A Quantitative Ultrastructural Study of the Development of Bluetongue Virus in Madin–Darby Bovine Kidney Cells

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SUMMARY

Samples of Madin–Darby bovine kidney cells were taken for electron microscopy at various times after infection with bluetongue virus. A quantitative electron microscopic cell sampling technique was used in observing ultrathin sections of the cell population, and various morphological features were tabulated. The appearance of virus within phagocytic vesicles and lysosomes, and the distension of the rough endoplasmic reticulum, were early ultrastructural changes resulting from the virus infection. Granular inclusions which were usually juxtanuclear and tubular structures were other features observed later in cells infected with bluetongue virus. Progeny virus particles were seen within granular inclusions, amidst tubular structures, and within cytoplasmic vesicles. There was agreement between the infectivity data from plaque counts of cell-associated virus and direct electron microscopic counts of cells containing intracellular virus, granular inclusions, and cytoplasmic tubular structures. The large tubules associated with bluetongue infection had a mean outer diameter of 47.2 nm, more than twice the size of classic cellular microtubules. The mean diameter of bluetongue virus measured in ultrathin sections and negatively stained preparations was 63 nm. Even at late stages of infection, small numbers of intracellular virus particles were observed with the electron microscope. This agreed with the low titres obtained from plaque counts of cell-associated virus. Bluetongue virus did not, therefore, accumulate in the cell and undergo a burst-like release. Instead, it appeared to be extruded from the cell as it was made. There was no evidence for virus release by budding from the plasma membrane, nor for the presence of an envelope around complete particles.

INTRODUCTION

Bluetongue virus causes a disease of sheep characterized by signs of generalized infection, including glossitis and laminitis. The virus is transmitted by, and replicates in, the culicoid fly, Culicoides variipennis (Foster, Jones, & McCrory, 1963; Jochim & Jones, 1966). The virus possesses double-stranded RNA (Verwoerd, 1969) and appears to be somewhat smaller than reoviruses (Els & Verwoerd, 1969) which it seems to resemble closely.

Bowne & Jochim (1967) did some preliminary work on the replication in primary lamb kidney cells and in the McCoy synovial cell line of bluetongue virus strain 8. This was, however, not a sequential study. Lecatsas (1968a) conducted a sequential study of blue-
tongue virus strain I0 in BHK 21 cells. He showed the uptake and penetration of bluetongue virus into the cell via phagocytic vesicles and lysosomes. Later in infection, he observed cytoplasmic tubular structures, dense granular inclusions containing virus particles, and complete particles which were released through discontinuities in the plasma membrane. Whether he made random or systematic ultrastructural observations of infected cell populations was not reported. In addition, quantitative observations to determine the percentages of those cells which exhibited a given feature were not reported, nor was a comparison made between the electron microscopic findings and kinetic data from a growth curve of the virus in BHK 21 cells.

The purpose of this study was to compare the ultrastructural development of cloned bluetongue virus strain 8 in the Madin-Darby bovine kidney cell line with published reports of bluetongue virus and reovirus replication in various cell systems, using a quantitative electron microscopic cell sampling technique. These results will be compared with the dynamics of virus multiplication seen in the virus growth curve.

METHODS

Infection of cell cultures. Confluent monolayers in 25 cm.² plastic flasks were inoculated with enough virus to give an input m.o.i. of 8 p.f.u. per cell. Virus was allowed to adsorb for 1 hr at room temperature; after this, the monolayers were washed twice to remove unadsorbed virus. Eagle's MEM (5 ml.) with Earle's salts without serum were added to each flask as maintenance medium during incubation.

One-step growth curve. At various intervals after infection, 5 ml. of maintenance medium were removed from two flasks and stored at 4 ° until assayed for released virus. Simultaneously, the cells were removed from the flask using a sterile rubber policeman. The cells were suspended in 2 ml. of maintenance medium without serum, frozen and thawed twice to release cell-associated virus, and stored at 4 ° until assayed. A plaque assay was used to determine the titres of released and cell-associated virus. Plaque counts were made after incubation at 34 ° for 7 days. A 1/5000 neutral red solution was added to cultures to enhance plaque visualization before plaque enumeration. Three replicate plates/dilution were used to compute the mean plaque count.

Electron microscopy. For negative staining, the supernatant fluid from four 75 cm.² plastic flasks containing infected cells was collected and clarified by centrifugation in a model RC-2B Sorvall Refrigerated Centrifuge at 7500 g and 4 ° for 10 min. The virus in the clarified supernatant fluid was sedimented by centrifugation in a Spinco Model L2-65B Preparative Ultracentrifuge at 105,000 g for 2 hr. The resulting virus pellet was resuspended in sterile distilled water and mixed with 2 % potassium phosphotungstate at pH 6·8. The stained preparation was placed on carbon-filmed 400 mesh grids for observation.

For thin sectioning, infected cell monolayers were scraped from 25 cm.² plastic flasks, then washed twice with phosphate-buffered saline (Dulbecco & Vogt, 1954) at 10 and 30 min., and 2, 6, 8, 11, 13, 16, 18, 24 and 28 hr after inoculation. They were fixed in buffered glutaraldehyde followed by post-fixation in buffered osmium tetroxide. ‘In block’ staining with uranyl acetate was done overnight. After dehydration in graded ethanol dilutions, the cells were embedded in Araldite 6005-Epon 812 plastic following Mollenhauer's (1964) procedure. The controls were prepared in the same manner. Sections were cut using glass or diamond knives on a Sorvall Porter-Blum MT-2 ultramicrotome and then placed on Index I (Ladd Research Industries, Inc., Burlington, Vt.) grids filmed with carbon-stabilized Formvar. Post-staining was with uranyl acetate followed by lead citrate (Reynolds, 1963).
All specimens were examined with an RCA EMU-4A electron microscope operating at 50 kV.

Since the Madin–Darby cell line is composed of relatively homogeneous epithelioid cells (Madin & Darby, 1958), an electron microscopic cell sampling technique was used to observe the changing sequence of cellular events with time. In addition to the cells systematically observed, sections of other cells were also scanned randomly at lower magnifications. The sampling method was as follows: a map was made of the cells in a single good section which was chosen according to the following criteria: (1) the section was intact and not folded so as to obscure cells, and (2) it contained at least 30 to 40 cells. Thirty or more cells containing a nucleus in the plane of section were carefully examined at 40,000 × instrument magnification, and the various morphological features were tabulated. In each category the cell sections were scored for presence or absence of the feature. We assumed that in a given section there would be a random sample of cells from the homogeneous Madin–Darby cell population which was originally in two 25 cm.² plastic flasks. Once the sampling was begun, each cell was taken in sequence as it appeared on the screen when the specimen was traversed, with the exception of those cells which did not contain a nucleus or were partially obscured by grid bars. In this manner, bias was avoided. We assumed that an approximately median representative section of cells which contained nuclei had been made. In addition, by restricting the sampling to a single section containing 30 or more cells, a given cell would not be sampled twice.

A Fullam (Schenectady, New York) carbon replica of a diffraction grating (54,800 lines per inch) was used for magnification calibration of the microscope. Virus particles and other structures were measured from negatives using a Nikon model 6C optical comparator.

RESULTS

Virus size and structure

Bluetongue virus strain 8 was measured both in negatively stained preparations and ultrathin sections (Table 1). The outer diameter of the negatively stained particle was 63.6 ± 1.6 nm., while that of the virus particle in ultrathin section was 63.8 ± 1.1 nm. The virus possessed large, hollow capsomeres, exhibited cubic symmetry, and had no envelope. In sectioned material, the particle appeared to consist of an outer coat, and centrally placed, dense nucleoid. The diameter of the nucleoid was 22.7 ± 0.8 nm. (Table 1). In favourable sections, the virus outer coat seemed to exhibit discrete projections (Fig. 6a) which may have corresponded to capsomeres.

The uninfected cell

Madin–Darby bovine kidney cells possess a large nucleus which contains a prominent nucleolus (Fig. 1). The cytoplasm contained many profiles of rough endoplasmic reticulum, mitochondria, and a few vacuoles. Occasionally a Golgi complex was observed near the nucleus. Polysomes consisting of five or six ribosomes in the plane of section were abundant in the cytoplasm. There were also some very fine cytoplasmic filaments in these cells. The plasma membrane of the cells was convoluted by many small cytoplasmic processes, which are a common feature in cultured cells. Madin–Darby control cells contained no latent virus contaminants.

Virus growth curve

The eclipse phase for the released virus was approximately 3 hr. From five to 15 hr the released virus remained constant. After 15 hr there was a secondary tenfold rise in the
concentration of released virus. No release of cell-associated virus occurred until 7 hr after inoculation, and the titres of the cell-associated virus were maximal after 21 hr (Fig. 2).

**Early events in infection**

Ten min. after inoculation it was possible to see adsorbed virus on the cell surface. Occasionally, phagocytic vesicles were observed forming near adsorbed virus. Thirty min. after inoculation virus was seen intracellularly either within phagocytic vesicles near the plasma membrane (Fig. 3) or within lysosomes. Virus nucleoids also were observed within lysosomes at this time. From 30 min. to 2 hr after inoculation lysosomes containing virus particles or nucleoids were seen near the nucleus. This was the site of granular inclusion body formation later in infection. Distension of the rough endoplasmic reticulum was first observed 2 hr after inoculation, and this was present throughout infection in all the cells which were examined (Fig. 6a, 7a).

**Table 1. Size comparison of bluetongue virus with reoviruses**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Negatively stained</th>
<th>Ultrathin sections</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Outer diameter (nm.)</td>
<td>Internal diameter (nm.)</td>
</tr>
<tr>
<td>Bluetongue strain 8 (present work)</td>
<td>63.6 ± 1.6* (n = 135)</td>
<td>—</td>
</tr>
<tr>
<td>Bluetongue strains 8, 254, 262, 318 (Bowne &amp; Ritchie, 1970)</td>
<td>45 to 55</td>
<td>60 to 70</td>
</tr>
<tr>
<td>Bluetongue type 10 (Els &amp; Verwoerd, 1969)</td>
<td>53.8 ± 1.6 (n = 100)</td>
<td>—</td>
</tr>
<tr>
<td>Bluetongue isolated from S. African outbreak (Owen &amp; Munz, 1966)</td>
<td>60</td>
<td>—</td>
</tr>
<tr>
<td>Bluetongue strains 8 and Cy (Studdert et al. 1966)</td>
<td>—</td>
<td>53</td>
</tr>
<tr>
<td>Reovirus type 1 Rhim &amp; Melnick, 1961</td>
<td>—</td>
<td>75</td>
</tr>
<tr>
<td>Hassan, Rabin &amp; Melnick, 1965</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Reovirus type 2 Loh, Holli &amp; Soergel, 1965</td>
<td>77.2 ± 2.8 (n = 143)</td>
<td>45.9 ± 2.0 (n = 27)</td>
</tr>
<tr>
<td>Anderson &amp; Doane, 1966</td>
<td>45</td>
<td>48</td>
</tr>
<tr>
<td>Reovirus type 3 Vasquez &amp; Tournier, 1962</td>
<td>59.5 ± 2.5 (n = 100)</td>
<td>32.5 ± 1.5 (n = 50)</td>
</tr>
<tr>
<td>Harford et al. 1962</td>
<td>—</td>
<td>—</td>
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</tbody>
</table>

* 99% confidence interval for the mean.

**Later events in infection**

Granular inclusions and large cytoplasmic tubules seemed to be associated with virus replication. Dense, finely granular, juxtaplasmic inclusions, within which the virus appeared to replicate, were first observed in 3% of the cells 8 hr after inoculation (Fig. 4). From 8 to 16 hr after inoculation, the number of cells containing granular inclusions increased sharply until approximately 72% contained inclusions. Incomplete particles consisting of only a nucleoid were often present within these entities alone, or along with the complete virions (Fig. 5a). Later in infection, several granular inclusions were observed scattered throughout
the cytoplasm of infected cells (Fig. 7a). These latter inclusions appeared to contain only complete virus particles.

The second structure associated with bluetongue infection was a large cytoplasmic tubule with a mean diameter of $47.2 \pm 1.2$ nm. (Table 2). This is more than twice the size of classic cellular microtubules (Fig. 6b). The macrotubules were arranged either in small parallel bundles (Fig. 7b) or in a disorganized array near complete virus particles or granular.

Fig. 1. Uninfected Madin-Darby bovine kidney cells. These cells possess a cytoplasm containing many mitochondria, rough endoplasmic reticulum cisternae and ribosomes, and a large nucleus containing a prominent nucleolus.
inclusions (Fig. 5b). These large tubules possessed no apparent structures within their lumen or coating their outer surface. Some macrotubules extended for a considerable length in the plane of section, while others appeared to be quite short. Large cytoplasmic tubules were first observed in cells 11 hr after inoculation (Fig. 8). The number of cells containing macrotubules increased sharply from 11 to 16 hr after inoculation with approximately 70% of the cells exhibiting large cytoplasmic tubules after this time.

Complete progeny virus particles were seen intracellularly beginning at 6 hr after inoculation when 3% of the observed cells contained them (Fig. 9). The percentage of cells exhibiting intracellular virus (excluding virus within lysosomes) increased sharply from 6 to 13 hr after inoculation. From the 13th hr after inoculation, 85% of the observed cells contained intracellular virus. When the data from an infectivity assay of cell-associated virus were plotted with data from electron microscopic direct observation of intracellular virus, there was good agreement between the two curves (Fig. 10).
Progeny virus particles were seen within granular inclusions (Fig. 5b), dispersed in the cytoplasm near the nucleus (Fig. 5a), within cytoplasmic vesicles, in groups near the plasma membrane during later stages of infection (Fig. 6a), or extracellularly (Fig. 6a). In early stages of infection virus particles appeared to be more localized in one area of the cytoplasm.

![Graph](image)

Fig. 4. Quantitative electron microscopic observations of granular inclusions in Madin-Darby cell cultures infected with bluetongue virus.

Table 2. Comparison of macrotubules with microtubules

<table>
<thead>
<tr>
<th></th>
<th>Macrotubule outer diameter (nm.)</th>
<th>Microtubule outer diameter (nm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slatterback, 1963</td>
<td>—</td>
<td>15-25</td>
</tr>
<tr>
<td>Madin–Darby cell microtubules (present work)</td>
<td>—</td>
<td>21.2 ± 1.2* (n = 25)</td>
</tr>
<tr>
<td>Bluetongue virus</td>
<td>47.2 ± 1.2 (n = 100)</td>
<td>—</td>
</tr>
<tr>
<td>Present work</td>
<td>Absent</td>
<td>—</td>
</tr>
<tr>
<td>Bowne &amp; Ritchie, 1970</td>
<td>Approx. 50 nm., but described as microtubules</td>
<td>—</td>
</tr>
<tr>
<td>Lecatsas, 1968a</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>Reovirus type 1</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>Jenson et al., 1965</td>
<td>Equals virus</td>
<td>—</td>
</tr>
<tr>
<td>Lecatsas, 1968b</td>
<td>Approx. 50 nm., but described as microtubules</td>
<td>—</td>
</tr>
<tr>
<td>Reovirus type 2</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>Anderson &amp; Doane, 1966</td>
<td>Approx. 50 nm., but described as microtubules</td>
<td>—</td>
</tr>
<tr>
<td>Reovirus type 3</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>Tournier &amp; Plissier, 1960</td>
<td>35</td>
<td>—</td>
</tr>
<tr>
<td>Gomatos et al., 1962</td>
<td>Equals virus</td>
<td>—</td>
</tr>
<tr>
<td>Harford et al., 1962</td>
<td>Equals virus</td>
<td>—</td>
</tr>
<tr>
<td>Dales, 1963</td>
<td>—</td>
<td>18 to 23</td>
</tr>
</tbody>
</table>

* 99% confidence interval for the mean.
However, as the infection progressed cells were observed which contained several foci of virus particles (Fig. 7a). Even at late stages of infection, neither paracrystalline virus inclusions nor the accumulation of large numbers of virus particles were observed intracellularly. This is in agreement with the low titres for cell-associated virus obtained from plaque counts when compared to released virus.

Fig. 5(a). A juxtanuclear granular inclusion containing incomplete bluetongue virus particles (IV) 28 hr after infection. In the cytoplasm near the nucleus may be seen two complete particles (V).

Fig. 5(b). A juxtanuclear granular inclusion containing complete bluetongue virions 48 hr after inoculation. Cross-sections as well as longitudinal sections of the macrotubules associated with bluetongue infection, and fine cytoplasmic filaments may be seen in the cytoplasm nearby.

From 8 hr after inoculation, the percentage of cells containing multivesicular bodies varied from 57 to 97%, whereas only 25% of the controls exhibited these bodies. We do not know the significance of this observation.

**Virus release**

We did not observe budding as a means of virus release in several hundred infected cells and virus release by extrusion was observed very infrequently. From 13 hr after inoculation 3 to 7% of the cells appeared to be releasing virions by extrusion. Once progeny virus began to be released, there was no way to differentiate between the extrusion of progeny virus and
Ultrastructure of bluetongue virus

Fig. 6(a). Madin-Darby cells 28 hr after infection with bluetongue virus. In the cytoplasm may be seen distended rough endoplasmic reticulum cisternae, a juxtanuclear granular inclusion containing incomplete virus particles, and complete particles near the plasma membrane. Note the projections (P) radiating from some of the extracellular bluetongue virus particles.

Fig. 6(b). Insert: a higher magnification of the area marked in Fig. 6a. The size difference between cellular microtubules (MT) and the macrotubules associated with the infection (T) may be seen here.
Fig. 7(a). A Madin-Darby cell 28 hr after infection with bluetongue virus. In the cytoplasm may be seen distended endoplasmic reticulum cisternae (ER), several granular inclusions (I), and scattered groups of bluetongue virus particles (V).

Fig. 7(b). Insert: a higher magnification of the area marked in Fig. 7a, showing longitudinal (LT) and cross-sections (CT) of macrotubules.
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readsorption of released progeny virus. From 13 hr after inoculation, 60 to 77% of the cells contained virus particles near the plasma membrane. Since most of these particles were not within cytoplasmic vesicles, we assumed that they represented progeny virus about to be released rather than adsorbed progeny virus which had not been uncoated. We observed what we considered to be virus release by extrusion, but found no evidence for accumulation of virus within the cell and subsequent burst-like release. We observed few intracellular virus

Fig. 8. Quantitative electron microscopic observations of macrotubules in Madin–Darby cell cultures infected with bluetongue virus.

Fig. 9. Quantitative electron microscopic observations of intracellular virus in Madin–Darby cell cultures infected with bluetongue virus.
particles, even at late stages of infection. The released virus curve plotted from plaque counts was much higher than the cell-associated virus curve; suggesting that bluetongue was released shortly after intracellular maturation. Extracellular virus particles were more often in small groups than singly outside the cell. Infrequently, several extracellular particles were observed enclosed within a membranous vesicle.

![Graph showing percentage of cells exhibiting virus over time](image)

**Fig. 10.** Comparison of infectivity assay data for cell-associated bluetongue virus with electron microscopic direct observation of intracellular bluetongue virus in Madin–Darby cells. ■—■, cells exhibiting intracellular virus when viewed with the electron microscope; ○--○, cell-associated virus titres.

**Features not associated with virus infection**

Careful observations were made of the nuclei and mitochondria of infected cells in all samples examined. No nuclear changes or dense mitochondrial inclusions resulting from the virus infection were seen in any samples.

The number of cells containing fine, kinky cytoplasmic filaments (Fig. 5b) appeared to increase during the course of infection so that after 11 hr, 73 to 93% of the cells contained filaments. However, similar filaments were present in 43% of uninfected Madin–Darby cells. Because they were so small, it was difficult to see the filaments, especially in a cytoplasm which was dense and filled with ribosomes and cellular organelles. It was therefore difficult to ascertain if the increase in the number of infected cells containing filaments was a result of cytoplasmic degeneration, which made them easier to observe, or virus replication.

**DISCUSSION**

The early events observed during bluetongue virus infection described here are in agreement with the ultrastructural observations of Lecatsas (1968a). In Lecatsas's and in our studies, adsorbed virus was seen on the cell surfaces 10 min. after inoculation. Phagocytic vesicles containing virus particles were observed during the next 30 min., and finally virus nucleoids or complete particles were seen within lysosomes between 30 min and 2 hr. These early events closely resemble those previously reported for reovirus type 3 in L cells (Silverstein & Dales, 1968).
Ultrastructure of bluetongue virus

Our observation of rough endoplasmic reticulum distension beginning 2 hr after inoculation agrees with the work reported by Lecatsas (1968a).

When stained with an anti-bluetongue virus fluorescent antibody conjugate, infected cells exhibited juxtanuclear fluorescence. By ultrastructural examination of infected cells, inclusion bodies were usually observed in the juxtanuclear position. These inclusions consisted of a dense, finely granular matrix containing bluetongue virus particles. We suggest that the juxtanuclear fluorescence corresponds to the location of the inclusion bodies. Using primary lamb kidney cells, Bowne & Jochim (1967) also observed such juxtanuclear fluorescence which corresponded to their cytoplasmic virus-containing granular inclusions seen with the electron microscope. Granular inclusions were first observed 8 hr after inoculation in our study. The percentage of cells containing inclusions increased abruptly between 11 and 16 hr after infection, then remained constant. These findings were in agreement with the intracellular virus curve determined by electron microscopy. Furthermore, incomplete as well as complete virions were observed within these inclusions. Incomplete virions were not seen in any other cytoplasmic location. Lecatsas (1968a) also observed granular inclusions, but neither drew attention to their frequent juxtanuclear location, nor observed incomplete virus particles within them. Bowne & Jones (1966) noted granular inclusions containing complete particles and also incomplete particles which consisted of either just a nucleoid or only an empty capsid within salivary gland cells of experimentally infected Culicoides variipennis. These granular inclusions appear to be the matrix for progeny virus replication. Granular inclusions also have been observed in infections with reovirus types 1 (Jenson et al. 1965) and 3 (Tournier & Plissier, 1960).

Dales (1963) reported the observation of reovirus type 3 replication in association with spindle tubules. The tubular structures observed by Dales were in the size range of spindle tubules. Before this, in three studies on reovirus type 3 replication tubules were reported having a diameter of 35 nm. (Tournier & Plissier, 1960) or about the size of the virus (Gomatos et al. 1962; Harford et al. 1962) in infected cells (Table 2). Subsequent papers on reoviruses (Lecatsas, 1968b; Anderson & Doane, 1966) and bluetongue virus (Lecatsas, 1968a) have referred to these large tubules as spindle tubules or microtubules citing the report by Dales (1963). Investigators have not attempted to measure these entities in order to distinguish them from classic cellular microtubules. When measured from their micrographs, the tubular structures described by Lecatsas (1968a, b) and Anderson & Doane (1966) are not in the size range of microtubules. Instead, their tubules measure about 50 nm. in diameter, which is similar to the tubules of 47 nm. observed in our study. Bowne & Jones (1966) described a ‘mesh-work of filaments or vesicles’ in the cytoplasm of infected Culicoides variipennis salivary gland cells; these structures appear to be similar to the large tubules described above. However, Bowne & Ritchie (1970) reported the absence of tubules in their primary lamb kidney cells infected with bluetongue virus. Lecatsas (1968a) saw macrotubules as early as 8 hr after inoculation, while our first observation of these structures was at 11 hr. Our work shows that these macrotubules occur only in cells infected with bluetongue virus. Consequently, we postulate that they are associated with the virus infection and await verification by future experimentation. Since the electron staining properties of the macrotubules are not similar to nucleic acid, they possibly may be composed of soluble antigen or another virus protein.

In our study, complete virus particles were seen as early as 6 hr after inoculation in a small percentage of cells, while Lecatsas (1968a) first observed complete particles at 12 hr. Two other points of disagreement between our study and his involve the location of inclusion bodies and the nature of fine cytoplasmic filaments. We attempted, but failed, to observe
dense inclusion bodies within mitochondria during any stage of infection with bluetongue virus. He did observe such structures. Our second point of disagreement with his report concerns the nature of fine cytoplasmic filaments. Lecatsas (1968a) believes that these filaments, which he claims are attached to virus nucleoids, are virus nucleic acid strands. However, he reported that the filaments were not digested by RNase. Our contention is that since fine cytoplasmic filaments are a normal component of both BHK 21 and Madin–Darby cells, it will be difficult to determine if these filaments are different from the ones observed in infected cells, and if so, what their role is in infection. According to Doane (1966) contend that in reovirus type 2 replication, these filaments appear to be incorporated into the virus outer coat. However, Dales, Gomatos & Hsu (1965) failed to find evidence for reovirus type 3 antigen in the filaments associated with the replication of that virus.

Data from the growth curve study indicate that bluetongue virus does not accumulate in the cell and undergo a burst-like release characteristic of mammalian reoviruses. Instead, it is released from the cell as it is made. The failure to accumulate intracellular virus may explain the lack of paracrystalline virus inclusions which have been observed in cells infected with reovirus types 1 (Jenson et al., 1965), 2 (Anderson & Doane, 1966), and 3 (Tournier & Plissier, 1960).

Virus release appears to be by extrusion rather than by budding. However, Bowne & Ritchie (1970) report that bluetongue virus is released by budding in their primary lamb kidney cells. Although some investigators have reported that a small number of bluetongue virions have a lipoprotein envelope (Owen & Munz, 1966; Bowne & Jones, 1966; Bowne & Ritchie, 1970), other workers have reported that it is chloroform-resistant and does not possess a lipid-containing envelope (Studdert, 1965; Svehag, Leendertsen & Gorham, 1966; Studdert, Pangborn & Addison, 1966; Els & Verwoerd, 1969; Lecatsas, 1968a). Virus release by budding incorporates a portion of the cell's lipoprotein plasma membrane around the particle; however, no enveloped particles were observed in our study. Therefore, virus release appears to be by extrusion rather than budding. Since some progeny virus particles were observed within intracellular vesicles, it is possible that extrusion of virus within a vesicle would give the appearance of a pseudo-envelope surrounding the extracellular particle. The observation of such extracellular particles was rare in this study as well as in previous reports (Lecatsas, 1968a; Els & Verwoerd, 1969).

Lecatsas (1968a) noted complete virus particles extracellularly near small discontinuities in the plasma membrane and stated that liberation of the virus was through these discontinuities. However, he published no micrograph illustrating this method of virus release. In our study, the percentage of cells exhibiting short discontinuities in their plasma membranes was 30 to 40 %, from 11 to 28 hr after inoculation as compared to the controls which did not exhibit such discontinuities. However, because it is sometimes difficult to differentiate between a glancing section of the plasma membrane and a true discontinuity, we have not attached much significance to these observations. It may be that virus extrusion occurs rapidly, thereby resulting in the low frequency of observations of this event (3 to 7 % in this study). Furthermore, it is very likely that in order to preserve its cellular integrity, the plasma membrane of a cell would fuse fairly rapidly after the extrusion of a particle through it.

Our value of 63 nm. (Table 1) for the diameter of bluetongue virus agrees with the 60 nm. obtained by Owen & Munz (1966) and 60 to 70 nm. which was the size group most frequently observed by Bowne & Ritchie (1970). However, our value is in disagreement with the 53.8 nm. obtained by Els & Verwoerd (1969) and 53 nm. given by Studdert et al. (1966). It may be worthwhile to point out that the methods which were used to measure the virus
and to calibrate the electron microscope were identical to those used by Els & Verwoerd (1969). This discrepancy in size is not surprising since there also is disagreement among investigators over the mean diameter of negatively stained reovirus type 2 particles (Table 1). Table 1 also illustrates the similarity in size values obtained by Anderson & Doane (1966) for negatively stained preparations and ultrathin sections of reovirus type 2. Similarly, the 59.5 nm diameter given by Vasquez & Tournier (1962) for negatively stained reovirus type 3 is very close to the 60 nm obtained by Harford et al. (1962) for the virus in ultrathin section. In negatively stained preparations of bluetongue virus, no double-layered capsid typical of mammalian reoviruses was observed either by Els & Verwoerd (1969) or by us.

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