

## OBSERVATIONS ON A STRAIN OF BLUETONGUE VIRUS BY ELECTRON MICROSCOPY

N. C. OWEN<sup>(1)</sup> AND E. K. MUNZ <sup>(2)</sup>, Veterinary Research Institute,  
Onderstepoort

For many years bluetongue has been known as an important and often fatal virus disease of sheep in South Africa, (Henning, 1956). As a result of extensions of the disease to countries outside Africa considerable interest in this disease has recently developed. The biological characteristics of the virus and its transmission by the biting midges have been studied extensively, but very little information on its bio-physical properties are available (Du Toit, 1944; Howell, 1963; Foster, Jones & McCrory 1963).

The particle size of infective bluetongue virus has been determined by ultrafiltration and ultracentrifugation and was found to have an average diameter of 100 to 150 m $\mu$  (Polson, 1948). Although no electron micrographs have as yet been published Studdert (1965) refers to unpublished data in which he mentions the absence of a distinct envelope in the structure of the particles.

This article describes a technique of partial purification of bluetongue virus and the electron microscopic structure of particles of a South African strain of the virus.

### MATERIALS AND METHODS

#### *Virus strain*

A strain of bluetongue virus isolated from a recent outbreak of the disease in the Kroonstad area of the Orange Free State was employed in this study. The virus was passed once through a bluetongue susceptible Merino sheep and then isolated in embryonated eggs and adapted to growth on primary lamb kidney cell cultures as described by Haig, McKercher & Alexander (1956).

#### *Cell cultures*

Primary lamb kidney cell cultures were prepared by overnight trypsinization according to the method of Bodian (1956). The growth medium consisted of Hanks B.S.S. with 0.5 per cent lactalbumin, 0.01 per cent yeastolate and 10 per cent bovine serum. During virus propagation, serum was omitted from the medium.

Bulk antigen (800 ml) was prepared by seeding monolayers in Roux flasks with the 4th passage level of tissue culture adapted virus at a final concentration of approximately  $10^8$  T.C.I.D.<sub>50</sub> per ml. The culture fluid with suspended cells was harvested on the third day just prior to complete cell destruction.

<sup>(1)</sup> Present address: Faculty of Agriculture, University of Natal, Pietermaritzburg (South Africa)

<sup>(2)</sup> Guest worker from Bayerische Landesimpfanstalt, München (Germany) under a grant of the Deutsche Forschungsgemeinschaft

### *Purification*

The cellular material was sedimented by centrifugation at 800g for 10 minutes in a Mistral 4L refrigerated M.S.E. centrifuge operating in the cold at 4° C. After careful aspiration of the supernatant fluid, the pelleted cells were dispersed in 10 ml sterile distilled water and homogenized for two minutes in an "Ultra-Turrax" homogenizer to release possible cell-bound virus. The supernatant fluid ( $S_1$ ) and homogenized cells were then pooled and treated with 0.01 per cent trypsin at 37° C for 30 minutes. The remaining cell debris was removed by a further centrifugation at 800g for ten minutes in the cold. The supernatant fluid ( $S_2$ ) was then spun at 40,000g for 60 minutes in the 21-Rotor of the model L Spinco ultracentrifuge to pellet the virus. The pellets so obtained were resuspended in 20 ml of phosphate buffer (0.02M), pH 7.0, again homogenized and finally clarified at 800g for five minutes.

A caesium chloride density gradient was prepared in each of twelve 40-Rotor centrifuge tubes according to the method described by Fischer, Cline & Anderson (1964) for the angle rotor. The virus suspension was, however, not floated on top, but introduced into the centre of each gradient by incorporation into the 30 per cent zone. By so doing, the centrifugation time could be considerably reduced (Polson & Levitt, 1963). The gradients were spun at 60,000g for 150 minutes at 4° C and the rotor was allowed to stop without applying the brake.

A narrow opaque zone, 0.5 cm in width and 2.5 cm from the bottom of each tube was encountered. This band was carefully aspirated from each of the twelve tubes by means of a specially mounted syringe and the fractions were then pooled. In order to remove the caesium chloride, the material was subjected to three cycles of differential centrifugation at 60,000g for 60 minutes and 800g for five minutes respectively. Between each high speed centrifugation, the pellets were resuspended in 0.002 M phosphate buffer, pH 7.0, while the final pellet was concentrated in 0.5 ml of this diluent.

### *Negative staining*

The virus suspension was mixed with a small drop of 0.5 per cent bovine plasma albumin on a glass slide. The resulting mixture was then placed on a carbon-coated copper grid and stained with a 5 per cent phosphotungstic acid (PTA) solution (pH 7.0) as described by Munz & Owen (1966).

### *Electron microscopy*

Electron micrographs were taken in a Philips-E.M. 200 microscope operating at 100 KV and magnification of 15,000 to 70,000 diameters. The negatives were further enlarged photographically. Size calculations were made by comparison with diffraction grating replicas photographed at the same magnification.

## RESULTS

Several roughly spherical particles were observed either with or without a distinct envelope. The majority, however, were without envelopes and appeared to be what is referred to as "naked" virus. The virus particles further consist of hollow, angular morphological units radiating out from a central spherical structure. The particles shown in Fig. 1 (a) and 1 (b) appear to have been penetrated by phosphotungstic acid and represent the so-called "empty" capsids of which only a few were encountered. The particle in Fig. 1 (a) is distinctly hexagonal in outline probably representing an icosahedron viewed along an axis of threefold symmetry.

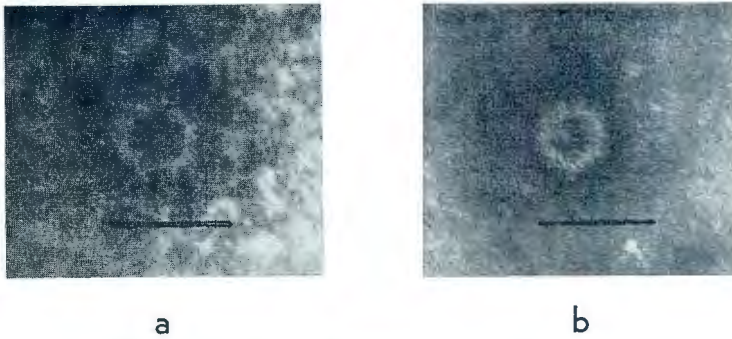


FIG. 1.—Bluetongue virus strained with phosphotungstic acid and showing the “empty” capsids. Note hexagonal shape of (a)  
Magnification: 150,000 $\times$ . Bar = 1000 $\text{\AA}$

In Fig. 2 (a) and 2 (b) two particles are presented which show the morphological units (capsomeres) as having a tubular nature, the centres of which are filled with phosphotungstic acid. Fig. 2 (a) is characteristic of the “naked” particles while in Fig. 2 (b) there is a distinct envelope present.

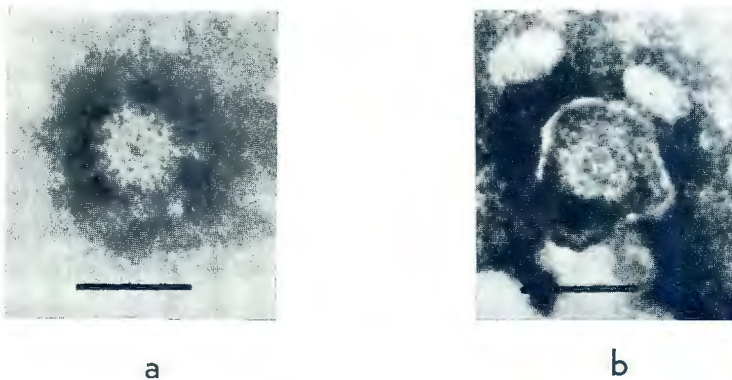


FIG. 2.—Bluetongue virus strained with phosphotungstic acid. (a) “Naked” virus. (b) Enveloped virus. Magnification: 150,000 $\times$ . Bar = 1000 $\text{\AA}$

The particles in Fig. 3 (a) and 3 (b) have been photographed at the higher instrument magnification and show the orientation of the morphological units. The capsomeres in Fig. 3 (a) can be seen as elongated hollow structures radiating from the central body, this being particularly clear along the periphery of the particle. Fig. 3 (b) has been included as it shows some evidence of an icosahedral structure. A possible facet has been demarcated by means of arrows at its points of fivefold symmetry. The edges of the facet each consists of four hollow capsomeres.

OBSERVATIONS ON A STRAIN OF BLUETONGUE VIRUS BY ELECTRON MICROSCOPY

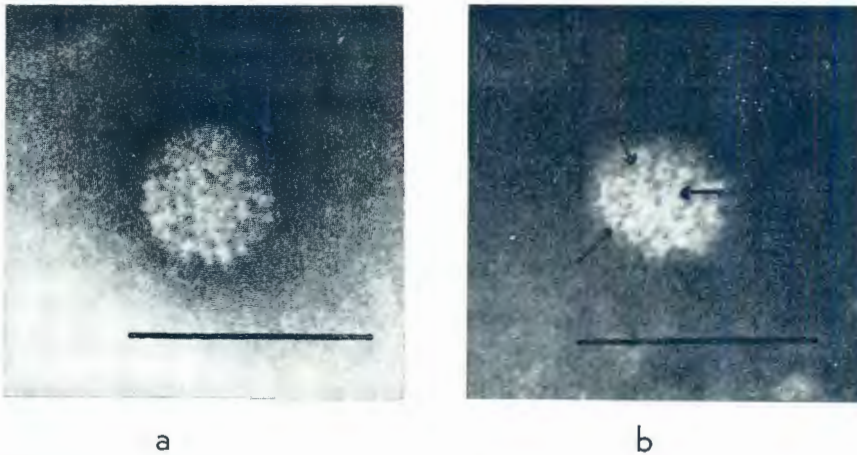


FIG. 3.—Bluetongue virus strained with phosphotungstic acid. (a) Elongated capsomeres. (b) Arrows indicate possible facet. Magnification: 350,000 $\times$ . Bar = 1000 $\text{\AA}$

The particle shown in Fig. 4 has been considerably enlarged photographically and may represent an icosahedron viewed on the face of fivefold symmetry. Again four distinct capsomeres can be observed at the base of at least two of the triangular facets.



FIG. 4.—Bluetongue virus strained with phosphotungstic acid; seen along axis of fivefold symmetry. Magnification: 450,000 $\times$

The diameter of the “naked” virus encountered was approximately 60  $m\mu$ , while those with an envelope had an overall diameter of approximately 100  $m\mu$ .

DISCUSSION

It has been possible in the course of this investigation to demonstrate some of the morphological features of a virulent strain of bluetongue virus. Under the conditions of these experiments both “naked” and enveloped virus particles were encountered.

The "naked" virus appears to have features closely resembling those of African horsesickness as reported by Polson & Deeks (1963). Some evidence has been presented to suggest that bluetongue virus is an icosahedron. By applying the formula  $10x(n-1)^2 + 2$  (Horne & Wildy, 1961), assuming  $x = 1$ , and in this case  $n = 4$ , the number of capsomeres is calculated to be 92. This figure is the same as that suggested for horsesickness virus, based on the method of counting all the visible capsomeres and multiplying by two.

By using the infectivity as a criterion in particle size determination, Polson (1948) calculated the diameter of bluetongue virus to be 100–150  $m\mu$ . If the envelope observed in these investigations is essential for infectivity the observed overall diameter of the enveloped particles (100  $m\mu$ ) is in close agreement with the earlier finding. Unfortunately, it was not possible to examine fractions from the entire gradient to establish whether the "naked" virus had been fractionated from the enveloped virus as a result of different densities. The significance of the envelope with regard to infectivity will have to form the basis of further investigations.

The phenomenon of "full" and "empty" capsids has been observed amongst others with laryngotracheitis virus (Watrach, Hanson & Watrach, 1963), herpes virus (Watson, Russel & Wildy, 1963) and polyoma virus (Crawford, Crawford & Watson, 1962). It has been suggested that the "empty" capsids result from a penetration of the phosphotungstate. However, in view of the findings of workers with the pox and herpes viruses (Müller & Peters, 1963; Nagington, Newton & Horne, 1964; Munz & Owen 1966; Herzberg, Lang, Reuss, Dahn & Plescher, 1964), it appears that the ratio of "full" to "empty" capsids is determined by the pH of the staining material.

#### SUMMARY

Tissue culture propagated bluetongue virus was partially purified and examined in an electron microscope with the negative staining technique. Virus particles either with or without a distinct envelope were observed. In some cases the capsids appeared to be "empty". The capsid was found to have an average diameter of 60  $m\mu$ , while the overall diameter of the enveloped particle was approximately 100  $m\mu$ .

Some evidence of an icosahedral shape was presented and the number of capsomeres was estimated at 92. The capsomeres appear to have a hollow, tubular nature. The findings have been discussed in relation to some other viruses.

#### ACKNOWLEDGEMENTS

The authors wish to thank the Council for Scientific and Industrial Research, (Pretoria) for the use of the electron microscope as well as Mr. J. Murphy for his help in this respect.

#### REFERENCES

- BODIAN, D., 1956. Simplified method of dispersion of monkey kidney cells with trypsin. *Virology*, Vol. 2, 575–577.
- CRAWFORD, L. V., CRAWFORD, E. M. & WATSON, D. H., 1962. The physical characteristics of polyoma virus. I. Two types of particles. *Virology*, Vol. 18, 170–176.
- DU TOIT, R. M., 1944. The transmission of bluetongue and horsesickness by *Culicoides*. *Onderstepoort J. Vet. Sci. Anim. Ind.* Vol. 19, 7–16.
- FISCHER, W. D., CLINE, G. B. & ANDERSON, N. G., 1964. Density gradient centrifugation in the angle head rotors. *Analytical Biochemistry*, Vol. 9, 477–482.

## OBSERVATIONS ON A STRAIN OF BLUETONGUE VIRUS BY ELECTRON MICROSCOPY

- FOSTER, N. M., JONES, R. H. & McCRORY, B. R., 1963. Preliminary investigations on the insect transmission of bluetongue in sheep. *Amer. J. Vet. Res.*, Vol. 24, 1195-1200.
- HAIG, D. A., MCKERCHER, D. G. & ALEXANDER, R. A., 1956. The cytopathogenic action of bluetongue virus in tissue culture and its application to the detection of antibodies in the serum of sheep. *Onderstepoort J. Vet. Res.*, Vol. 27, 171-177.
- HENNING, M. W., 1956. Animal Diseases in South Africa. S. Africa; C.N.A.
- HERZBERG, K., LANG, D., REUSS, K., DAHN, R. & PLESCHER, C., 1964. Über das Verhalten der Filamente des Kanarienvirus und der Capsomeren des Varizellen-Zoster-virus gegenüber Lösemitteln und Fermenten. *Zbl. f. Bakt. I. Orig.* Vol. 195, 133-143.
- HORNE, R. W. & WILDY, P., 1961. Symmetry in virus architecture. *Virology* Vol. 15, 348-373.
- HOWELL, P. G., 1963. Emerging diseases of animals. FAO Agricultural Studies, No. 61, 1963.
- LWOFF, A., HORNE, R. & TOURNIER, P., 1962. A system of viruses. *Cold Spring Harbor Symp. Quant. Biol.*, Vol. 27, 51-55.
- MÜLLER, G. & PETERS, D., 1963. Substrukturen des Vaccinevirus, dargestellt durch Negativkontrastierung. *Arch. Ges. Virusforsch.* Vol. 13, 435-451.
- MUNZ, E. K. & OWEN, N. C., 1966. Electron microscope studies on lumpy skin disease virus type "Neethling". *Onderstepoort J. Vet. Res.* Vol. 33,
- NAGINGTON, J., NEWTON, A. A. & HORNE, R. W., 1964. The structure of orf virus. *Virology*, Vol. 23, 461-472.
- POLSON, A., 1948. The particle size of the bluetongue virus as determined by ultra-filtration and ultracentrifugation. *Onderstepoort J. Vet. Sci. Anim. Ind.* Vol. 23, 137-148.
- POLSON, A. & DEEKS, D., 1963. Electron microscopy of neurotropic African horsesickness virus. *J. Hyg. Camb.*, Vol. 61, 149-153.
- POLSON, A. & LEVITT, J., 1963. Density determination in a preformed gradient of caesium chloride. *Biochim. Biophys. Acta*, Vol. 75, 88-95.
- STUDDERT, M. J., 1965. Sensitivity of bluetongue virus to ether and sodium deoxycholate. *Proc. Soc. Exptl. Biol. Med.* Vol. 118, 1006-1009.
- WATRACH, A. M., HANSON, L. E. & WATRACH, M. A., 1963. The structure of infectious laryngotracheitis virus. *Virology*, Vol. 21, 601-608.
- WATSON, D. H., RUSSELL, W. C. & WILDY, P., 1963. Electron microscopic particle counts on herpes virus using the phosphotungstate negative staining technique. *Virology*, Vol. 19, 250-260.