The Morphology of Vesicular Stomatitis Virus (Indiana C) Derived from Chick Embryos or Cultures of BHK21/13 Cells

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SUMMARY

The virus of vesicular stomatitis is shown to exist as a system of several particle forms. Two major particles, the 'bullet' and 'cap', appear in electron micrographs in various states as outer structural layers are absent or lost. High infectivity is not associated with particles of one form. The distribution of particle forms is apparently determined by cultural conditions and is not significantly modified by the preparative procedures employed.

The morphology of the several components of the virus system is described.

INTRODUCTION

The virus system of vesicular stomatitis (VSV) has recently been studied by several groups (Table 1) and there is general agreement that the characteristic infective component is bullet-shaped, about 160 m μ long and 60–70 m μ in diameter, with one flat and one round end. Scant attention has been given to a variety of other particle forms which have often been interpreted as artifacts caused by preparation techniques. The present study is part of an attempt to characterize the several structures revealed by electron microscopy and to relate these to the initial virus system and to the diversity of its biological properties. A brief preliminary note on these observations has been published (Bradish & Kirkham, 1964).

METHODS

The infective fluids used in these experiments were obtained from chick embryos or rolled monolayer cultures of baby hamster kidney cells (BHK21/13: Macpherson & Stoker, 1962). Vesicular stomatitis virus of the Indiana Serotype and strain Ind. C was used throughout.

Titration of infectivity on BHK21 monolayers. The infectivity of experimental samples was determined by plating 0.2 ml. each of a series of appropriate dilutions on to washed monolayers of BHK21 cells formed in 2 or 3 days on 7 cm. Petri dishes. Each dilution was plated in duplicate or triplicate. After an adsorption period of 30 min. at 37° the monolayers were overlaid with 5 ml. 1% Difco Noble agar in maintenance medium (Eagle's medium + 5% bovine serum + 5% Difco

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Table 1. *A summary of data on the virus system of vesicular stomatitis*

Author	Virus serotype	Origin of infective material	Method of study	Mean dimensions in $m\mu$		Density of infective component ($g./cm^3$)	Other observations
				Bullet	Cap		
(1) Chow <i>et al.</i> (1954)	New Jersey	Egg fluids	Electron microscopy of shadowed preparations	210×60 (rods)	—	—	Characteristic rods with hair-like projections and trailing filaments
(2) Bradish <i>et al.</i> (1956)	New Jersey and Indiana	Egg fluids	Electron microscopy of shadowed preparations Analytical centrifugation	175×69 (rods)	65 (granules)	—	Diversity of particle forms and association of infectivity with rod. Also 7–20S components associated with complement-fixing activity and trace infectivity
(3) Reczko (1960)	Indiana	Chick chorio-allantoic membrane	Electron microscopy of PTA stained preparations	147×46	58 (330S)	1.18	Recognition of asymmetry of bullet and cap and of presence of trailing filaments and possibly of transverse striations. Virus particles in cells of chorion layer
(4) Stone <i>et al.</i> (1961)	New Jersey	HeLa cells and mouse embryo brain	Electron microscopy of PTA stained preparations	170×60	60 (circular cross-sections)	—	'Test-tube' particles in cytoplasm and in vesicles
(5) Howatson & Whitmore (1962)	Indiana	Earles's L cells	Electron microscopy of PTA stained preparations	175×68	Absent	—	Development of virus particles in cell: more detailed morphology
(6) Prevec & Whitmore (1963)	Indiana	Earles's L cells	Density gradient separation with ^{32}P labelled components	—	—	1.20	Non-infective component of density $1.18-1.19 g./cm^3$
(7) Hackett (1964)	New Jersey	Primary chick fibroblasts	Electron microscopy of PTA stained preparations	170×65	82×65	—	Autointerference in relation to caps
(8) Bradish & Kirkham (1964)	Indiana	BHK 21 cells and egg fluids	Electron microscopy of PTA stained preparations	165×65	65×65	—	Confirmation of presence of caps in fresh, infective preparations
(9) Warrington (1965)	Indiana	BHK 21 cells and egg fluids	Density gradient separation with biological assay	—	—	1.18–1.22	Host-imposed modifications of density of bullet and cap and a complexity of non-infective and complement fixing components of greater density
(10) Present study (Bradish & Kirkham, 1966)	Indiana	BHK 21 cells and egg fluids	Electron microscopy of PTA stained preparations	154×61	66×61	—	Distribution and morphological detail of several forms of bullet and cap in fresh infective preparations

tryptose phosphate broth + 40 units/ml. each of 'Mycostatin' and 'Neomycin') and incubated for 2 days at 37° in an atmosphere of 5% (v/v) CO₂ in air. The sharply defined plaques of 3–5 mm. diameter were counted after staining the monolayers for about 1 hr with neutral red in isotonic saline (0.05 mg./ml.). Counts of between 10 and 100 plaques/plate were expressed as plaque-forming units/ml. undiluted sample (p.f.u./ml.). The diluent and washing medium throughout was isotonic saline buffered to pH 7.6 by 0.005 M-phosphate-phosphate solution (PBS).

Preparation of infective fluid from chick embryos. The shells of fertile 7- or 8-day-old eggs were drilled in the position of the heads of the embryos and again at the air sacs. Suction applied at the latter holes then caused the chorio-allantoic membranes to drop so that 0.03 ml. embryonic fluid of infectivity 10⁶ p.f.u./ml. could be applied by syringe through the first holes. The holes were then sealed with Sellotape and the eggs incubated for 24 hr at 37°. Fewer than 4% of embryos survived to this time. The eggs were then placed for 2 hr at 4° to allow contraction of the blood vessels of the membranes. Chorio-allantoic and amniotic membranes were removed with sterile forceps and scissors and the fluids by Pasteur pipette.

The pooled allantoic and amniotic fluids, after clarification to remove cell debris, indicated an infectivity of 10⁸–10⁹ p.f.u./ml. and were immediately suitable for electron microscopy. Such samples were stored at 4° and were diluted to 10⁶ p.f.u./ml. to provide the seed for further passage in embryos. Seed infectivities above 10⁶ p.f.u./ml. caused the death of the embryos before the release of further virus.

The allantoic and amniotic membranes were suspended in PBS (0.1 ml./membrane) by grinding thoroughly with washed sand in a chilled mortar. Further dispersion of the sand and membrane mixture by ultrasound (Dawe transducer no. 1163 and generator no. 1143A at 25–75 kcyc./sec.) continued for 10 min. at 10–20° and provided a gain in infectivity of about tenfold. The membrane dispersion was finally clarified by centrifugation (3000 rev./min. for 10 min.) to provide a sample of infectivity 10¹⁰–10¹¹ p.f.u./ml. Such samples were less suitable for electron microscopy than the embryonic fluids since micrographs showed a significant content of membrane fragments which were not readily separated by centrifugation.

Preparation of infective fluid from rolled monolayer cultures of BHK 21 cells. These monolayers were grown at 37° in cylindrical glass bottles which were rolled continuously about the cylindrical axis at 1 rev./3 min. Each 3 l. bottle of diameter 13 cm. and length 29 cm. provided a monolayer surface of about 1200 cm². The bottles were seeded with 7 × 10⁷ BHK 21 cells previously stripped from monolayers grown in Roux bottles. These cells were suspended in 250 ml. Eagle's medium (cf. Titration of Infectivity) and produced a confluent monolayer of about 6 × 10⁸ cells after rolling for 2 days in the 5% (v/v) CO₂ in air mixture. Gas equilibration was allowed by loose bottle caps of aluminium foil.

After 2 or 3 days the growth medium was withdrawn and the confluent, cylindrical monolayers were washed with calcium- and magnesium-free PBS and then inoculated with 5 ml. of the egg membrane extract of infectivity 10⁹–10¹⁰ p.f.u./ml. After adsorption of virus to the rolling monolayers for 30 min. at 37° the inoculum was removed, the monolayers washed again with PBS and then finally covered by 20 ml. of Eagle's medium without serum or broth.

Samples withdrawn at hourly intervals showed that the release of virus from the

continuously rolling monolayers was complete in 7–9 hr. (Fig. 1). Most samples for later work were collected at 7–8 hr in order to minimize contamination by the later detachment of cells and the release of cell debris. By about 9 or 10 hr the cell sheets were completely stripped from the glass. The terminal infectivity was about 10^{11} p.f.u./ml. and 10–100-fold higher than that of the inoculum. The infective unit yield was regularly 500 p.f.u./cell or more.

Fig. 1. The release of infectivity from a rolled monolayer culture of 6×10^8 BHK21 cells after infection with 20 p.f.u./cell of vesicular stomatitis virus (strain Ind. C) of egg origin.

Purification and concentration of infective fluids. In some cases the infective fluids from chick embryos or cultures of BHK21 cells were partially purified by single cycles of centrifugation in the Spinco Model L ultracentrifuge. The major components of the virus system were deposited by spinning for 15 min. at 25,000 rev./min. in the 10 ml. tubes of the L40 rotor. Pellets of about 2 mm. diam. were resuspended in 2 ml. 0.04 M-phosphate buffer, pH 7.6. These resuspensions were finally clarified by acceleration to 1500 rev./min. and then to rest in the L40 tubes. Sampling operations in subdued light and recoveries of infectivity were generally as described by Bradish, Brooksby & Dillon (1956).

Electron microscopy. Samples to be examined in the electron microscope were stained with a heavy metal solution (Brenner & Horne, 1959). Phosphotungstic acid (PTA) was the most successful although uranyl acetate and phosphomolybdic acid were also used and yielded consistent information. An equal volume of 1% PTA was mixed with the virus sample and mounted immediately on to carbon films on microscope grids. The carbon films were previously formed on collodion films (1% nitrocellulose in amyl acetate) subsequently removed by chloroform vapour. The Siemens Elmiskop I electron microscope was operated at 80 and 100 kV with magnifications of up to $\times 80,000$.

RESULTS

Structure and dimensions

Table 2 summarizes the data obtained in a number of representative experiments on the Ind. C. strain of vesicular stomatitis virus derived from chick embryos or rolled monolayer cultures of BHK21 cells. The data relate to the six or more major forms regularly encountered in electron micrographs of this virus system. The highly schematic diagram of the virus (Fig. 2) assists the present description.

The largest frequent particle form is the bullet or rod which may appear as full, empty or stripped with one round and one flat end. Full bullets (Pl. 1, fig. 1) are uniformly light in the print and frequently show an axial hollow of about $26\text{ m}\mu$ diameter and lipid fringe of surface projections (Howatson & Whitmore, 1962) of about $9\text{ m}\mu$ depth. The axial hollow, when present, is of irregular length and diameter and may be confused with areas on the particle surface from which the limiting envelope has been detached. When clearly defined the axial hollow may be up to $80\text{ m}\mu$ long.

Table 2. *Number and mean dimension ($\text{m}\mu$) of major particle forms in virus system of vesicular stomatitis*

Form and dimension	Bullet									Sphere or spherical section	
	Full			Stripped			Full cap			Diam. No.	
	Length	Diam.	No.	Length	Diam.	No.	Length	Diam.	No.		
Expt. 1	151.9	55.8	50	189.1	45.9	20	71.1	54.3	17	54.1	21
Expt. 2	151.5	62.0	82	177.7	47.2	91	64.7	58.3	84	64.2	76
% of form	—	—	30	—	—	25	—	—	23	—	22
Expt. 3	153	62.5	3	—	—	None	67.8	62.9	51	61.1	26
% of form	—	—	4	—	—	0	—	—	64	—	32
Data of fig. 3 & 4	156.3	61.8	198	—	—	—	—	—	—	—	—
Overall mean	154.4	60.9	333	179.8	46.7	111	66.5	59.4	152	61.8	123

Full virus particles are not penetrated by PTA, and have a mean length of $154\text{ m}\mu$, a mean diameter of $61\text{ m}\mu$ and an axial ratio of 2.54 (Table 2). These dimensions include the limiting membrane but not the fringe of surface projections. The distributions of lengths and mean diameters for 198 bullets observed at instrumental magnifications from $\times 20,000$ to $\times 80,000$ are shown in Figs. 3 and 4. The fringe was well defined in 102 of these particles and gave a mean thickness of $8.7\text{ m}\mu$. When the fringe is included the mean dimensions for the bullet become $172 \times 78\text{ m}\mu$ and these dimensions should be compared with the early data obtained by electron microscopy of metal shadowed preparations (Table 1). It may be noted that the mean diameter at the flat end ($61.3\text{ m}\mu$) is significantly greater than that at the round end ($60.5\text{ m}\mu$). This difference ($P < 0.001$ by Bahrens-Fisher test) will be discussed later.

'Empty' bullets retain the form and dimensions of the full bullets but, through greater penetration of PTA, appear darker in the print and show greater detail of structure. Four distinct layers may now be recognized: fringe, dense-limiting envelope, helical shell and reticular core (Pl. 1, fig. 2). The dimensions of the full and

Fig. 2. Schematic diagram of major component (virion) of the virus system of vesicular stomatitis showing possible types of extrusion. A: fringe of surface elements; B: dense limiting envelope; C: helical shell of capsomeres (Capsid); D: core material with or without axial hollow.

empty bullets are determined by those of the limiting envelope and the helical shell only becomes apparent when this envelope is partially or totally stripped (Pl. 1, fig. 1, 2, and Pl. 2, fig. 3). The stripped bullet, or naked helix, is about 180 mμ in length and 47 mμ in diameter (Table 2), and shows the turns of the helix as 34 cross-bands or striations. The significantly greater length of the stripped bullet may be a consequence of the release of the helix from the limiting envelope. The smaller diameter of the stripped bullet as compared with the full bullet indicates a mean thickness of 7 mμ for the dense limiting envelope; a dimension which is confirmed by the multilayer structure shown by many empty bullets (Pl. 1, fig. 1, 2).

The mean spacing of the 34 striations of the bullet indicates a helical pitch of 4.5 mμ. If the clearly beaded structure of these striations may be interpreted as 4–5 mμ particles in helical array, then on the count of 12 particles per half-turn there are about 820 morphological units of 4–5 mμ in the complete helical shell of the bullet. This shell appears as the third structural layer in 'empty' or sufficiently

stripped particles (Pl. 1, fig. 1, 2). Thus far the structure of the bullet is broadly similar to that described by other authors (Table 1), although many details and dimensions are significantly different.

In addition to the three forms of the bullet, three cap or 'spherical' forms are also observed. The cap is essentially a short bullet which, like the bullet, may appear in full or empty form (Pl. 2, figs. 4, 5). Table 2 shows that the cap has a mean length of $66.5\text{ m}\mu$ and a mean diameter of $59.4\text{ m}\mu$. The distribution of cap lengths is shown in Fig. 3. These dimensions include the limiting envelope but not the lipid fringe. The identity of the diameters of the sphere, cap and bullet shows the close relationship between these forms (Fig. 4). The relatively rare but regular observation of rods having two flat ends (Pl. 1, fig. 1; Pl. 2, fig. 3) and a length of $170\text{--}230\text{ m}\mu$ and of other forms having two round ends and a length of about $230\text{ m}\mu$ indicates that the frequent bullet and cap are members of an array of related forms. Bullets of

Fig. 3

Fig. 4

Fig. 3. Distribution of lengths of full bullets and caps in preparations of vesicular stomatitis virus from BHK21 cells. The dimensions relate to the dense limiting envelope and exclude the fringe of surface projections.

Fig. 4. Distribution of diameters of full bullets, full caps and spherical particles for preparations from BHK21 cells quoted in Fig. 3. The dimensions are those of the limiting envelope and not of the surface projections.

about twice the normal lengths are also observed (Pl. 2, fig. 3). The apparent sphere of mean diameter $62\text{ m}\mu$ (Table 2) is interpreted as an end-on view of a suitably disposed cap. Such caps may appear to be full or empty and in the latter case the penetration of PTA allows identification of the four structural layers as distinct concentric rings (Pl. 2, fig. 4). Although the outer fringe may be absent in some cases, the dense limiting envelope, the helical shell and the reticular core, with or without axial hollow, are well defined and confirm the dimensions for the other forms quoted earlier (Table 2). Caps from which both fringe and limiting envelope have been stripped have not been identified as distinct structures.

In addition to the larger particle forms discussed above several substructures are observed and one of these may be an aggregation of the fringe or lipid elements released from the surface of virus particles (Pl. 2, fig. 5). This appears as a compact coil covering a circular area of up to $120\text{ m}\mu$ diameter and with a structure and spacing similar to that of the projecting fringe of intact bullets and caps. The

slightly greater diameter of the bullet at the flat end and the frequent extrusion of hair-like and balloon-like processes from the flat ends of bullets and caps suggests a fragility at this end (Pl. 3, fig. 6). Hair- and balloon-like processes may be distinct since both may be observed on the same particle (Pl. 3, fig. 6). The hair-like process appears to be derived from the reticular core (4th structural layer) and the balloon-like process from the helical shell (3rd structural layer). The particulate nature of the helical shell is duplicated in the balloon-like process and this suggests the breaking-away of clusters of the 4–5 m μ subunits. (Pl. 3, fig. 6; Pl. 4, fig. 8). These processes are observed only at the flat ends of cap and bullets and are distinct from the peeling away of the fringe and limiting envelope which may be observed at any part of the particle surface. The present observations are inconsistent with the suggestion of Howatson & Whitmore (1962) that the hair-like filaments and membranous processes are detached portions of the host-cell membrane with attached cytoplasmic material. In micrographs of the circular cross-section of particles with well-defined axial hollows the fourth structural layer or reticular core still remains as a distinct ring within the helical shell (Pl. 2, fig. 4). In this respect the axial material becomes a fifth structural layer. The circular form shown by Howatson & Whitmore (1962) also illustrates this degree of complexity.

Distribution of particle forms

In the present representative surveys every particle form in the micrograph fields (Figs. 3, 4) has been classified and measured. The pooled data from several experiments (Fig. 3) show the distinct distributions of the lengths of caps and bullets. Although there are some long caps and some short bullets the length ranges do not overlap and the data confirm the presence of the two major particle forms and not a continuum of lengths determined by the random disruption of a single long bullet form. The distributions of the diameters (Fig. 4) of the limiting envelopes of bullets, caps and spheres are identical in range and mean (Table 2) and are consistent with the relationships discussed later.

Typical data (Expts. 1 and 2 of Table 2) show that the clarified fluids from cultures of BHK21 cells held for 8 hr after infection with vesicular stomatitis virus yielded full bullets, stripped bullets, caps and spheres in approximately equal numbers. Since caps and spheres are interpreted as aspects of the same form it follows that bullets and caps are almost equally frequent. Most virus suspensions derived from pooled embryonic fluids gave a similar distribution. In contrast, some supernatant fluids collected after 4–6 hr from infected BHK21 cells showed (Expt. 3 of Table 2) almost all caps (96 %) and very few bullets (Pl. 4, fig. 7). Alternatively, some preparations from BHK21 cells or egg fluids showed almost all bullets with very few caps (Pl. 4, fig. 8). Since all of these preparations were associated with high infectivity or a yield pattern of the type shown in Fig. 1 it clearly cannot be concluded on this evidence that only the bullet is infective or that the cap is either a product of adverse manipulation or an interfering antigen. The factors determining a particular distribution of particle forms have not yet been defined.

In justification of the classification of the 'sphere' as a particular aspect of the cap rather than of the bullet it may be noted that in preparations derived from BHK21 cells and embryonic fluids the numbers of bullets and caps varied independently, whereas spheres and caps occurred in almost equal numbers regardless

of the count of bullets. This behaviour is consistent with the random settling of the cap on end-on or side-on aspect and with the preferential side-on settling of the majority of bullets. Some caps are seen in intermediate positions showing both cap and sphere aspects (Pl. 2, fig. 4, 5). The situation in a thin section of a pellet of bullets would be quite different since the initial random array would ensure that 'bullet' sections, circular cross-sections and taper-sections at other angles would occur with appropriate frequencies. This type of micrograph has been presented by Howatson & Whitmore (1962).

DISCUSSION

Although the virus of vesicular stomatitis has been much studied the experimental methods employed have varied so widely that divergence of detail and interpretation must be expected. The relevant data are gathered in Table 1 and are numbered chronologically for convenience in reference. In the earliest observations (Table 1; 1 and 2) the specimens were metal shadowed for electron microscopy and it must be assumed in view of the significant coating thickness that dimensions may be overestimated by up to 5 m μ . This uncertainty is increased to up to 20 m μ by the later observation (Table 1; 3-5) of the fringe of surface projections which must have been included to some extent in the 'shadowed dimensions' but not in the later 'PTA dimensions' defined by the limiting envelope. The near identity of some mean dimensional data (Table 1; 2 and 5) obtained by different methods in electron microscopy must therefore be regarded as coincidental.

On the basis of the available distributions of the dimensions of the envelopes of PTA-stained bullets (Table 1; 5 and 10) there is a significant difference between the mean lengths observed by Howatson & Whitmore (175 m μ) and the present authors (154 m μ): each distribution barely includes the mean of the other. This difference may arise from the experimental procedures or be a reflection of modifications imposed by the hosts employed. Significant host imposed modifications of density of infective components have been observed in this laboratory by Warrington. (Table 1; 9). The mean dimensions of the bullet or rod obtained by analytical ultracentrifugation and in the present study by electron microscopy are in close agreement. (Table 1; 2 and 10). At the morphological level the data of several groups agree closely and support the general structure (Reczko, 1960; Stone, Sellers & Hiramoto 1961; Howatson & Whitmore, 1962) shown schematically in Fig. 2.

More important distinctions between the different interpretations arise in relation to particle forms other than the bullet. In the early studies (Table 1; 2) of shadowed preparations two particle forms were recognized, the rod and the spherical granule, and these are now identified as the bullet and the cap in PTA stained preparations. The presence of these two components in initial or fractionated samples was confirmed by analytical centrifugation which revealed distinct peaks at 625S and 330S. Howatson & Whitmore (Table 1; 5) reported only bullets in their preparations and suggested that spherical forms were not distinct entities but corresponded to the remains of bullets broken during prolonged storage or centrifugation: they did not observe caps in their preparations and the spherical forms were regarded as 'blebs' or bullets viewed end-on. Although caps may be generated to some degree by adverse manipulation (Table 1; 2) there is little doubt that this is a minor process in the range of experiments described here. Hackett (Table 1; 7) similarly found that

spherical components and caps were not produced in preparations which had lost 90 % of initial infectivity through storage. On the other hand many of the present preparations of high infectivity contained a significant or even predominant concentration of well-defined caps with very few bullets when examined immediately and without fractionation of any kind. Such caps showed a narrow distribution of lengths which is distinct from that of the bullet and cannot be regarded as a continuum of degraded material.

The observation by Hackett (Table 1; 7) that spherical components and caps were found in fresh preparations after many cycles of growth in primary chick fibroblasts supports the present view that the cap, like the bullet, is one of several particle forms which may be released by the infected cell. Initial infective preparations may contain bullets and caps in any proportion although the conditions in culture which control this release have not been determined.

Although vesicular stomatitis virus cannot be passaged continuously in roller cultures of BHK 21 cells without severe depression of infectivity yield by an auto-interference effect this has not been found to be associated with an increased concentration of caps or aberrant bullets. The present data do not therefore incriminate the cap as the major factor in the auto-interference effect, as suggested by Hackett.

Howatson & Whitmore (Table 1; 5) suggested that the flat end of the bullet is due to the clean breaking-off of this form at the moment of release from the cell, but it is difficult to reconcile this mechanism with the doubly flat-ended and doubly round-ended forms which are observed rarely but regularly. Howatson & Whitmore also interpreted the trailing filament or hair-like extrusion as a portion of the cell membrane with attached cytoplasmic material, but this appears to be inconsistent with the several distinct types of extrusion process and the identification of at least one of these with the substance of the virus particle.

It is concluded that the virus of vesicular stomatitis is a system of several distinct forms and that we are unable at present to identify any one of these as the only primary particle or the only infective component. These particle forms are probably released as such from the cell in distributions determined by cultural conditions and the origin and state of the cell.

The variety of particle forms associated with the virus system of vesicular stomatitis is related to an equally complex range of biological properties. The reduction of infectivity by centrifugation or heating, or by treatment with solvents or acid is accompanied by a loss of the major particle forms, the bullet and cap. The remaining slower sedimenting material (sedimentation coefficient 7–30 S) is active as a specific antigen in the complement fixation test (Bradish *et al.* 1956), as an antigen in the blocking of the neutralizing activity of homologous rabbit antisera and as an antigen in the production of neutralizing antibody in rabbits (Ferrier & Bradish, to be published). These data suggest, as in the case of similar studies on the virus system of foot and mouth disease, that the slower sedimenting components include virus-specific protein elements which are derived from the structure of, or are precursors of, the major infective components. These protein elements may be identified with the 4–5 m μ particles of the helical shell. The function of other structural elements is less clear. Howatson & Whitmore (1962) indicated a relationship between the surface elements of the released bullet and those of the parent cell

but the process by which virus penetrates and infects the cell has not been interpreted at the morphological level. The ribonucleic acid (Chamsy & Cooper, 1963; Levine & Olson, 1963) of the virus particle is presumed to be associated with the dense reticular core (4th structural layer) or axial material and this, when released into the medium by extrusion or disruption, may give rise to the slowly sedimenting trace infectivity ($\sim 20S$) reported by Bradish *et al.* (1956).

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Survey electron micrograph ($\times 110,000$) showing various types of particle and extrusion in the virus system of vesicular stomatitis. Note: A, full bullets; B, empty and partially stripped bullets; C, caps in full or partially stripped state; D, doubly flat-ended rods with axial hollow at each end; E, dense limiting envelope as second structural layer. F, striations (helical shell) of third structural layer in 'empty' bullets.

Fig. 2. Micrograph showing full (A) and empty or stripped bullets (B). In the latter the four structural layers of Fig. 2 appear in apparent section. ($\times 170,000$)

PLATE 2

Fig. 3. Micrograph showing full (A) and empty or stripped bullets (B), bullets of abnormal length (C), rods with two flat ends and two axial hollows (D), full caps (E) and empty caps with striations of third structural layer (F). ($\times 120,000$.)

Fig. 4. Micrograph showing full (A) and empty or stripped caps (B) with extrusions and axial hollows. Caps in 'end-on' presentation (C) show the structural layers of Fig. 2 as concentric rings: the fringes of surface projections are not clear in the print so that the particles are defined by the dense limiting envelope (second structural layer). ($\times 100,000$.)

Fig. 5. Micrograph showing full (A) and empty or stripped caps (B). Caps in inclined aspect (C) give an oblique view of the flat end and its concentric rings of structural layers (cf. Pl. 1, fig. 2 and Pl. 2, fig. 4). Note the coiled structure (D) of about five turns resembling material from the projecting fringe and limiting envelope. ($\times 110,000$.)

PLATE 3

Fig. 6. Micrograph showing various types of extrusion from bullets and caps. Note full or partially stripped bullets with hair- and balloon-like processes (A) at flat end and detachment of projecting fringe and dense limiting envelope (B) elsewhere on surface. Note also particulate nature of striations in stripped bullets (C) and of material derived from these (D). ($\times 170,000$.)

PLATE 4

Fig. 7. Survey micrograph showing 96 % caps and spheres in supernatant collected at 6 hr from infected roller culture of BHK21 cells. ($\times 40,000$.)

Fig. 8. Survey micrograph showing various types of extrusion from full and stripped bullets as in Pl. 3, fig. 6. Note also almost completely stripped particles (A) in apparent section showing remnants of dense limiting envelope and hair-like extrusion from third structural layer (helical-shell). ($\times 69,000$.)

Note added in proof

Since preparation of this script further papers have appeared on the morphology and autointerference effect of VSV. Huang & Wagner (1965), Wagner & Huang (1966), using VSV-Indiana in Krebs-2 mouse ascites cells, found that infective bullets and 'small non-infective particles' banded in sucrose at different densities and that both switched-off the synthesis of cellular RNA and interferon, even after ultraviolet irradiation. Hackett, Madin & Schaffer (1966), using VSV-Indiana grown in primary chick fibroblasts, separated infective bullets from non-infective caps and found that both interfered with the multiplication of infectivity. This property was destroyed by heat, u.v.-irradiation or sonication. These inconsistencies require the definition of the properties of the several distinct particle forms within each density band.

The data of Warrington (1965) and McCombs, Benyesh-Melnick & Brunschwig (1966) show that distinct interpretations may arise from the use of different assay methods, density gradient media or host systems. The latter group, using VSV-Indiana in human embryo lung fibroblasts, found three infectivity peaks in caesium chloride gradients: 'normal' bullets (density 1.19 g./ml.), bent-forms (1.22 g./ml.) and strands of 15 m μ diameter, probably nucleoprotein, with trapped bullets and bent forms (1.26 g./ml.). The abundance of strands and bent-forms and absence of caps may reflect a property of the host system and experimental conditions. The 15 m μ strand may arise as a filamentous extrusion from the 3rd and 4th structural layers (Fig. 2) and is consistent with the slowly sedimenting (Bradish *et al.* 1956) and rapidly diffusing (Warrington, 1965) infective component.

The distinct morphological group of VSV-like agents has been discussed by Bell (1966). The observation that other arboviruses also fall within this group, although of distinct bullet length and number of striations, indicates that the arboviruses are not a group of morphologically similar agents (Andrewes, 1965).

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