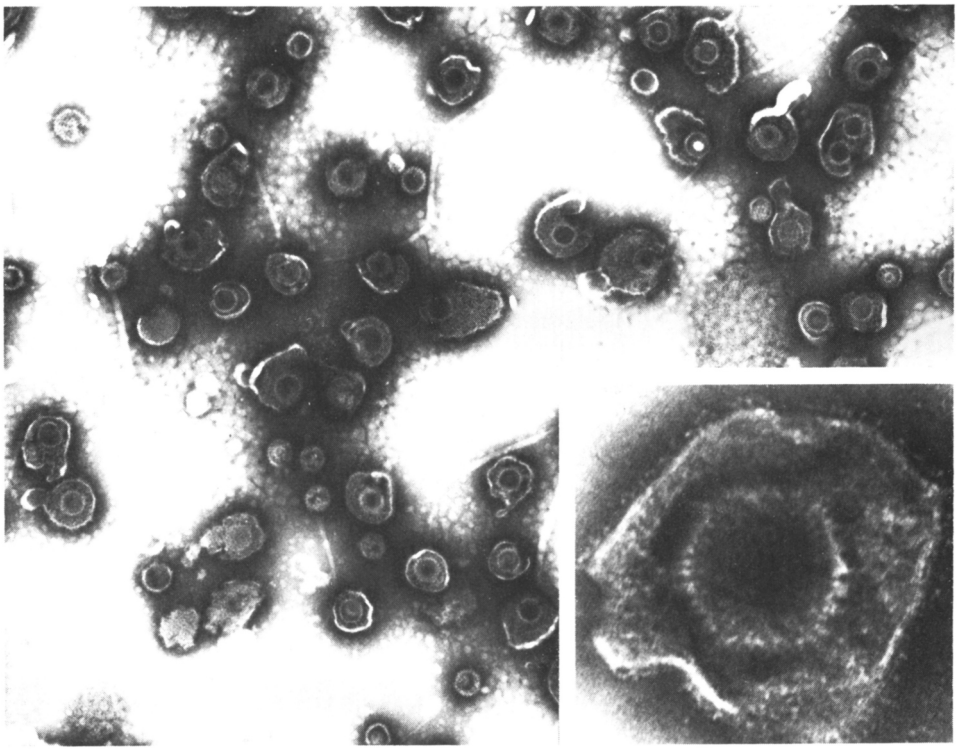

FIG. 2. Negatively stained preparation of purified IBRV. ×17,000. Inset. Minute projections on the envelope of a matured virion ×100,000.

have been used for rate-zonal centrifugation and have yielded similar results. Potassium tartrate was chosen because of its lower viscosity, thereby facilitating the preparation of gradients.

It is evident that a single cycle of rate-zonal centrifugation is sufficient to prepare purified IBRV. Using this method, purified virus suspensions with titer of 1×10^8–10^9 PFU/ml can be routinely obtained. Buoyant density analysis of banded virus revealed that IBRV belongs to that class of herpesviruses with low buoyant densities. The buoyant density of isopycnically banded IBRV in potassium tartrate gradient was measured to be 1.22 g/cm^3 at 25°, while the buoyant densities of equine abortion virus and Lucke's virus in potassium tartrate were 1.18 g/cm^3 and 1.20 g/cm^3, respectively (10, 11). Pertoft (12) reported in 1970 that IBRV possessed a buoyant density of 1.08–1.09 g/cm^3 in silica gel gradients.

Electron microscopic examinations of the purified IBRV revealed particle homogeneity of the virus preparation. Most virions possessed envelopes and the presence of minute projections were noted on the surface of virus envelopes which were particularly noticeable under higher magnification.

Summary. A purification scheme for infectious bovine rhinotracheitis virus utilizing rate-zonal centrifugation in a 10–40% potassium tartrate gradient was described. The density of IBRV in the potassium tartrate gradient was found to be 1.22 g/cm^3. Electron microscopic examination of purified virus preparations revealed homogeneous populations of enveloped virions with minute projections on the envelope surface.

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