Buoyant Density of Bovine Viral Diarrhea Virus (36511)

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The purification and buoyant density of bovine viral diarrhea virus (BVDV) have been studied by several investigators. One report placed the buoyant density of four strains of BVDV (NADL, C24V, CG-1220, and NY1) grown in primary embryonic bovine cells at 1.15 g/cm^3 (1), while a second group of investigators reported the buoyant density of BVDV, strain C24V, propagated in calf testicle cells to be 1.115 g/cm^3 (2).

In this paper, using methods similar to those described for rubella virus (3, 4), we report the buoyant density of BVDV, strain NADL, produced in fetal bovine bone marrow cells.

Materials and Methods. Virus. The National Animal Disease Laboratory (NADL) strain of BVDV was used throughout this investigation. The virus was kindly provided by Dr. A. J. Hackett, Naval Biomedical Research Laboratory, Oakland, CA, and was plaque purified three times in our laboratory.

Cells. Fetal bovine bone marrow (BBM) cells, FB4BM, provided by Dr. John W. Kendrick, Department of Clinical Sciences, University of California, Davis, CA, were cultivated in Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal calf serum.

Growth curve. Three ounce prescription bottles (Brockway Glass Co. Inc.) were seeded with BBM cells suspended in Eagle's medium with 10% fetal calf serum. When monolayers of cells had formed, they were washed twice with Eagle's medium and inoculated with BVDV at a multiplicity of infection of approximately one. Adsorption of the virus was carried out at 37° for 1 hr, after which time 4.0 ml of Eagle's medium plus 5% fetal calf serum was added. At 12 hr intervals infected cultures were assayed for total, intracellular, and extracellular virus. Samples were centrifuged at 500 rpm for 5 min

TABLE I. Concentration Steps for Bovine Viral Diarrhea Virus.

Conc steps	Quantity (ml)	PFU/ml	Total PFU	Recovery (%)
Infective tissue culture fluid	20	3.0×10^6	6.0×10^7	100
Clarified tissue culture fluid	20	1.5×10^6	3.0×10^7	~ 50
Discontinuous gradient 20–50% sucrose				
Fraction 1	20	1.0×10^3	2.0×10^4	~ 0.033
2	2.5	9.0×10^6	2.2×10^7	~ 37
3	1.0	1.5×10^6	1.5×10^6	~ 2.5
4	3.0	1.1×10^6	3.3×10^6	~ 5.5

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and the supernatant fluid was assayed for extracellular virus. The cell pellets were washed 3 times with TEN buffer and frozen-thawed once to release intracellular virus. Infectious virus titers were determined by plaque assay in BBM cells using 1.5% agar in Eagle's medium containing 5% fetal calf serum.

Labeling of viral RNA. Monolayers of BBM cells grown on 16 oz Brockway prescription bottles were inoculated with approximately 6×10^6 plaque-forming units (PFU) following procedures described above. At 24 hr postinoculation actinomycin D was added to give a concentration of 0.1 μg/ml. Two hours later 250 μCi of ^{3}H-uridine (0.5 mCi/ml; sp act, 30 Ci/mmole; Schwarz/Mann) was added to each bottle. Extracellular virus was harvested at 72 hr postinfection.

Concentration and buoyant density. Virus was harvested from 16 oz Brockway bottles by collecting the infected tissue culture fluid. No effort was made to obtain intracellular virus. Culture fluid from infected cells was clarified by centrifugation at 5900*g* for 10 min at 4° to remove cell debris.

A discontinuous two-step gradient was formed in a 1 × 3 in. cellulose nitrate tube by depositing 4.0 ml of a 50% (w/w) solution of sucrose, in 0.01 *M* Tris-hydrochloride, 0.001 *M* EDTA, and 0.1 *M* NaCl (pH 7.4; TEN buffer), onto the bottom of the tube and overlaying it with 5.0 ml of a 20% (w/w) buffered sucrose solution. The 20% sucrose solution was then gently overlayed with 20 ml of clarified tissue culture fluid and centrifuged at 25,000 rpm for 2 hr at 4° in a Spinco SW 25.1 rotor. Two intense light scattering bands could be detected, one in the region of 20% sucrose and another at the 50% sucrose interface. The bands were collected and assayed for infectivity. Most of the infectivity was found to reside in the region of 20% sucrose (Table I and Fig. 2).

Twenty milliliters of a virus stock grown in the presence of ^{3}H-uridine was processed by identical methods. The 20% sucrose band material from both labeled and unlabeled viral stocks were pooled, diluted, and layered onto a preformed continuous 10–35% sucrose density gradient. Gradients were centrifuged in a Spinco SW 25.1 rotor at 25,000 rpm for 24 hr at 4° and 1 ml fractions were collected using an Isco automatic fraction collector.[2] Density profiles of gradients were determined by weighing 100 μl aliquots of consecutive fractions at room temperature.

Determination of radioactivity. Acid-precipitable counts were determined by placing 100 μl aliquots from each fraction on separate strips of Whatman chromatography pa-

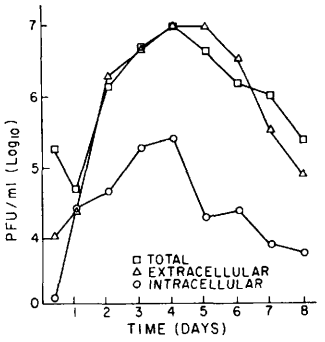

FIG. 1. Growth curve of bovine viral diarrhea virus, strain NADL, in bovine bone marrow cells.

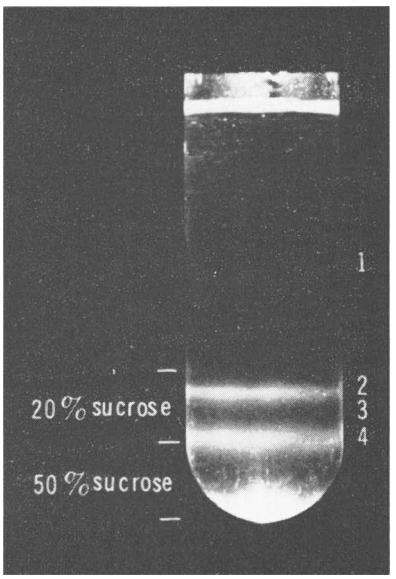

FIG. 2. 20–50% sucrose discontinuous gradient.

[2] Model D density gradient fractionator, Instrumentation Specialties Co. Inc., 4700 Superior, Lincoln, NB 68504.

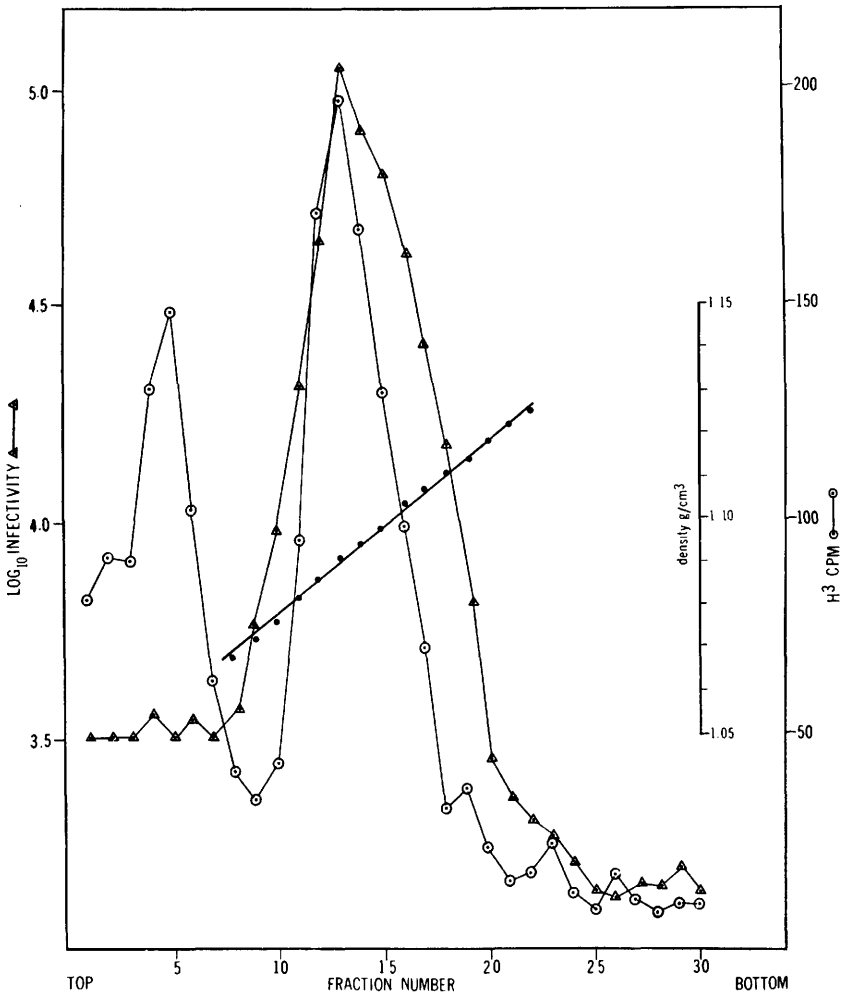

FIG. 3. Distribution of BVDV infectivity (PFU) and ^{3}H-uridine radioactivity from fractions of a 10–35% sucrose density gradient.

per, which when dry were placed into cold (4°) 5.0% perchloric acid (PCA). After 20 min, strips were washed with cold PCA and placed into cold 95% ethanol for 10 min. They were then washed once with cold 95% ethanol, allowed to dry, and placed into scintillation vials. After adding 10 ml of Permablend-toluene (Packard Instruments Company, Inc.) scintillation fluid, radioactivity was measured in a Beckman liquid scintillation spectrometer.

Results. Growth curve. A growth curve of BVDV, strain NADL, in BBM cells (Fig. 1) showed that the extracellular viral titer was equal to or greater than the total viral yield and that both the intracellular and extracellular viral yield reached its maximum 96 hr postinoculation. The intracellular viral yield is much lower than the extracellular viral titer and represents only about 1% of the total viral yield. From this it can be seen that the extracellular viral titer represents about 99% of the total viral yield.

Concentration and buoyant density. Centrifugation of clarified tissue culture fluid onto a 20–50% sucrose discontinuous gradient resulted in a concentration of the virus by almost tenfold at the region of 20% sucrose (Table I). Two light scattering bands were detected (Fig. 2), however, most of the

infectivity was associated with the band in the region of 20% sucrose.

A representative density gradient run is shown in Fig. 3. Under the conditions employed we found BVDV preparations consisting of infectious units with buoyant densities ranging from 1.075 to 1.115 g/cm^3. However, the majority of infective virions were found to have a buoyant density in sucrose of approximately 1.092 g/cm^3.

Discussion. Castrucci, Cilli and Gagliardi (5) showed that the maximum viral yield occurs about 96 hr after infection, but only total virus was determined. Our results indicate that the peak of virus production was at 96 hr postinfection, as was that of Castrucci, Cilli and Gagliardi, but surprisingly the extracellular viral titer was as high as the total viral yield. The constant release of BVDV from infected cells allows the virus to be harvested without the necessity of freezing and thawing the cells and it also gives a starting suspension of BVDV that contains less cellular debris. These results are similar to those reported in an earlier study (6) in which the titer of extracellular virus was found to be higher than intracellular virus, although the total viral yield was not determined for comparison.

The high extracellular viral titer combined with the very low intracellular yield, the slow rise of extracellular viral titer, and long maintenance of peak viral production suggest that the virus is probably being released from the cells by budding or by extrusion of cytoplasmic vacuoles. Preliminary experiments in our laboratory based on electron microscopic observations, seem to support this suggestion.

Isopycnic centrifugation of diluted 20% sucrose band material through a continuous sucrose density gradient produced peak infectivity at a buoyant density of about 1.092 g/cm^3. This value is more compatible with that of Maess and Reczko (2) who reported BVDV, strain C24V, to have a buoyant density of 1.115 g/cm^3 in potassium tartrate than with Ferneilius (1) who reported the buoyant density of BVDV as 1.15 g/cm^3 in sucrose, potassium tartrate and cesium chloride. The buoyant density which we report for BVDV in BBM cells is similar to that reported for rubella virus (3). It has been suggested that both should be placed in the Togavirus group (7).

From the correspondence of radioactivity and infectivity (Fig. 3) we conclude that ^{3}H-radioactivity is associated with complete BVD virions and is not due to cosedimenting cellular contaminants. Material containing ^{3}H-radioactivity is also shown to be at the top of the gradient. The finding that there is only slight infectivity in this region, suggest three possibilities; (i) the material is free viral RNA; (ii) it represents incomplete virus; or (iii) it is a host cell RNA constituent. The first two explanations are more likely, since cells were treated with actinomycin D before being labeled.

Summary. Bovine viral diarrhea virus (BV DV), strain NADL, was purified by removing extracellular fluid from infected cultures at 96 hr postinoculation and clarifying by low speed centrifugation. Clarified fluid was concentrated by centrifugation onto a two-step 20–50% sucrose discontinuous gradient. Most of the infectivity was associated with a light scattering band in the region of 20% sucrose. ^{3}H-Uridine labeled and unlabeled viral band material was then pooled, diluted, and isopycnically banded through a sucrose density gradient. Infectious BVDV was recovered from fractions between 1.075 and 1.115 g/cm^3. The main peak of infectivity and radioactivity was found at a density of 1.092 g/cm^3.

1. Ferneilius, A. L., Arch. Gesamte Virusforsch. **25**, 48 (1968).

2. Maess, J., and Reczko, E., Arch. Gesamte Virusforsch. **30**, 39 (1970).

3. Cusumano, C. L., Proc. Soc. Exp. Biol. Med. **122**, 461 (1966).

4. Sedwick, W. D., and Sokol, F., J. Virol. **5**, 478 (1970).

5. Castrucci, G., Cilli, V., and Gagliardi, G., Arch. Gesamte Virusforsch. **24**, 48 (1967).

6. Gillespie, J. H., Madin, S. H., and Darby, N. D., Cornell Vet. **53**, 276 (1963).

7. Horzenik, M., and Maess, J., Arch. Gesamte Virusforsch. **33**, 306 (1971).

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