Biophysical Studies of Vesicular Stomatitis Virus

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ABSTRACT

McCOMBS, ROBERT M. (Baylor University College of Medicine, Houston, Tex.), MATILDA BENYESH-MELNICK, AND JEAN P. BRUNSCHWIG. Biophysical studies of vesicular stomatitis virus: J. Bacteriol. 91:803–812. 1966.—The infectivity and morphology of vesicular stomatitis virus (VSV) were studied after density gradient centrifugation in cesium chloride (CsCl), potassium tartrate (KT), and sucrose. Centrifugation in CsCl revealed two equally infectious bands corresponding to densities of 1.19 and 1.22 g/ml, and a third (density, 1.26 g/ml) band of low infectivity. Two bands (densities of 1.16 and 1.18 g/ml) were observed in the KT gradient, in which the lighter band contained most of the infectivity. Centrifugation in sucrose resulted in a single broad infectious band (density, 1.16 g/ml). The typical rod-shaped VSV particles were found mainly in the lighter bands obtained in CsCl (1.19 g/ml) and KT (1.16 g/ml) and in the single sucrose gradient band (1.16 g/ml). Bent particles equally as infectious as the rod-shaped particles were a constant finding in the CsCl preparations, and were observed mainly in the second band (density, 1.19 g). Numerous strands 15 mμ wide were found in the third CsCl (density, 1.26 g/ml) and the second KT (1.18 g/ml) bands. Similar strands could be liberated from VSV particles after treatment with deoxycholate. Internal transverse striations were found to be a regular feature of VSV particles examined with the pseudoreplication-negative-staining technique. For crude virus stocks, the physical particle-to-infectivity ratio ranged from 73 to 194. Several morphological similarities between VSV and myxoviruses were observed, including 10 mμ surface projections, pleomorphic morphological forms, and 15 mμ seemingly nucleoprotein strands.

The morphology of vesicular stomatitis virus (VSV) has been studied by several investigators (4, 5, 9) using either the negative staining procedure of Brenner and Horne (2) or the modification by Howatson and Whitmore (5). The virus particles were shown to be rod-shaped, rounded at one end and flat at the other, and measuring about 65 by 175 mμ. Other structural details that were observed included surface projections, a central axial hollow extending through a portion of the particle from the flat end, and occasionally internal transverse striations. A buoyant density of 1.20 g/ml in cesium chloride (CsCl) (40-hr centrifugation) has been reported for VSV (8).

In the present study, VSV was found to band in a different fashion after density gradient centrifugation in CsCl for short (3.5 hr) and long (24 hr) periods of time. The morphology and quantification of the particles after density gradient centrifugation and after chemical treatment was studied by use of the pseudoreplication-negative staining procedure as described by Smith and Melnick (12). Some of the particles recovered from the density gradient bands, as well as particles obtained after chemical treatment, exhibited a striking similarity to typical myxoviruses.

MATERIALS AND METHODS

Tissue culture. Serially propagated human embryonic lung (HEL) fibroblasts were used. These cells were grown in Eagle’s medium with 10% fetal bovine serum and 0.075% sodium bicarbonate (for cells in stoppered vessels) or 0.225% sodium bicarbonate (for cells in petri dishes in a 5% CO₂ atmosphere). Primary monkey kidney (MK) cells grown in 1-oz prescription bottles with the use of the procedures and the M-H medium described by Melnick (6) were also employed.

Virus. The Indiana serotype of VSV was kindly supplied by Werner Henle. Virus stocks were prepared in HEL fibroblasts. Infected monolayers showing 80 to 90% cytopathic effects were frozen and thawed twice, and the crude suspension was pooled and clarified by low-speed centrifugation. The resulting supernatant fluid was stored at -90 C. The virus for density gradient centrifugation and chemical treatment was concentrated 10- to 20-fold and was partially purified by two cycles of differential ultracentrifuga-
tion (19,620 × g for 15 min; 54,500 × g for 60 min) and resuspended in 0.05 m tris(hydroxymethyl)amino-
methane (Tris) buffer (pH 7.4).

Infectivity assay. VSV was titrated by plaque ass-
ay by use of either MK monolayers in 1-oz prescrip-
tion bottles or HEL monolayers in 60-mm petri dis-
hes. Decimal virus dilutions in 0.05 m Tris buffer were
used. For assays in MK cells, 0.1 ml of virus was
added to drained monolayers and, after absorption at
37 C for 1 hr, the cultures were overlaid with 5 ml of
Earle's salt solution containing 0.4% sodium bicar-
bonate, 0.0017% neutral red, 0.1% skim milk, and
1.5% agar (Difco) (14). The HEL monolayers were
first washed with Tris buffer and then infected with 0.5
ml of virus. After 1 hr of incubation at 37 C, they
were overlaid with 5 ml of Eagle's medium containing 5%
fetal bovine serum, 0.9% agar, and 0.225% sodium bicarbonate. A second overlay (3 ml) of the same com-
position, but with neutral red (final concentration 1:25,000), was added after 48 hr, and the plaques were
counted after overnight incubation. Since assays on
MK and HEL monolayers yielded identical results,
they were used interchangeably throughout this study.

Density gradient centrifugation. VSV was banded by
density gradient centrifugation in cesium chloride (CsCl), potassium tartrate (KT), potassium citrate
(KC), and sucrose. In general, 0.5 ml of the partially
purified and concentrated virus was layered over 4.5
ml of preformed gradient and centrifuged at 99,972
× g for 3.5 hr at 4 C in the SW 39 rotor of a Spinco
model L2 ultracentrifuge. The bands obtained were
removed either from the side of the tube by use of a
syringe and needle (25 g), or by puncturing the bottom
of the tube with a Buchler piercing unit (Buchler
Instruments, Inc., Fort Lee, N.J.) and collecting the
drops. In the latter procedure, 80 to 90 fractions of
5 drops each were collected. The density of the bands
was computed by measurement of the refractive index in an Abbe 3L refractometer, and the density was
extrapolated from standard curves obtained by use of
data from the International Critical Tables.

Chemical treatment. Partially purified VSV prepar-
ations were treated with trypsin, sodium deoxy-
cholate, or sodium lauryl sulfate under various condi-
tions (see Results). Immediately after incubation with
the reagent, specimens were prepared for electron
microscopy.

Electron microscopy. Two methods of specimen
preparation were employed. The first was that de-
scribed by Brenner and Horne (2), in which an equal
volume of 2% phosphotungstic acid (PTA) at pH
7 and virus suspension were mixed. A drop of this
mixture was placed on a collodion-coated steel grid
(200 mesh), and the excess was removed after several
minutes. The second method was the pseudoreplica-
tion method described by Smith and Melnick (12)
and later modified to include osmic acid fixation (11).
A drop of the virus suspension was first allowed to
dialyze to dryness on 2% agar and was then immedi-
ately fixed in osmic acid vapor for 7 min. Paraloid
(0.75% in amyl acetate) was spread over the surface
containing the virus and, after drying, the film was
stripped from the agar by floating on a 0.2% aqueous
solution of PTA (pH 7) containing 0.05% sucrose.

The film was picked up on a steel grid and air-dried
prior to electron microscopic examination. All photo-
micrographs were taken with a Hitachi 11A electron
microscope at instrument magnifications of 5,000 and
15,000.

Particle counts were initially made by the sedi-
tmentation technique (10) with the use of the modi-
fication for negative staining (12). Later, it was found
that the droplet pseudoreplication method, without
sedimentation, yielded reproducible particle counts
(see Results), and this technique was also used for
the enumeration of particles in the density gradient
bands.

RESULTS

Morphology of VSV. The two methods of nega-
tive staining with PTA gave significantly different
results. The particles seen in Fig. 1a were stained
by the procedure of Brenner and Horne (2), and
had the morphology previously described by
others (4, 5, 9). The surface projections were evi-
dent, and the PTA had penetrated only a central
hollow area extending part way from the flat end
of the particle. When the pseudoreplication
method of Smith and Melnick (12) was used, the
particles had a different appearance (Fig. 1b). The
size of the particles prepared by this method was
65 by 175 nm, in good agreement with that previ-
ously reported. Considerably more internal
detail, in particular the internal transverse stria-
tions, was routinely seen, probably because of
penetration of the PTA. Such internal structural
detail was observed by the above-mentioned
authors mainly in particles that were examined
after prolonged incubation at 4 C and that were
considered to be partially disrupted.

The technique of pseudoreplication was em-
ployed for all of the following electron micro-
scopic examinations.

Virus particle quantification. A comparison of
particle counts determined by the sedimentation
method and the droplet method was carried out.
Two different unconcentrated stocks of VSV were
used. The infectivity titers were 1.6 × 10⁸ plaque-
forming units (PFU) per ml for stock A and 1.5 × 10⁸
PFU per ml for stock B. For the sedimentation
method, the formula described by Smith and
Melnick (13) was applied for the calculation of
the particle concentration per milliliter of virus
suspension: particles/ml = (average number of
particles/field) × (1/dilution) × (1/height of
column) × (1/unit area in cm²) = (average num-
ber particles/field) × (1/dilution) × (1.25) ×
(1.2 × 10⁴).

The calculations for the droplet method were as
follows. It was found in repeat measurements
that 1 ml of virus suspension contained 50 drops of
the virus suspension used for the examina-
tions. Thus, the following formula was applied:

\[ \text{particles/ml} = \left( \frac{\text{average number of particles/field}}{\text{unit area in cm}^2} \right) \times \left( \frac{1}{\text{dilution}} \right) \times (50) \times \left( \frac{1}{100 \text{ particles/milliliter}} \right) \times (1.2 \times 10^8). \]

A good correlation was found between the counts obtained and the values determined by the two methods in two experiments with virus stock A and one experiment with virus stock B. Stock A yielded \(3.1 \times 10^8\) particles per milliliter by the sedimentation method and \(2.1 \times 10^8\) particles per milliliter by the droplet method. The values determined for stock B were \(1.1 \times 10^8\) and \(1.6 \times 10^8\) particles per milliliter, respectively. Because of its simplicity, the droplet method was employed in all further experiments.

The ratio of physical particles to infectivity (PFU) calculated from these data varied from 73 to 194, with an average of 126.

**Density gradient centrifugation of VSV.** In preliminary experiments, virus was incubated at 4°C for 3.5 hr with the appropriate compound used for making the gradient, at a concentration corresponding to the approximate buoyant density of the virus. About 50% infectious virus was lost after incubation with CsCl, KT, and KC. A better recovery (79 to 100%) was obtained after incubation with sucrose. Gradients prepared in Tris buffer yielded sharper bands as compared with those prepared in water, and thus were utilized throughout the centrifugation experiments.

The bands obtained after equilibrium density gradient centrifugation (3.5 hr at 99,972 \(\times\) g) in CsCl, KT, and sucrose are illustrated in Fig. 2. Three bands were obtained in CsCl, two bands in KT, and a single band in sucrose. The corresponding buoyant density of each band is indicated in the figure. The bottom bands in both the CsCl and KT had a granular appearance.

The distribution of infectivity in fractions collected from the bottom of the tube with the three gradients is illustrated in Fig. 3, 4, and 5. The peaks of infectivity shown in these figures corresponded to the visible bands seen in Fig. 2. Results of density gradients with KC were similar to those found in KT, and will not be presented.

The separate bands in all three gradients were collected from the side of the tube and examined in an electron microscope. The morphological forms observed in each of the three CsCl bands are presented in Fig. 6. The first band (density, 1.19 g/ml) contained the typical rod-shaped virus particles, as well as some “bent” particles that appeared to be broken almost exactly in half and folded together (Fig. 6a). The second band (density, 1.22 g/ml) consisted almost entirely of the bent particles (Fig. 6b). The third band (density,
1.26 g/ml) contained strands, about 15 μm in diameter, as well as some rod-shaped and bent particles trapped within the strands (Fig. 6c), which probably caused the granular nature of the band as seen in the centrifuge tube.

In KT, band 1 (density, 1.16 g/ml) contained intact, rod-shaped particles and some bent forms, whereas band 2 (density, 1.18 g/ml) contained masses of strands which had entrapped many particles, thus bearing a great similarity to the appearance of the third CsCl band. In the sucrose gradient, the single band obtained (density, 1.16

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**FIG. 2.** CsCl, potassium tartrate and sucrose density gradients of VSV (3.5-hr centrifugation at 99,972 × g) and the buoyant density of the bands obtained.

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**FIG. 3.** Distribution of VSV infectivity in fractions after density gradient centrifugation in cesium chloride (3.5-hr centrifugation at 99,972 × g). Symbols: ○ = density; ● = infectivity.

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**FIG. 4.** Distribution of VSV infectivity in fractions after density gradient centrifugation in potassium tartrate (3.5-hr centrifugation at 99,972 × g). Symbols: ○ = density; ● = infectivity.

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**FIG. 5.** Distribution of VSV infectivity in fractions after density gradient centrifugation in sucrose (3.5-hr centrifugation at 99,972 × g). Symbols: ○ = density; ● = infectivity.

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A quantitative correlation between infectivity and particle counts of the density gradient bands,
FIG. 6. Electron micrographs of portions of the band occurring at (a) density 1.19 g/ml in CsCl, (b) density 1.22 g/ml in CsCl, and (c) density 1.26 g/ml in CsCl. X 35,000 (inserts, X 150,000).
collected from the side of the tube, was carried out. An average of the results of two experiments is given in Table 1. CsCl band 1 contained 48% of the total infectivity recovered in the three bands; band 2 contained 44% and band 3 contained 8%. Upon further centrifugation in CsCl, for 24 hr, much of the infectivity previously contained in band 1 was found in the second band. In KT, band 1 contained 36% of the infectivity recovered and band 2 contained 64%. Of particular interest were the particle counts made from pseudoreplicas of droplet preparations taken from each band. There was a high degree of correlation between the per cent infectivity (PFU) and the per cent total particles recovered from the individual bands. From the data presented in the two last columns in Table 1, it appeared that both the rod-shaped and bent particles were equally infectious. This is illustrated clearly in the CsCl experiments. After 3.5 hr of centrifugation, the infectivity was highest in the first band. This band also contained 54% of the total particles counted, with nearly equal numbers of rod-shaped and bent forms. However, after 24 hr of centrifugation, 63% of the infectivity and 51% of the total particles were found in the second band. The increase in particles consisted of an increase in the bent forms rather than the rod-shaped forms.

**Morphological degradation of VSV.** The effect of several chemical agents on the morphology of VSV was studied. Partially purified virus was incubated in the presence of 0.05% trypsin (30 min at 37 C) with approximately 50% loss of infectivity. The particles appeared devoid of the 10-m, surface projections or spikes (Fig. 7a), as compared with the control, nontreated preparation (Fig. 7b). However, there seemed to be a trypsin-resistant area at the rounded end of the particles, as seen in the insert of Fig. 7a. No other morphological alteration of the particles was observed by this treatment.

Complete disruption of VSV particles, leaving only the 15 m, strands, was achieved using 0.05% sodium deoxycholate (30 min at 37 C; Fig. 7c). Evidence that these strands form an integral component of the virion is illustrated in Fig. 8, in which strands can be seen emanating from virus particles obtained from the third band in CsCl or from the second band in KT. The strands were associated with the partially disrupted particles, with most strands originating from the inner portion of the particle. When these strands, obtained with the deoxycholate treatment, were further treated with trypsin, they were no longer detectable in the electron microscope, indicating a complete digestion by the enzyme. Preliminary results indicated that neither ribonuclease nor deoxyribonuclease altered the morphology of these strands.

Treatment of VSV particles with 0.5% sodium lauryl sulfate (10 min at room temperature) resulted in complete disruption of the particles; electron microscopy revealed that the preparation was devoid of either particles or strands.

**Occurrence of myxovirus-like forms in VSV preparations.** The electron microscopic examination of VSV preparations, in particular those

<table>
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<th>PFU</th>
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<td>1.26</td>
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<td>Potassium tartrate</td>
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* At 99,972 × g.
† Per cent = no. per band / total no. recovered × 100.
treated with CsCl, revealed particles closely resembling myxoviruses. Several of these particles are illustrated in Fig. 9. Particle “a” had the usual rod-shaped appearance, while particles “b” and “c” seemed to be assuming the pleomorphic shape characteristic of myxoviruses. The morphology of particle “d” appeared indistinguishable from that of typical myxoviruses, including pleomorphic shape, surface projections, and even an ill-defined internal helix.

**DISCUSSION**

Reczko (9), Howatson and Whitmore (5), and Hackett (4) were the first to study in detail the morphology of VSV by use of the negative staining technique of Brenner and Horne (2). In their preparations, typical virus particles were penetrated by PTA only in the area of the central axial hollow, and the internal structure was seldom visible, although the surface projections were clearly outlined. The failure of the PTA to penetrate intact VSV particles seemed to be due to the presence of a lipid membrane surrounding the particles (8). With the pseudoreplication method employed in this study, the parlodion used to strip the virus from the agar surface was applied as a solution in amyl acetate. This solvent probably solubilized enough of the outer lipid to allow the PTA to penetrate.
Particle counts obtained by the sedimentation and the droplet methods were in agreement, thus indicating that the droplet method can be used as a rapid and simple means of quantification. The average ratio of physical particles to PFU obtained for the two unconcentrated virus stock suspensions was 126, varying from 73 to 194. Hackett (4) taking into consideration only unpenetrated intact particles, reported a ratio close to 10 for potent VSV preparations. With the pseudoreplication technique used in our study, all particles were penetrated by the PTA and the ratio obtained was based on total counts of intact particles.

VSV was previously banded in CsCl by Prevec and Whitmore (8), who reported a single band of infectivity having a density of 1.20 g/ml, but they did not study the morphology of the particles in this band. After 3.5 hr of centrifugation of VSV, we found that the peaks of infectivity in bands 1 and 2 were approximately equal, but, upon further centrifugation for 24 hr, the second band (containing bent forms) increased in content over the first band (Table 1). If centrifugation was continued for 40 hr, as done by Prevec and Whitmore (8), the second band would probably increase in quantity so that the first and third bands would be obscured, thus giving the appearance of a single, broad band.

The recovery of three bands of infectivity from CsCl density gradients, compared with a single band from sucrose gradients, may be explained on the basis of the morphology of the particles recovered. As a result of centrifugation in CsCl, the VSV particles were partially disrupted into bent forms (seen in band 2) that may retain their
infectivity, and were completely disrupted into 15 mμ strains (seen in band 3). KT also caused the disruption of particles into bent forms and the 15 mμ strands. This gradient salt, however, was not able to resolve the rod-shaped and the bent forms into different bands. The high percentage of infectivity (64%) found in the second band was due to large numbers of virus particles trapped in the masses of strands.

Even though VSV had been widely used, it remains an unclassified animal virus. Mussgay and Suarez (7) suggested that VSV was related to the arboviruses because of its multiplication in mosquitoes, maturation at the cell membrane, and the presence of lipid as an integral viral component. However, Howatson and Whitmore (5) suggested that the surface projections seen on the virus particles closely resembled those of the myxoviruses. In our study, three distinct similarities between VSV and the myxoviruses were noted. The first and most common was the spikes (10 mμ in size) seen on the VSV particles. In addition, the spikes could be removed with trypsin, as has been reported for some myxoviruses (1, 3). The second similarity was the occurrence of myxovirus-like forms in the preparations, and the third similarity was the strands present in the bottom bands and after treatment with deoxycholate. The viral origin of these strands is well illustrated in Fig. 8. The strands measured about 15 mμ in diameter, which is similar to that of the nucleoprotein strands reported for the paramyxoviruses (15).

**Fig. 9.** VSV particles (from CsCl-treated preparations) showing varying degrees of myxovirus-like structure. X 150,000.

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**LITERATURE CITED**


