MECHANISMS CONTROLLING THE INFECTION OF *CULICOIDES* BITING MIDGES WITH BLUETONGUE VIRUS

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ABSTRACT

The mechanisms controlling the transmission of bluetongue virus (BTV) by vector *Culicoides* species were studied using immunohistochemistry, virus titration assays, *in vitro* transmission tests, viral binding protein analyses and transmission electron microscopy.

After infection with BTV by intrathoracic (IT) inoculation, 100% of *C. variipennis* individuals from a susceptible colony developed a fully disseminated infection and transmitted the virus through their saliva. However only 35.4% of midges were persistently infected after ingestion of an infectious blood meal, while only 12.1% of persistently infected midges transmitted the virus through their saliva. The titres of BTV were about $10^{5.0}TCID_{50}$/midge [Standard error of means (SEM) of log-transformed data=0.15, n=1400] in IT inoculated midges and varied from 0.32 to $10^{5.0}TCID_{50}$/midge in orally infected individuals. Only those midges containing $\geq 10^{3.0}TCID_{50}$ of BTV could transmit the virus through their saliva. The following patterns were observed in orally (persistently) infected individuals: 1) virus was restricted to the anterior and posterior midgut, and the foregut-midgut junction; 2) virus replicated in the gut cells, disseminated into the haemocoel but could only be detected in a few sporadic fat body cells beyond the gut; 3) virus escaped from the gut cells into the haemocoel and replicated in some secondary organs/tissues but at low levels; 4) a fully disseminated infection was observed and virus replicated in the haemocoel and secondary organs/tissues, including the salivary glands, at high levels. The infection of the gut can be divided into two main types: 1) virus replication in gut cells ranging from very low to higher levels but with virus spread throughout the cytoplasm of the infected cells; 2) virus positive reaction restricted to endosome-like structures in the cytoplasm of some gut cells.

BTV was detected in the anterior and posterior midgut, foregut-midgut junction, fat body, ganglia, salivary glands and ommatidia of the compound eyes of some infected midges. No virus was ever found in the hindgut cells, muscles, Malpighian tubes and oocytes/nurse cells of the ovaries.

BTV infection of the salivary glands of *C. variipennis* was shown to follow a typical pattern. Virus entered the acinar cells from the haemocoel passing through the basement membrane, then localised and replicated in virus inclusion bodies (VIBs) in the cytoplasm of acinar cells. Mature progeny virus particles were released into acini, then transported through intermediate ducts and accumulated in crystalline arrays in the lumen of the major secretory ducts. No virus was released back into the haemocoel through the basement membrane; nor was virus released back into acinar cells from the acini.

Nervous tissue of *C. variipennis* is one of the most susceptible tissues to BTV. Ultrastructural observation showed characteristics of BTV replication, including formation of VIBs, large amounts of progeny virus particles and tubules, in infected thoracic ganglia.

A 60-kD viral protein adhered to both BHK-21 (mammalian) cells and a *Culicoides* cell line, KC cells. A 44-kD BTV viral protein, co-migrating with nonstructural protein NS2, adsorbed to BHK-21 cells but not to KC cells, while a 39.6 kD viral protein, co-migrating with major inner capsid protein VP7, adhered only to KC cells but not to BHK-21 cells.

CONTENTS

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CHAPTER 1
LITERATURE REVIEW

1.1 INTRODUCTION

Bluetongue (BT) is a non-contagious, infectious arthropod-borne viral disease of ruminants caused by bluetongue virus (BTV). All species of ruminant are susceptible to infection but severe disease is usually seen only in certain breeds of domestic sheep (Hutcheon 1902; Thomas and Neitz 1947; Bowne 1971; Erasmus 1975; Hourrigan and Klingsporn 1975; Gorman 1990). The virus has a wide geographical distribution between latitudes 40°N and 35°S and has been found in the Americas, Africa, Asia and Australia (Mellor 1990). Presence of the virus within a country can lead to serious losses both directly in terms of disease and indirectly due to disruption in international trade in bovine and ovine products.

1.2. BLUETONGUE VIRUS

1.2.1 Structure and Classification of BTV

Bluetongue virus is the prototype of the *Orbivirus* genus in the family Reoviridae (Holmes *et al* 1995). The virus is non-enveloped, and has an icosahedral structure which approximates 68 nm in diameter by negative contrast electron microscopy, and 86 nm by cryo-electron microscopy (Hewat *et al* 1992). The intact virus particle is composed of a double-layered protein coat surrounding a genome of ten segments of double-stranded RNA, each of which encodes at least one viral protein (Verwoerd 1969; Verwoerd *et al* 1972). The inner capsid layer, or core, contains 5 distinct proteins: two major proteins VP3 and VP7, and three distinct minor proteins VP1, VP4, and VP6/VP6a. The outer capsid layer is composed of two major proteins, VP2 and VP5, and in some cases has been reported to be associated with a third minor component, NS2 (Mertens *et al* 1987). Sixty copies of VP3 form the subcore upon which the VP7 capsomers are located (Huismans *et al* 1987a; Hyatt and Eaton 1988). There are 780 molecules of VP7 which form 260 triangular spikes protruding 5 nm from the base of the inner shell, and comprising the characteristic ring-shaped capsomers seen on the surface of the BTV core

particle (Huismans *et al* 1987a; Hyatt and Eaton 1988; Loudon and Roy 1991; Prasad *et al* 1992; Hewat *et al* 1992). There is also some evidence that VP7 is not completely shielded by the outer capsid and recent three-dimensional analysis of the BTV virion, using cryoelectron microscopy and image processing techniques, has revealed that a portion of the VP7 molecules is exposed at the surface of virions (Hewat *et al* 1992). Twenty VP7 spikes (which contain group-specific antigens) are accessible on the surface of the virus and can be recognized by VP7 specific antibodies (Hyatt and Eaton 1988).

Three non-structural proteins (NS1, NS2, NS3/NS3a) have also been identified in BTV infected cells (Mertens *et al* 1987; Verwoerd *et al* 1972; Van Dijk and Huismans 1988; Huismans and Els 1979). NS1 forms the tubules associated with BTV replication in infected cells (Huismans and Els 1979). NS2 has been reported to be associated with the outer capsid layer of the BTV particles (Mertens *et al* 1987). It also binds single- stranded RNA and is a major constituent of the virus inclusion bodies (VIB) seen in infected cells (Hyatt *et al* 1993). NS3/NS3a have been found to be released from infected cells with fragments of plasma membrane and are thought to be involved in the release of BTV particles from infected cells prior to cell lysis (Hyatt *et al* 1991a,b, 1993).

To date, 25 distinct serotypes of BTV have been identified, on the basis of neutralisation assays with type-specific antiserum, although a variety of other tests have also been used to differentiate BTVs (Howell 1970; Howell and Verwoerd 1971; Verwoerd *et al* 1979; Gorman *et al* 1983; Davies *et al* 1992). Individual animals infected with a single serotype of BTV produce a solid immunity against reinfection with that serotype but not other serotypes (Neitz 1948; Howell 1960, 1969; Jeggo and Wardley 1985). The geographical distribution of individual BTV serotypes is reviewed in Section 1.3.

Being a virus with a segmented genome, BTV is capable of genetic reassortment. Previous studies have shown that genome reassortment occurs when BTV of two different strains or different serotypes are injected into a natural host animal (Samal *et al* 1987a, b; Stott *et al* 1987). No such result has yet been reported in relation to BTV-vector interaction.

1.2.2 Replication of BTV

The replication of BTV has been studied mainly in mammalian cell lines (BHK21, Vero, MDBK) although some work has also been carried out in insect cells (Mertens *et al* 1984, 1987; Howell *et al* 1967; McPhee *et al* 1982; Hyatt *et al* 1989, 1993). In mammalian cells, BTV replication is typically short term and cytocidal, while in insect cells it is long-term and persistent, generally without cytopathic effect.

The major events in BTV replication are: adsorption and penetration; uncoating; formation of replicative complexes, viral inclusion bodies (VIB); formation of tubules; and movement of the virus to, and release from, the cell surface (Eaton *et al* 1990; Gould and Hyatt 1994).

Firstly, virus particles attach to specific receptor sites on the cell surface and are then taken up by endocytosis and penetrate the cell membrane (Dales 1973; Hyatt *et al* 1989). Efficient neutralisation by antibodies to outer capsid proteins can occur at the cell surface, preventing receptor mediated, endocytosis and subsequent internalisation of BTV (Brookes *et al* 1993). In general, with BTV and other reovirus infections, it has been shown that the endosomes fuse with lysosomes where the outer coat of the virus is partially disrupted (modified) by lysosomal proteases (Sturzenbecker *et al* 1987; Eaton and Hyatt 1989). Subviral particles or cores are released into the cytoplasm by crossing the lysosomal membrane (Tyler and Fields 1985), a process involving complete removal of the outer coat before the synthesis of viral RNAs and proteins (Watanabe *et al* 1968). However, contrary to these findings, Eaton *et al* (1990) reported that the majority of BTV particles are released from endosomes into the cell cytoplasm prior to fusion with lysosomes. This may indicate that the mechanism of cell entry by BTV is independent of lysosomal enzymes.

Subsequent to these events, removal of the outer protein layer activates a fully conservative viral-associated RNA polymerase, and RNA capping enzymes which transcribe the ten segments of dsRNA into 10 mRNAs. These mRNAs are, in turn, translated into at least seven structural and three non-structural viral proteins (Mertens *et*

al 1984; Huismans *et al* 1987b). VIBs develop in the early stages of the infection and are believed to be the major sites of viral RNA and protein synthesis, and subsequently of virus particle assembly (virus factories) (Eaton and Hyatt 1989; Thomas *et al* 1990; Hyatt *et al* 1991b; Roy 1992; Brookes et al 1993).

Like other double-stranded RNA viruses, transcription (defined as the synthesis of viral (+) strand RNA) takes place within the parental core particles. The new (+) strands are extruded from the core particles and are translated to make viral proteins. The same (+) strands are also packaged with the virus structural proteins to make nascent sub-viral, or replicase particles. These particles mature via a process that involves synthesis of complementary negative strands on the ten positive strand RNA templates, thus reforming the dsRNA segments of the progeny virus genome. Once the new progeny or core particles have been formed, they are also able to synthesise positive strand RNA copies of the genome. Addition of a layer of new outer coat proteins completes the virus replication cycle (Wickner 1993) and the virus is then destined for export. Like other reoviruses, a majority of the newly synthesized BTV particles remain cell associated.

Subsequent to assembly, the progeny virus particles that are produced early in infection, are either released by budding, which involves the temporary acquisition of a lipid membrane, or by an unknown mechanism of extrusion, without acquiring a membrane. These two kinds of release are believed to occur in insect cells and also at the early stage of infection in mammalian cells (Eaton *et al* 1990; Hyatt *et al* 1989, 1991a, 1993). During late stages of infection, virus is released from mammalian cells as a result of cell lysis. However, BTV infection in insect cells is persistent without cell lysis. The release of BTV particles from infected cells has been shown to involve trans-membrane transport mediated by NS3/NS3a, a process which may occur during BTV exit from both mammalian and insect cells (Hyatt *et al* 1991a, 1993). A much higher level of NS3/NS3a synthesis has been observed in insect cells than in mammalian cells (M. Jennings and PPC Mertens, personal communication).

Upon release, BTV can reinfect the same cell, a process defined as superinfection (Hyatt *et al* 1989). The subsequent release of more transcriptase-active cores into the cytoplasm

and the generation of more VIBs would then effectively increase the multiplicity of infection and may accelerate the whole replication process.

NS1 is a major nonstructural protein synthesized in BTV infected cells and polymerizes to form tubules. These tubules, 68 nm in diameter, are a characteristic structure in the cells infected with BTV and other orbiviruses (Huismans 1979; Huismans and Els 1979; Urakawa and Roy 1988). The function of these tubules is so far unknown and virus particles have not to date been reported to be associated with them (Gould and Hyatt 1994). However, it has been suggested that NS1 is associated with virus particles and VIBs (Eaton *et al* 1988).

1.3. THE EMERGENT DISEASE

BT in Africa was first reported by Hutcheon (1902) and Spreull (1905). The virus was initially thought to be confined to Africa until 1943 when an outbreak of BT was recognised in Cyprus. Subsequently, Gambles (1949) reported that there had been a series of BT epizootics outside Africa; in Cyprus in 1924, 1943 and 1977, in Palestine in 1943 and in Turkey in 1944, 1946 and 1947 respectively (Mellor 1990). More recently, BT has also been reported in Israel (Komarov and Goldsmit 1951) and in Western Turkey in 1977-1979 (Yonguc *et al* 1982; Yonguc 1987).

The confirmation of BT in the USA was associated with an extensive epizootic in 1948. Since then BT has been recognized in sheep in several parts of the United States, including Texas, California, New Mexico, Wyoming, Idaho and Florida, and 5 BTV serotypes (serotype 2, 10, 11, 13 and 17) have been isolated from sheep, cattle or *Culicoides* (Hardy and Price 1952; McKercher *et al* 1953; Gibbs *et al* 1983a, 1983b; Gibbs and Greiner1983; Gorman 1990). BTV was isolated for the first time in the USA from sheep in California in 1952. Recent genetic analyses indicate that several BTV serotypes could have had a long evolutionary history in North America (Heidner *et al* 1991). In the United States, the disease is enzootic in the southwestern region, occurs in sporadic outbreaks in the central and southeastern areas, and is rare or absent in the north-central and northeastern states (Metcalf *et al* 1981, Johnson 1992).

In 1956/57, a major epizootic of BT in sheep began in Portugal and extended into Spain causing 179,000 deaths with a mortality rate up to 75% within the first 4 months (Manso-Ribeiro *et al* 1957). The last case was recorded in 1960 (Anon 1960). No clinical cases or serological evidence of virus activity have been reported from either Spain or Portugal since then. The virus circulation in the environment was apparently successfully blocked through a coordinated campaign of quarantine, slaughter, and compulsory annual vaccination. This is one of the few recorded instances of BTV circulation being stopped in a geographical area. The virus is not currently considered to be enzootic in the European continent although BT epizootics have also occurred in Greece in 1979 [Lesbos] and 1980 [Rhodes](Vassalos 1980; Mellor *et al* 1983; Gibbs and Greiner 1994; Mellor 1995).

In 1958, BT was reported in Western Pakistan in a flock of Rambouillet sheep previously vaccinated and imported from Utah, USA (Howell 1969). An outbreak of BT in goats and sheep also occurred in Pakistan in 1960 and in Maharashtra State of India in 1961 (Sapre 1964). Subsequently BT was not observed in this area in India for almost 20 years. However, in 1981, epizootics occurred in sheep throughout the region and also involved other parts of India. The morbidity rate varied from 3.7% to 43.2% during the years of 1981-82, 1983-84, 1985-86 and 1988-89. During interval years no cases of the disease were reported (Kulkarni *et al* 1991). BT outbreaks are now reported annually from the states of Andhra Pradesh, Karnataka, Maharashtra and Tamil Nadu (P.S. Mellor, personal communication). BTV therefore seems to be enzootic in this region.

Before 1977, Australia was considered to be an area free of BTV. However, eight serotypes of the virus have subsequently been isolated, mainly from healthy cattle or from insects, in the Northern Territory of Australia. Of these serotypes, three have so far been recorded only in Australia (serotype 20, 21 and 23) and five have also been found in other countries (serotype 1, 3, 9, 15 and 16) (St George *et al* 1978, 1980; Gard *et al* 1987a, 1987b). Apart from one clinical case in a sentinel sheep flock on a research station, no clinical disease has been reported in the field. This is possibly because in Northern Australia cattle are the main domestic ruminant whereas sheep, the most susceptible animal to BT disease, are not farmed. No evidence of BTV has yet been found in

Southeast or Southwest Australia where the sheep industry is dominant. However, there is evidence that BTV 1 and BTV 21 are widely spread in Queensland, and small numbers of sheep and goats have been detected with natural infections with these serotypes in enzootic areas with high cattle density (Flanagan *et al* 1993).

A pattern similar to that seen in Australia also appears to be emerging from BTV studies in 11 Caribbean and Central American countries. Although there is no evidence of clinical BT in this region, at least 100 strains and 6 serotypes of BTV have been isolated from healthy animals (Gibbs *et al* 1983a; Homan *et al* 1990; Gorman 1990; Greiner *et al* 1993; Mo *et al* 1994).

Serological evidence for the presence of BTV and/or virus isolation has also been reported from Japan (Miura *et al* 1980), Papua New Guinea, Malaysia and Indonesia (Sellers 1981; Miura *et al* 1982; Sendow *et al* 1991; Hassan 1991). Eight BTV serotypes have been isolated from wide areas of Indonesia although clinical disease has only been reported once, in imported Suffolk sheep in Java (Sendow *et al* 1986, 1993). In China, the virus was first isolated in 1979 in Yunnan Province (Zhang *et al* 1989). Clinical cases have now been reported in 4 provinces and serological evidence has shown that the virus has a wide distribution in at least 29 provinces in the country (Zhang *et al* 1989; Hu *et al* 1989).

The geographical distribution of BTV in the world is shown in Table 1.1.

Table 1.1 Geographical distribution of BTV

Continent/region	Serotypes of BTV isolated
Africa	1-16, 18, 19, 24, 25
Asia	1-4, 7, 9, 10, 12, 16, 17, 20, 21, 23
Australia	1, 3, 9, 15, 16, 20, 21, 23
Europe	4, 10
North America	2, 10, 11, 13, 17
South America and Caribbean	1, 3, 4, 6, 8, 12, 17

1.4. TRANSMISSION OF BTV

BTV is an arbovirus (arthropod-borne virus) and transmission from infected to susceptible mammalian hosts by arthropod vectors is therefore a critical facet in the life cycle of this virus, particularly in terms of its distribution, seasonal incidence, rate of spread, susceptible hosts and also in regard to control.

1.4.1 The host animals

All species of ruminant appear to be susceptible to BTV infection but the consequences of the infection vary. Severe disease is usually seen only in certain breeds of domestic sheep and some species of deer (Gibbs *et al* 1983a, 1983b; Gibbs and Greiner 1983; Herniman *et al* 1983; Gorman 1990; MacLachlan *et al* 1992; Weiser-Schimpf *et al* 1993). In cattle the disease is usually asymptomatic although there may be an extended period (weeks) of viraemia lasting for as long as 100 days (Owen *et al* 1965; Luedke *et al* 1982, MacLachlan *et al* 1987; MacLachlan 1995). Serological studies have shown that in some areas a large proportion of the wild ruminant population seem to be natural hosts for BTV and the virus has been isolated from two distinct enzootic areas in the United States, Southwest USA and Florida (Davies 1980; Erasmus 1980; Thorne *et al* 1988).

BTV-associated disease has been observed in a free range population of topi [*Damaliscus korrigum* (Ogilby)] in Uganda and also a captive eland [*Tragelaphus strepsiceros* (Pallas 1766)] in the USA (Well 1962; Hoff *et al* 1973). Mortality and abortion affecting domestic dogs have been reported recently following the use of a BTV contaminated vaccine (Anita *et al* 1994). Recent studies have also shown evidence of BTV infection among African carnivores (Alexander *et al* 1994).

1.4.2 The vectors

BTV is transmitted by certain species of biting midge belonging to the genus *Culicoides* (Diptera: Ceratopogonidae). In nature it is maintained through a series of alternating cycles of replication between its *Culicoides* vectors and susceptible ruminant hosts (Du Toit

1944; Walker and Davies 1971; Braverman and Galun 1973; Braverman *et al* 1985; Mellor *et al* 1984a, 1984b; Mellor 1990, 1995). Out of 1,000 plus *Culicoides* species in the world (Boorman 1988), only 17 have been connected with BTV transmission (Mellor 1990), suggesting that *Culicoides* can be divided into two distinct groups, susceptible and unsusceptible species. Susceptibility is likely to be a complex situation under genetic control and even within a known vector species a variable proportion of individuals will not be susceptible (Foster and Jones 1979; Mellor 1990, 1995; Tabachnick 1991, 1992; Mecham and Nunamaker 1994).

To date, only six species of *Culicoides*, namely *C. variipennis* (Coquillett 1901), *C. imicola* (Kieffer 1913), *C. fulvus* (Sen and Das Gupta 1959), *C. actoni* (Smith 1929), *C. wadai* (Kitaoka 1980), *and C. nubeculosus* (Meigen 1830) have actually been proven to transmit the virus after oral infection although this number is likely to increase in the future (Greiner *et al* 1985; Standfast *et al* 1985; Mellor 1990).

1.4.3 Distribution of the vector species of *Culicoides*

So far it has been shown that BTV and vector species of *Culicoides* have a wide geographical distribution around the world, approximately between latitudes 40°N and 35°S, and 50°N and 30°S respectively (Mellor 1990; Sellers 1991). In many areas the species of *Culicoides* transmitting BTV have already been identified although in some areas the major vector species remain to be determined.

Culicoides imicola, first shown to transmit BTV from infected to susceptible sheep in South Africa (Du Toit 1944), is now considered the major vector throughout Africa and the Middle East. In these areas outbreaks of BT seem to be invariably related to the presence of this species of midge. Numerous isolations of BTV have been made in many countries in this region (Du Toit 1944; Navai 1971; Walker and Davies 1971; Braverman and Galun 1973; Jennings *et al* 1983; Mellor *et al* 1984a, 1984b; Braverman *et al* 1985; Wirth and Dyce 1985; Shimshony 1987; P.S. Mellor personal communication 1991). In addition, BTV has been isolated from *C. tororoensis* (Khamala and Kettle 1971) and *C. milnei* (Austen 1909) in Kenya (Walker and Davies 1971) and from *C. obsoletus* (Meigen

1818) in Cyprus (Mellor and Pitzolis 1979). However, further evidence to link any of these three species of *Culicoides* to BTV transmission has not been obtained and it seems likely that they are of only local or minor significance in the epidemiology of BTV in the region.

Although BT in sheep has been recorded in Pakistan (Sarwar 1962; Sapre 1964; Howell and Verwoerd 1971), India (Sapre 1964; Bhambani and Singh 1968; Uppal and Vasudevan 1980) and serological evidence of the virus has been observed in Japan, Papua New Guinea, Malaysia and Indonesia (Miura *et al* 1980, 1982; Sellers 1981), in respect of the vectors in these areas, BTV has only been recovered from a single pool of *Culicoides* of the *Avaritia* subgenus which contained *C. fulvus* and *C. orientalis* (Macfie 1932) in Indonesia (Sendow *et al* 1993). There is no further published work relating to the isolation of BTV from any species of *Culicoides* from other parts of these areas. However several species of *Culicoides* [*C. wadai, C. fulvus, C. brevitarsis* (Kieffer 1917), *C. oxystoma* (Kieffer 1910) and *C. actoni*] that are known or suspected vectors of BTV in Australia are distributed widely across Southeast Asia (Wirth and Dyce 1985; Kurogi *et al* 1987, 1989; Sukarsih *et al* 1993). *Culicoides imicola* also occurs across the region having been recorded in Iran (Navai 1971), India (Dyce and Wirth 1983) and Laos (Howarth 1985). BTV has not apparently been isolated from *Culicoides* in China although suspected vector species of *Culicoides* are widely distributed in that country (quoted by Zhang *et al* 1993).

In Australia, BTV was first isolated in 1977 from a pool of *Culicoides* collected in the Northern Territory during 1975. Of the eight serotypes of the virus which have now been identified in Australia (St George 1985; Gard 1987a, 1987b), two have so far been isolated from *Culicoides* species, including *C. fulvus* and *C. brevitarsis* (St George and Muller 1984; Standfast *et al* 1985). In the laboratory, *C. wadai, C. actoni C. peregrinus* (Kieffer 1910) and *C. oxystoma* support BTV replication after oral infection, and *C. fulvus* and *C. actoni* have transmitted the virus between sheep (Cybinski *et al* 1980; Standfast *et al* 1985). *Culicoides wadai, C. fulvus* and *C. actoni* exhibited higher experimental infection rates than *C. brevitarsis* when feeding on infected sheep. All of these four species are closely related to *C. imicola* although their relative importance in the transmission of BTV remains to be determined. Of seven species of *Culicoides* suggested

as potential vectors of BTV in Australia, only *C. brevitarsis* has a distribution that covers almost all of the northern cattle breeding areas and stretches as far south as the sheep breeding areas (Standfast *et al* 1985). The latest study in Australia suggests that both *C. brevitarsis* and *C. wadai* are capable of supporting the circulation of BTV 16 and BTV 23 in the field (Bellis *et al* 1994).

Culicoides variipennis is the major vector species of BTV throughout the USA and in the Okanagan Valley in Canada, with numerous isolates of virus from field collected midges (Sellers 1981; Jones *et al* 1981; Weiser-Schimpf *et al* 1993). However, *C. variipennis* does not occur in southern Florida, the Caribbean region, most parts of Central America and all of South America, areas where BTV has also been detected (Sellers 1981; Homan *et al* 1985; Walton *et al* 1984; Gibbs and Greiner 1983). In these areas, *C. insignis* (Lutz 1913), *C. pusillus* (Lutz 1913) and *C. filariferus* (Hoffman 1939)/*C. ocumarensis* (Ortiz 1950) are the most common species at those times of the year when BTV transmission occurs (Greiner *et al* 1992, 1993; Saenz *et al* 1994). *Culicoides insignis* has recently been incriminated as a BTV vector, through the isolation of BTV 2 from this species in Florida (Greiner *et al* 1984, 1985; Walton *et al* 1984) and in French Guyana (Lefevre 1988, quoted by Mellor 1995). It has also been shown to be capable of transmitting BTV in southern Florida (Tanya *et al* 1992). Other vector competence studies on North American midges showed that *C. debilipalpis* (Lutz 1913) and *C. venustus* (Hoffman 1925) would be inefficient BTV vectors in the field, because these species exhibited very low oral infection rates even under ideal experimental conditions (Jones *et al* 1983; Mellor *et al* 1985). Typically, BT outbreaks in USA occur in late summer and autumn, when vector populations of *C. variipennis* are high (Osburn *et al* 1981; Loomis *et al* 1985).

Before 1981 there was no information on the identity of BTV vectors in European countries, although BT outbreaks had occurred in Spain, Portugal and Greece (Manso-Ribeiro *et al* 1957; Mellor *et al* 1983; Vassalos 1980; Dragonas 1981). However in 1981, *C. imicola* was recorded in areas of Turkey adjacent to BTV affected Greek islands (Jennings *et al* 1983). Then in 1982 the species was discovered on the Greek island of Lesbos (Boorman and Wilkinson 1983), and in 1984 on Rhodes (Boorman 1986). At the other end of the Mediterranean *C. imicola* was recorded in Spain and Portugal during

1982 and 1984 respectively (Mellor *et al* 1983, 1985). It has been suggested by further research that *C. imicola* is the only major vector of BTV in the Mediterranean basin (Mellor 1987). However, a number of other potential vector species of *Culicoides* occur in this area. *Culicoides schultzei* (Enderlein 1908), a suspected vector in Australia (Standfast *et al* 1985), has been found in Greece (Mellor *et al* 1984b). *Culicoides obsoletus* (Meigen 1918), from which BTV has been isolated in Cyprus (Mellor and Pitzolis 1979), and *C. nubeculosus*, a laboratory vector of BTV (Jennings and Mellor 1988), both occur widely throughout the region. These three species may have been of only secondary importance in past outbreaks of BTV in the Mediterranean area. However, together they have a very wide distribution in Europe and should therefore be regarded as posing a potential threat during any future BTV outbreaks in this area (Mellor 1987).

1.4.4 Infection of *Culicoides* with BTV

As an arbovirus, BTV has to replicate both in its vertebrate and invertebrate hosts. Furthermore, in the invertebrate host (*Culicoides*), BTV must be capable of replication in a variety of tissues in order to facilitate transmission and maintain the virus. Successful infection of vector *Culicoides* with BTV, replication of the virus in the vector and subsequent transmission of the virus to a susceptible host animal are determined by the type or strain of virus, the characteristics of the vector and certain environmental factors (Jones and Foster 1974, 1978; Mullens 1992; Mecham and Nunamaker 1994; Mullens *et al* 1995).

It has been well established that in the wild, *Culicoides* are infected with BTV only when ingesting viraemic blood from an infected vertebrate host (Mellor 1990). There is no evidence of transovarial transmission of BTV through *Culicoides* (Jones and Foster 1971b; P.S. Mellor, personal communication). In the laboratory, however, artificial feeding techniques (Rutledge *et al* 1964; Mellor 1971; Braverman and Swanepoel 1981) and intrathoracic inoculation (Mellor *et al* 1974) have been used in studies on BTV infection of *Culicoides* and transmission of the virus.

1.4.4.1 The susceptibility of *Culicoides* to BTV

Vector species of arthropod rarely have a susceptibility rate (SR)* of 100% to an arbovirus and *Culicoides* are no exception. The SR of *Culicoides* to BTV can vary widely and is affected by various factors, including the serotype of the infecting virus, the species and population of *Culicoides*, and individual variability of the insects (Jones and Foster 1974, 1978; Mullens 1992; Tanya *et al* 1993; Mecham and Nunamaker 1994; Mullens *et al* 1995).

Studies on both laboratory and field-collected populations of *C. variipennis* have confirmed the importance of both viral and vector components on the oral susceptibility of the insect to BTV infection. Mecham and Nunamaker (1994) have shown statistically significant differences in the responses of two laborary colonies of *C. variipennis sonorensis* to two BTV serotypes. They demonstrated a higher infection rate (IR)** of the AA colony with BTV serotype 13 and a higher infection rate of the AK colony with BTV serotype 11. Within a vector species, such as *C. variipennis,* the population present during a BTV outbreak is most susceptible to the strains of the BTV serotype that are circulating, at the time, and is less susceptible to all other BTV serotypes (Jones and Foster 1978). Individual populations of *C. variipennis* in the USA have been shown to have SRs to different BTV serotypes varying from 0 to 69% (Jones and Foster 1978; Barber and Jones 1984; Tanya *et al* 1993). Even within a single field population of *C. variipennis*, SRs have been recorded as being highly variable (Mellor 1990). Jennings and Mellor (1987) also found that within an established laboratory colony of *C. variipennis*, the response to oral infection with a single serotype of BTV could vary widely between experiments and they recorded IRs ranging from 0 to 51.6%.

Of the environmental factors which may influence the infection rate of *Culicoides*, temperature is thought to be the most critical (Mullens 1992; Mullens *et al* 1995).

* SR: the proportion of a vector population capable of supporting infection, replication and transmission of an infectious agent after oral infection.

**IR: the proportion of a vector population actually infected with an infectious agent after oral infection

A recent study showed that the susceptibility of *C. variipennis sonorensis* to infection with BTV is under the control of a single genetic locus (Tabachnick 1991). Additional genetic loci may act as modifiers to regulate the major locus and the level of infection achieved with different virus serotypes (Jones and Foster 1974; Tabachnick 1991; Mecham and Nunamaker 1994). Similarly, studies on mosquitoes have also suggested that the susceptibility of *Aedes aegypti* (Linnaeus 1762) to yellow fever virus is likely to be governed by a single major gene and modifying minor genes, or a group of closely linked genes (Miller and Mitchell 1991).

1.4.4.2 Intrathoracic inoculation

In numerous studies, mosquitoes, and even those *Culicoides* not susceptible to arbovirus infection by ingestion of an infectious blood meal, can be infected by intrathoracic inoculation (IT) of the virus (Houk *et al* 1986; Mellor 1990; Kramer *et al* 1992). Direct inoculation of virus into the haemocoel of midges, by-passing the gut, the primary infection barrier in these refractory insects, results in rapid, initial virus replication, without the "lag-phase" seen after oral infection (Jones and Foster 1966; Foster and Jones 1979; Mellor 1990). Moreover, after inoculation, 100% of infected individuals develop a persistent infection which lasts for life. Virus transmission may occur after a period of 4 to 5 days compared with 10 to 14 days postinfection (pi) in orally infected midges (Jones and Foster 1966; Foster and Jones 1973).

When *C. variipennis*, the major Northern American vector of BTV, is inoculated intrathoracically with the virus, the virus replicates to a level of about 10^5 $TCID_{50}$ (50 percent tissue culture infection dose) per insect after a 4 day incubation period at 25^0C ($\pm 1^0C$). In orally infected *C. variipennis*, maximum virus titres do not develop until at least 7 days pi and transmission does not normally occur before 10 days pi at this temperature (Mellor 1990).

However the results of IT inoculation do not reflect the natural route of virus infection and subsequent to this method, virus may replicate in both susceptible and insusceptible individuals (Mellor *et al* 1974; Mellor and Boorman 1980). Nevertherless, IT inoculation

can be used as a good positive control when studying virus infection in arthropods and as a known positive sample in developing detection techniques for virus infection in insects.

1.4.4.3 Oral infection of BTV in susceptible Culicoides

Only female vector *Culicoides* transmit BTV from infected to susceptible animals (Fig. 1.1) since males are not haematophagous.

Culicoides variipennis is the only BTV vector for which detailed virogenic data exist although this is virtually all from titration studies of infected insects. Fully susceptible individuals of this species support virus replication in a manner similar to that represented schematically in Fig 1.2. Female *C. variipennis* are able to ingest approximately 10^{-4} ml blood. When membrane feeding upon viraemic blood containing 10^6 $TCID_{50}$ of virus per ml, each midge will take about 10^2 $TCID_{50}$ of virus. Over the next 24 hours the virus titre per individual decreases in what is known as the eclipse or partial eclipse phase. Replication in susceptible tissues then occurs and virus concentration rises to reach a plateau between days 7 and 9 post-infection, at a level of $10^{5\text{-}6}$ $TCID_{50}$ of virus per midge.

Fig. 1.1 A female *C. variipennis* after a blood meal.

This virus concentration is maintained for the rest of the insect's life. Transmission to a vertebrate host becomes possible at 10-14 days pi, subsequent to infection and replication of virus in the salivary glands (Bowne and Jones 1966; Chandler *et al* 1985; Foster *et al* 1963; Foster and Jones 1973; Luedke *et al* 1967). The initial decrease in virus titre is interpreted as being due to virus inactivation during digestion of the blood meal and while attachment, penetration and uncoating of BTV in the mid-gut cells is proceeding. The first increase in virus titre (day 3-4) probably corresponds to BTV growth in the infected gut cells and the second increase (day 10-14) could be due to further cycles of virus replication in the secondary target organs. The plateau region beyond 14 days pi probably represents a steady state of virus replication and inactivation (Foster and Jones 1979). Since arboviruses rarely cause the severe damage to insect cells that they cause to mammalian cells, virus replication would be expected to continue in insect cells for the duration of their life span, once all susceptible insect cells are infected. This also means that once infected midges become competent to transmit the virus, they will remain competent for the rest of their lives.

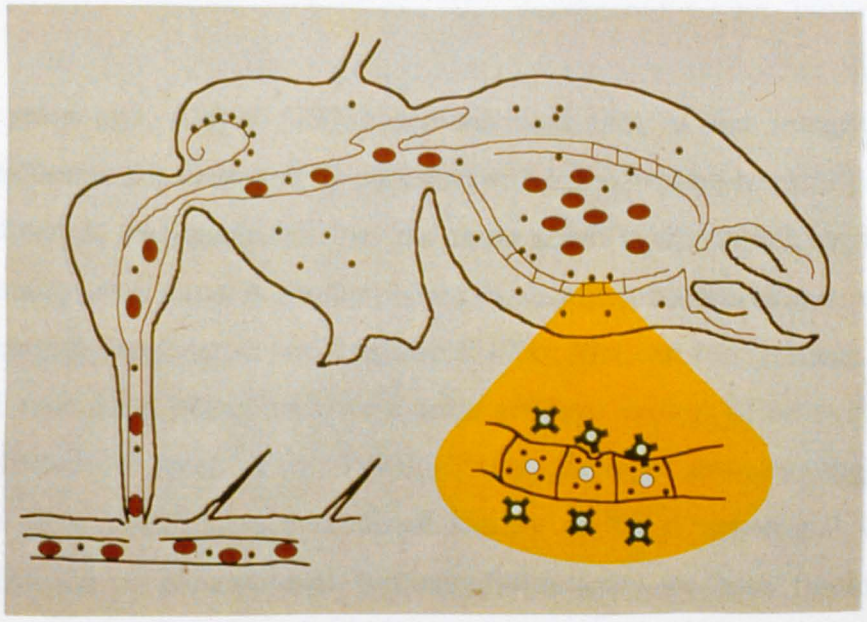

Fig. 1.2 Natural infection and dissemination of BTV in a vector *C. variipennis*.

● Red blood cells; • Virus particles

The amount of virus transmitted to susceptible animals during biting by an infected *C. variipennis* has not been accurately estimated but Mellor (personal communication) has recovered 3-20 $TCID_{50}$ of virus, after allowing individual BTV-infected *C. variipennis* to feed through a membrane on uninfected blood. Other authors have also reported that the bite of a single *C. variipennis* is sufficient to infect a susceptible sheep (Foster *et al* 1968).

The IR of *C. variipennis* by the oral route has been shown to be dependent on the concentration of virus in the blood meal and the susceptibility of the species (Jones and Foster 1971a). It has been reported that a single blood meal containing $3x10^6$ ELD_{50} (50 percent egg lethal dose) of virus per ml will produce an IR which is equal to the SR, i.e., the percentage of individuals in a population able to be orally infected (Jones and Foster 1971a).

1.5 TRANSMISSION BARRIERS TO THE INFECTION OF *CULICOIDES* WITH BTV

More than 60 years ago, Storey (1933) demonstrated that, if the integrity of the mesenteron of leafhoppers is disrupted by puncture with a needle, non-transmitting strains become transmitters. It was suggested that the mesenteron is the initial barrier to the transmission of maise-steak virus. A similar phenomenon with arboviruses and mosquitoes has also been demonstrated (Merrill and TenBroeck 1935; McLean 1955). It has also been observed that IT inoculated mosquitoes were more efficient vectors of arboviruses than those orally infected (Kramer *et al* 1993). These findings strongly suggest that susceptibility to virus infection is determined mainly at the mesenteronal level and indicates the presence of mesenteronal barriers. Subsequent to these finding, many hypotheses have been put forward to explain the basis of the gut-barrier to arbovirus infection in mosquitoes (Chamberlain and Sudia 1961; Chamberlain 1968; McLintock 1978; Murphy *et al* 1975; Tinsley 1975). The same situation probably also applies to the infection of *Culicoides* with BTV since 100% of a vector population of *Culicoides* have been shown to become persistently infected with BTV, subsequent to IT inoculation of the virus, while a much smaller proportion of the same population was persistently infected

after oral ingestion of BTV in a blood meal (Jones and Foster 1966, Foster and Jones 1973).

However, Chamberlain and Sudia (1961) found that *Anopheles quadrimaculatus* (Theobald 1911) cannot transmit eastern equine encephalomyelitis virus even though 79% of the females contain high concentrations of the virus in their bodies. Similar observations were made on the soft tick *Ornithodoros moubata* (Murray 1877) some populations of which can be persistently infected with certain strains of African swine fever virus (ASFV) although transmission occurs rarely (P.S. Mellor, personal communication). It has also been shown that only a proportion of persistently (orally) infected *C. variipennis* subsequently become competent to transmit BTV (P.S. Mellor, personal communication). It is therefore evident that though the mesenteron may play a key role as an infection barrier, it may not be the only site of interference with the normal infection and subsequent transmission of an arbovirus by an arthropod.

Although a considerable amount of work has been carried out on the transmission of arboviruses by arthropod vectors, the nature of the biochemical mechanisms underlying transmission are still poorly understood. Nevertheless it is becoming increasingly apparent that in non-vector insects, or even in some individuals within a vector species, a series of constraints exist which limit the ability of these insects to become infected with an arbovirus or to transmit the virus to susceptible animals, after ingestion of an infective blood meal. Therefore the vector competence of arthropods for arboviruses seems to be associated with and is controlled by complex and multiple barrier systems to virus dissemination within the insect's body. The hypothetical and conceptual barriers are summarized in Fig. 1.3, which shows the pathway of viral replication within a mosquito, from ingestion of a viraemic blood meal until the virus is transmitted orally or vertically.

1. Intact virus particles are ingested as a part of a blood meal from a viraemic host and are deposited in the mid-gut of the vector.

2. The virus attaches to the luminal surface of the mid-gut cells, penetrates the cell wall and replicates in the gut cells. Occasionally virus may bypass the gut cells and leak directly into the haemocoel.

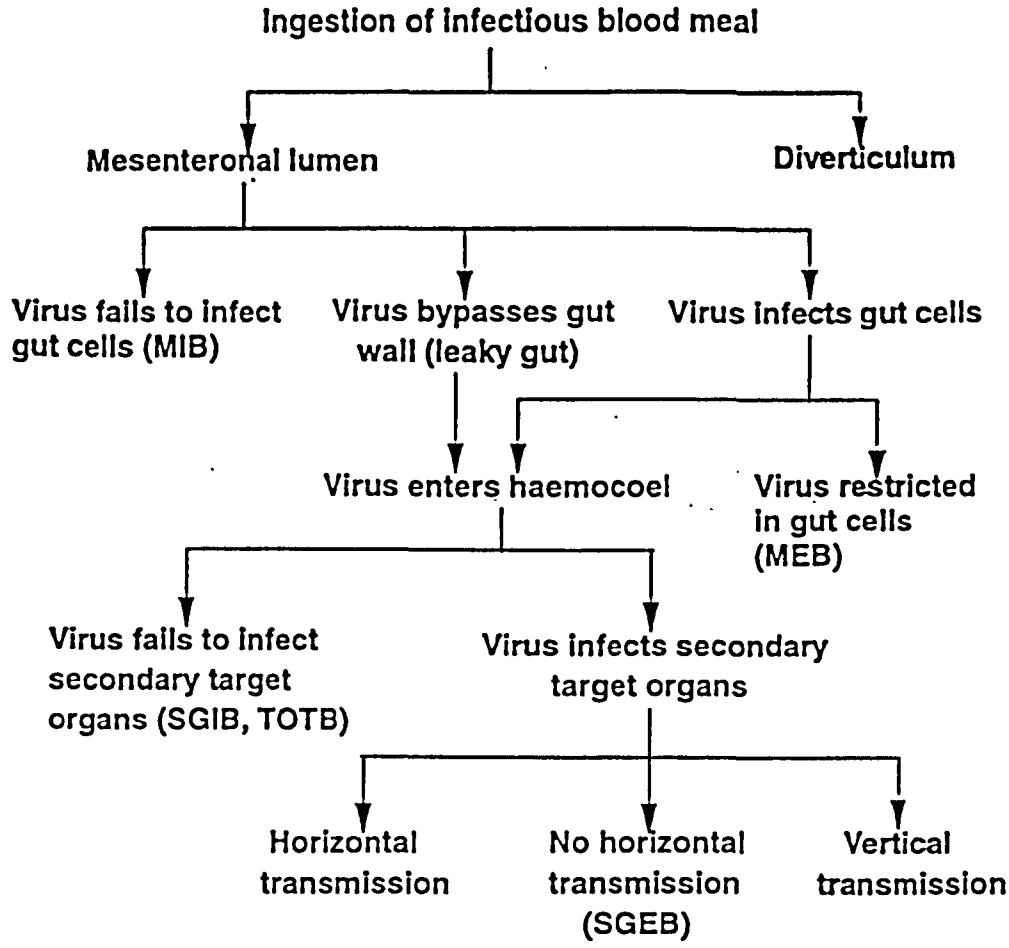

Fig. 1.3 Hypothesized and conceptual barriers to arbovirus infection of mosquitoes. MIB, mesenteron infection barrier; MEB, mesenteron escape barrier; SGIB, salivary gland infection barrier; SGEB, salivary gland escape barrier; TOTB, transovarial transmission barrier. (Adapted from Hardy *et al* 1983)

3. Progeny virus particles are released through the basement lamina of mid-gut wall into the haemocoel in which the secondary target tissues or organs, including salivary glands, are suspended. These are then infected.

4. Virus replicates in the salivary glands and release of virus from the glands may then result in virus transmission during biting activity on susceptible animals.

5. Virus may infect the ovary tissues and egg cells, which can lead to vertical transmission to progeny insects. The female insects infected by vertical transmission may then be able to transmit virus during biting on susceptible animals.

In a competent insect vector, BTV, like any other arbovirus, must follow this general cycle of events (P.S. Mellor, personal communication).

1.5.1 Mesenteronal infection barriers to BTV

A critical event in the transmission of arboviruses is the initial infection of the gut cells of the vector insects following ingestion of an infectious blood meal. For mosquito-borne viruses, infection is believed to begin in the abdominal or posterior midgut where most of the blood meal is deposited during engorgement (Chamberlain and Sudia 1961; McLintock 1978; Hardy *et al* 1983; Hardy 1988).

Like mosquitoes, the midgut of female *Culicoides* consists of a single layer of columnar cells and the posterior part of the midgut is the site both of nutrient absorption and the entrance of arboviruses. After ingestion, the virus is deposited in the midgut with the blood meal and the cells of the midgut are the first to be infected. It has been demonstrated that the midgut represents the primary barrier to infection and is of major importance in determining the susceptibility of *Culicoides* to BTV infection. This barrier is under genetic control (in mosquitoes and midges) and susceptible and refractory phenotypes can be selected (Miller and Mitchell 1991). The mesenteronal infection barrier (MIB) is associated with the initial interaction between the virus and the cells and the early events in the infection process (Chamberlain and Sudia 1961); a number of mechanisms controlling the MIB have been proposed .

1.5.1.1 Specific BTV receptor

The most persuasive suggestion postulates the presence or absence of specific BTV receptors on the luminal surface of the insect midgut cell wall. Infection of the midgut would thereby be initiated by the binding of BTV to the receptors. The biochemical nature of receptor sites for attachment of BTV to arthropod (or mammalian) cells has yet to be determined. However the specificity of the BTV-vector relationship does seem to support the presence of such a specific viral receptor.

1.5.1.2 Digestive enzymes in midgut

Recent studies have suggested that modification of the virus particles by treatment with proteolytic enzymes such as chymotrypsin or trypsin can affect their ability to initiate infection of *Culicoides* and experimental work has shown that this can enhance their infectivity for *Culicoides* cell culture by approximately 100-1,000 times (depending on the level of virus particle aggregation) (Mellor 1990; Mertens *et al* 1993; Mertens *et al* 1995). There is no direct evidence concerning the composition of *Culicoides* digestive enzymes, but many groups of haematophagous insects including mosquitoes, tsetse flies, tabanids and sandflies are known to secrete mixtures of proteases, especially chymotrypsin and trypsin, into the midgut (Champlain and Fisk 1956; Akov 1972; Gooding 1972; Spiro-Kern and Chen 1972; Briegel and Lea 1975; Thomas and Gooding 1976; Houseman 1980; McFarlane 1985; Clements 1992). It therefore would be surprising if this were not also the case with *Culicoides*.

These data suggest that digestive enzymes within the *Culicoides* gut could modify virus particles, whilst in the gut lumen, either enhancing or diminishing their ability to attach to the midgut cells. Mertens *et al* (1987) produced infectious subviral particles (ISVP) by treating intact BTV particles with chymotrypsin and trypsin, and core particles (CP) by uncoating either ISVPs or intact virus particles (IVP) *in vitro* by cation treatment. ISVPs showed a similar infectivity for mammalian cells (BHK-21) to that of IVPs, whereas CPs have only very limited infectivity for these cells (approximately 10^{4-5} times less infectious than either IVP or ISVP). All three types of BTV particle are orally infective to susceptible *Culicoides*. However IVP and CP showed similar levels of infectivity, while ISVPs were 100-500 times more infectious for *Culicoides* (Mertens *et al* 1995). Similarly, cleavage of a protein exposed on the surface of virions of La Crosse virus is necessary for initiating infection in mosquitoes (Ludwig *et al* 1989).

It is, therefore, suggested that the insect digestive enzymes could modify BTV particles so that the binding domain of the virus would be more effectively exposed to viral receptors on the luminal surface of midgut cells. Since VP2 has been implicated as being the main cell attachment/ neutralisation component in mammalian cell systems, it is

thought likely that it, or some other component of the outer coat, may play a similar role in the infection of insect cells with IVPs. However, as the outer coat is entirely missing from CPs it is probable that their ability to infect insect cells is mediated through an entirely different mechanism, possibly involving different viral protein (s) exposed on the surface of the BTV particles. It is therefore possible that there may be different BTV specific receptors on the mid-gut cells of susceptible *Culicoides*, which could function in response to the different types of BTV particles. If this is the case, in susceptible *Culicoides* the process of conversion of IVP to ISVP, and their subsequent binding to a large numbers of ISVP specific receptors could result in enhanced infection facilitated by some aspect of the digestive process. Other research has indicated that BTV and African Horse Sickness virus (AHSV) seem to share common *Culicoides* vector species (P.S. Mellor, personal communication). It is likely that the transmission mechanisms of these two viruses are similar, especially at the level of the initial interaction between the viruses and the midgut cells of the vector insects.

The susceptibility of *C. variipennis* to infection with BTV has been shown to be under the contol of a single genetic locus (Tabachnik 1991; Roberston and Tabachnick 1992) which may determine the presence or absence of the specific BTV receptor on the luminal cell surface of the gut epithelium.

1.5.2 Mesenteronal escape barrier (MEB)

The MEB reflects later events in viral replication and release from the infected midgut cells of vector insects. Research on mosquitoes has already shown that viral multiplication subsequent to oral infection is confined solely to the cells of the mesenteron in some females. The virus is unable to be released from the midgut and cannot initiate infection of secondary target cells elsewhere in the insect (Hardy *et al* 1983; Romoser *et al* 1987). Jennings and Mellor (1987) demonstrated that a MEB to BTV exists in *C. variipennis*. They found that the maximum titre of BTV in orally infected *C. variipennis* varies from less than $10^{1.0}$ to 10^5 $TCID_{50}$ of virus per insect. In those midges containing less than $10^{2.5}$ $TCID_{50}$ of BTV, the virus was completely restricted to the midgut cells and failed to disseminate to the secondary organs (Mellor 1990). These midges exhibited a MEB and

were incapable of operating as vectors. The nature of the MEB has not been established. However, ultrastructural observation on mosquitoes infected with western equine encephalomyelitis virus suggests that virus may not be maturing in MEB females. This assertion is based on the fact that in MEB females there is an an unusual accumulation of naked nucleocapsids along the basal margins of the mesenteronal epithelial cells (Houk quoted by Hardy *et al* 1983). There is no evidence to date to show that a similar phenomenon exists in *Culicoides*.

1.5.3 Salivary gland infection and escape barriers

The presence of salivary gland infection (SGIB) and salivary gland escape (SGEB) barriers have been suggested by Hardy *et al* (1983), based on studies of arbovirus-mosquito interaction. Observations supporting the existence of a SGIB indicate that no detectable viral infection of the salivary glands occurred in some mosquitoes orally infected with West Equine Encephalomyelitis (WEE) virus even though virus was present in the haemocoel (Kramer *et al* 1981; Hardy *et al* 1983). However, the concentrations of virus in the haemocoel were usually low (Hardy *et al* 1983). It was also observed that a SGIB to Rift Valley fever virus developed in female *Anopheles stephensi* (Liston) during the maturation of the insects, since the salivary glands supported virus replication in immature (larvae) mosquitoes but not in mosquitoes infected as adults (Romoser *et al* 1994). Factors important in the formation of a SGIB might include the physical barriers presented by the thoracic fat body which sometimes surrounds the salivary glands and/or the basal lamina of the salivary glands themselves (Weaver *et al* 1990); low levels of infectious virus in the haemocoel; or a lack of specific receptors. It is also well known that insects produce potent antimicrobial compounds that kill invading bacteria and it has been queried whether these or similar compounds, produced in vectors, can also protect the host against the viral pathogens that they transmit. In this context the cellular and humoral immune systems in insects may play an important role in inactivating virus within the haemocoel (Woodring 1985; Clements 1992; Lowenberger *et al* 1994), thereby limiting its dissemination to secondary target organs including the salivary glands.

It has also been demonstrated that some mosquitoes with infected salivary glands are unable to transmit the virus orally (Hardy *et al* 1983; Hardy 1988), suggesting that a SGEB may be exhibited in these insects. The existence of this barrier may be due to low levels of infectious virus produced in some salivary glands, low levels of virus secretion into the salivary gland ducts, a modulation of virus replication in the salivary glands or to virus-induced cytopathology of salivary glands (Hardy *et al* 1983; Hardy 1988). No study has yet been made of SGIB and SGEB to BTV in *Culicoides*.

1.5.4 Mesenteronal physical barrier

In mosquitoes, infectious virus has been found in haemolymph samples taken from some females within 4 hours of feeding (Hardy *et al* 1983; Hardy 1988). This supports the concept that ingested virus can enter the haemocoel of a small proportion of insects without initial replication in mesenteronal cells, which is the so-called 'leaky midgut' phenomenon. Furthermore there appears to be a relationship between the efficiency of the midgut mesenteronal physical barrier (MPB) and mosquito body size, in that small individuals have been shown to be more susceptible to a 'leaky gut' than large individuals. It has been implied that this may be connected to the thickness of the basal lamina, which appears to be linearly related to mosquito body size (Paulson and Hawley 1991; Grimstad and Walker 1991; Leake 1992). It has been observed that the basal lamina of the midgut presents a physical barrier or impediment to vesicular stomatitis virus dissemination within sandflies (Weaver *et al* 1992). However, a recent study failed to demonstrate a role for basal lamina thickness as a modulator of Dengue-1 virus dissemination across the midgut epithelium of *Ae. albopictus* (Skuse 1894) (Thomas *et al* 1993). No direct information on the 'leaky-gut' phenomenon has been published in relation to *Culicoides* species. However, a study of BTV-microfilariae co-infection in *Culicoides* supports the function of the gut wall as a physical barrier which can stop BTV breaking through to the haemocoel. *Culicoides nubeculosus* was unable to support BTV replication unless microfilariae were ingested simultaneously. The penetration of the gut barrier by microfilariae allowed BTV to enter and replicate in the haemocoel (Mellor and Boorman 1980). Similar results were observed in coinfections of chickungunya virus and microfilariae in mosquitoes, and electon-microscopy has identified the holes made by the

microfilariae that penetrated through the midgut epithelial layer (Zytoon *et al* 1993).

1.5.5 Transovarial transmission barrier

Vertical transmission of arboviruses has been observed in sandflies and mosquitoes (Beaty *et al* 1980; Turell *et al* 1982; Turell 1988; Comer *et al* 1990; Bosio *et al* 1992; Baquar *et al* 1993; Kramer *et al* 1993; Fulhorst *et al* 1994).

Transovarial transmission does not appear to occur with BTV in *C. variipennis* (Jones and Foster 1971b, Nunamaker *et al* 1990). In the laboratory BTV has not been detected in the progeny *C. variipennis* of parental midges infected with the virus (P.S. Mellor, personal communication). This suggests that there is a transovarial transmission barrier (TOTB) in *C. variipennis* which can prevent parental midges transmitting BTV directly to their progeny. The nature of TOTB in *Culicoides* is still unknown.

1.6 SUMMARY

Bluetongue is an arthropod-borne disease of ruminant animals and BTV is distributed across a vast area of the world. The virus is maintained in nature by an endless series of alternating cycles of replication in vector species of the biting midge, *Culicoides*, and various ruminant animal hosts. The susceptibility of *Culicoides* to infection with BTV and the ability of infected *Culicoides* to transmit BTV to susceptible animals are controlled by a complex series of intrinsic factors. These comprise a series of potential barriers to virus infection or dissemination within individual *Culicoides*. Because of these barriers not all female midges, even within a single vector species, are susceptible to infection with BTV, or if infected, are competent to transmit the virus.

In a competent vector, BTV can pass through all of the barriers and eventually be transmitted. Once ingested by a competent vector, the virus binds to the luminal surface of the midgut cells, enters these cells and replicates in them. The progeny virus particles are then released through the basement lamina into the haemocoel from where the secondary susceptible organs, including salivary glands, are infected. Alternatively in a

small number of individuals, the virus may, under certain conditions, "leak" directly from the gut lumen, through the gut wall and into the haemocoel and so infect other organs. Transmission of BTV can take place by biting activity of the insect subsequent to virus replication in salivary glands. The whole cycle from oral infection to transmission takes 10 to 15 days at 25°C. Once infected, a vector insect usually remains so and is able to transmit virus for the rest of its life.

In relation to the transmission of BTV, the midgut, salivary glands and ovaries of the vector insects are the location of the most important parts of the barrier system. It is known that the presence or absence of these barriers, which determine either susceptibility or resistance, are under genetic control. However, the nature of the biochemical mechanisms underlying such barriers are still poorly understood and remain to be elucidated.

1.7 PURPOSE OF THE PROJECT

The purpose of this project is to analyze the mechanisms which limit or facilitate the transmission of BTV by *Culicoides* biting midges by studying the interactions between the virus and *Culicoides* at both the cellular and the whole insect levels, using a variety of established methods and new techniques to study virus replication *in vitro* and *in vivo*, and to provide for the first time parallel quantitative and qualitative data.

CHAPTER 2
THE KINETICS AND LOCALISATION OF BLUETONGUE VIRUS INFECTION IN THE VECTOR, *CULICOIDES VARIIPENNIS*

2.1 INTRODUCTION

Culicoides variipennis, a proven BTV vector species, was used in this study. Immunohistochemical techniques were used for the first time to observe BTV replication and the localisation of BTV in different organs/tissues of *C. variipennis* after infection by inoculation or by oral ingestion of an infectious blood meal. Conventional virus titration assays were performed on infected *C. variipennis* to comparatively link these quantitative data with the localisation study. The objective of this study was to identify and characterise the barriers to BTV infection, dissemination and transmission in a vector species.

2.2 MATERIALS AND METHODS

2.2.1 Virus

BTV serotype 1 (BTV 1SA) was obtained from the Veterinary Research Institute, Onderstepoort, South Africa and is maintained in the Pirbright Laboratory, Institute for Animal Health (IAH), UK. The virus was passaged two times in chicken embryos and 7 times in baby hamster kidney (BHK-21) cells. The intact virus particles (IVP), infectious subviral particles (ISVP) and core particles (CP) of BTV 1SA were provided by Dr. P.P.C. Mertens also at IAH-Pirbright (Mertens *et al* 1987).

2.2.2 Tissue culture cells

Both BHK-21 cells and a *Culicoides variipennis* cell line (KC cells) were used in this study. The KC cell line was provided through the courtesy of S.J. Wechsler, Arthropod-borne Animal Diseases Research Laboratory, Wyoming, USA. BHK-21 cells were used for routine propagation and titration of BTV. Both BHK-21 cells and KC cells were used

to study the infectivity of the different BTV particles, for immunohistochemical (IHC) detection of virus replication and to detect expression of the BTV nonstructural protein NS3. BHK-21 cells were grown at 37°C in modified Eagle's medium containing 5% normal foetal calf serum (FCS) which had been heat inactivated at 56^{0}C for 30 minutes. KC cells were grown at room temperature (RT, 22±2^{0}C) in Schneider's insect medium (Sigma S9895) (Wechsler *et al* 1989) containing 10% FCS. For IHC detection, the cells were grown on coverslips (12 mm in diameter) in plastic *petri* dishes (60 mm). For virus titration, 96-well micro plates were used.

2.2.3 Infection of tissue culture cells

Suspensions containing 10^{2-3} BHK-21 $TCID_{50}$ of BTV per ml were prepared to infect cell cultures grown on coverslips. When the monolayers were almost confluent the medium was discarded, the cells were washed with phosphate-buffered saline (PBS) and were infected with 0.2 ml of the virus suspension mixed with 0.8 ml of fresh Eagle's medium (BHK-21 cells) or Schneider's medium (KC cells). The cells were then incubated at 37°C (BHK-21 cells) or RT (KC cells) for 30 min, before removing the viral suspension and washing the cells three times with Eagle's medium. The cells were then incubated at 37°C in Eagle's medium containing 2% FCS (BHK-21 cells) or at RT in Schneider's medium with 5% FCS (KC cells) respectively. As required, cells were rinsed twice in PBS and were fixed at various intervals postinfection (pi) in a series of time course experiments. For IHC detection cells were fixed for 5 min in prechilled acetone and air dried for 15 min at RT. Cells could be used for IHC detection at once or could be kept at -20°C in sealed plates. The medium was also collected and was used in virus titration experiments using the same time course series.

2.2.4 Antibodies and reagents for immunohistochemistry assay

A group-specific monoclonal antibody against the core protein VP7 of BTV (MAbA3) was provided by Dr. J Anderson of the IAH-Pirbright Laboratory (Anderson 1984). This MAb was used to detect replication of BTV 1SA in cells and in intact *Culicoides*. Polyclonal antibody against the NS3 polypeptide 10C (amino acids 37-46) (PAb10C) was provided

by Dr.A Wade-Evans also of the Pirbright Laboratory and was used to assay the expression of NS3 in cells infected with BTV 1SA. Horseradish peroxidase conjugated rabbit-anti-mouse and goat-anti-rabbit immunoglobulins were obtained from the DAKO Corporation (DAKOPATTS P260 and DAKOPATTS P448).

2.2.5 *Culicoides*

Culicoides variipennis, the major vector of BTV in North America, was used in this study. The insects were originally imported from the USA in 1967 and have been maintained as a self-sustaining colony in the insectaries of the IAH-Pirbright Laboratory for over 25 years (Boorman 1974). Adult midges are held in waxed cardboard boxes and maintained with 10% sucrose. Anaesthetised mice provide a blood meal source. Larvae are reared in enamel pans on a substrate of glass fibre in a medium which contains 4 ml of nutrient broth, 3/4 of teaspoon of grass meal and 1/4 teaspoon of Bemax (a proprietary wheat germ product, Wm. Lillico & Son Ltd, Surrey) per litre of dechlorinated water. The room temperature in the insectary is kept at 24° ± 1°C

2.2.6 Infection of *Culicoides* with BTV

Adult female *Culicoides* midges, 3 days old, were used in experiments and were infected with BTV by intrathoracic (IT) inoculation or by feeding on virus suspensions in blood.

For IT inoculation, all of the insects were injected with approximately $10^3 TCID_{50}$ of BTV 1SA (suspended in Eagle's medium) per midge by using fine glass needles connected to a semi-automatic inoculation device (Boorman 1975). The inoculated midges were then incubated at 25°C, collected at intervals after infection and stored at -70°C for titration and IHC assay.

For oral infection, blood meals were prepared using citrated normal sheep blood, diluted in the ratio of 3:7 with suspensions of BTV in Eagle's medium, so that the final suspension contained $10^{6-7} TCID_{50}$/ml of the virus. The midges were fed through a parafilm membrane on the blood-virus suspension maintained at 37°C by using the method of

Mellor (1971). All engorged female midges were transfered to cardboard boxes after feeding, provided with 10% sucrose and were incubated at 25°C until required. Female *Culicoides* fed a similar blood meal but without BTV were used as virus-negative controls.

2.2.7 Titration of virus

For titration, midges were ground individually in Eppendorf tubes using a motor-driven plastic pestle and were suspended in 0.1 ml of Eagle's medium. Medium collected from cell cultures, and the suspensions of *Culicoides* were diluted tenfold serially. Titration was carried out in 96-well microplates with confluent BHK-21 cell monolayers. Cytopathic effect (CPE) was used as a positive indicator of the presence of virus. $TCID_{50}$/ml was determined using the method of Karber (quoted by Whitaker 1972):

$\log TCID_{50} = m - \Delta (S - 0.5)$

m: log dilution containing the highest concentration of virus; Δ: log dilution factor; S: sum of proportion of positive cultures; 0.5: constant

2.2.8 Preparation of cryostat sections of *Culicoides*

For cryostat sectioning (Bancroft 1990), the midges were first dipped in 2% SDS for 1 minute and were then rinsed twice in PBS. After the liquid on the surface of insects had been removed with a piece of paper tissue, the midges were placed on a cork (20-30 midges per cork) 7 mm in diameter and then embedded in OCT embedding compound (Raymond A Lamb, Tissue-tek C-101.25), before being left at RT for 5 minutes. The block was frozen at -70°C and could then be used immediately or stored at -70°C. Alternatively, the block could be frozen rapidly by dipping in liquid nitrogen.

Before sectioning, the temperature of the microtome was set at -24±1^{0}C and the frozen block was put in the cryostat chamber for about 30 min until the temperature of the block had risen to the same level. Serial sections 10 μm thick were cut and mounted on microscope slides coated with polylysine (Sigma P1339), and air dried for 10 min at RT before fixation. For IHC staining, the sections were rinsed twice in PBS, fixed with prechilled acetone for 10 min and air dried at RT. They were then suitable for use at once,

or could be stored at -20°C in a sealed container.

2.2.9 Immunohistochemistry assay

The indirect immunoperoxidase technique (Taylor 1978, De Jone *et al* 1985, Bancroft 1990) was carried out as follows. The fixed cells or sections were rinsed twice in PBS (pH 7.3) and then incubated for 30 min at RT in blocking buffer (5% normal bovine serum in 1% milk-PBS, pH 7.3) with 0.3% H_2O_2 to stop non-specific reactions and to eliminate endogenous peroxidase activity of cells or insect tissues. After incubation for 90 min at 37°C in a 1:20 dilution of MAbA3 (or PAb10C 1:1,000) diluted in 1% milk-PBS, the slides were washed three times for 5 minutes at 37°C in PBS. The slides were then incubated for 30 min at RT with peroxidase conjugates, diluted 1:200 with 1% milk-PBS. After being washed extensively with PBS, the immunochemical reactions were developed for 10 min at RT with 0.5 mg/ml diaminobenzidine (DAB) solution containing 1 μl 30% H_2O_2 per ml. The reaction was stopped by washing with PBS for 5 min at RT. Haematoxylin (Merck Ltd 35060 4T) (Taylor 1978; Bancroft 1990) was used to counterstain the cells or sections. The cells or sections were dehydrated with gradient ethanol and then mounted using liquid Eukitt mounting medium (Merk Ltd. 36189 4G). Antigen-positive cells and tissues were identified by the presence of a rusty brown precipitate. The negative controls included virus-negative and antibody-negative cells or sections.

Statistical analyses, including calculation of mean, standard error of mean (SEM) and standard error of proportion (SEP*, see Page 50), t-test of mean and χ^2-test, were carried out using conventional statistical methods (Rowntree 1991; Clarke and Cooke 1992).

2.3 RESULTS

2.3.1 Infection of BHK-21 and KC cells with BTV IVP, ISVP and CP

Fig. 2.1 shows the results from 3 experiments of the replication curves of BTV 1SA in BHK-21 cells which were infected with IVP, ISVP and CP of BTV 1SA. The experiments

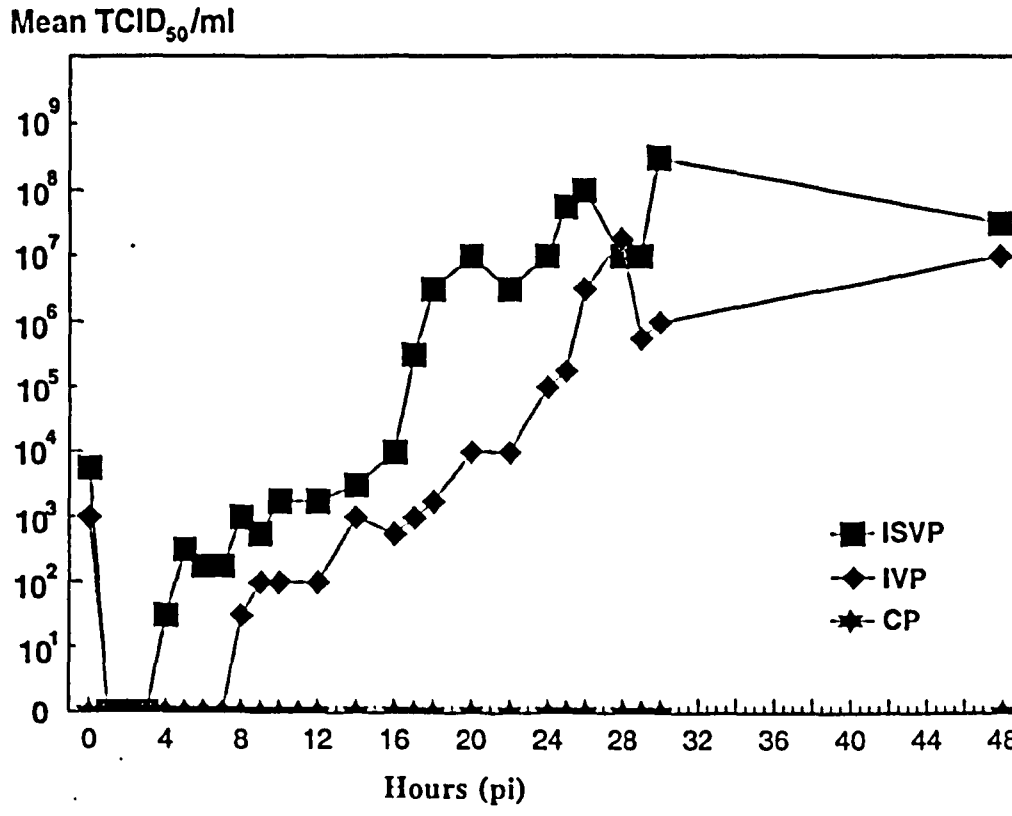

Fig. 2.1 Yield of progeny BTV in BHK-21 cells infected with IVP, ISVP and CP of BTV 1SA at 0-48 h postinfection. Data are derived from three experiments. IVP: intact virus particles; ISVP: infectious subviral particles; CP: core particles. Data and standard error are shown in appendix 1.

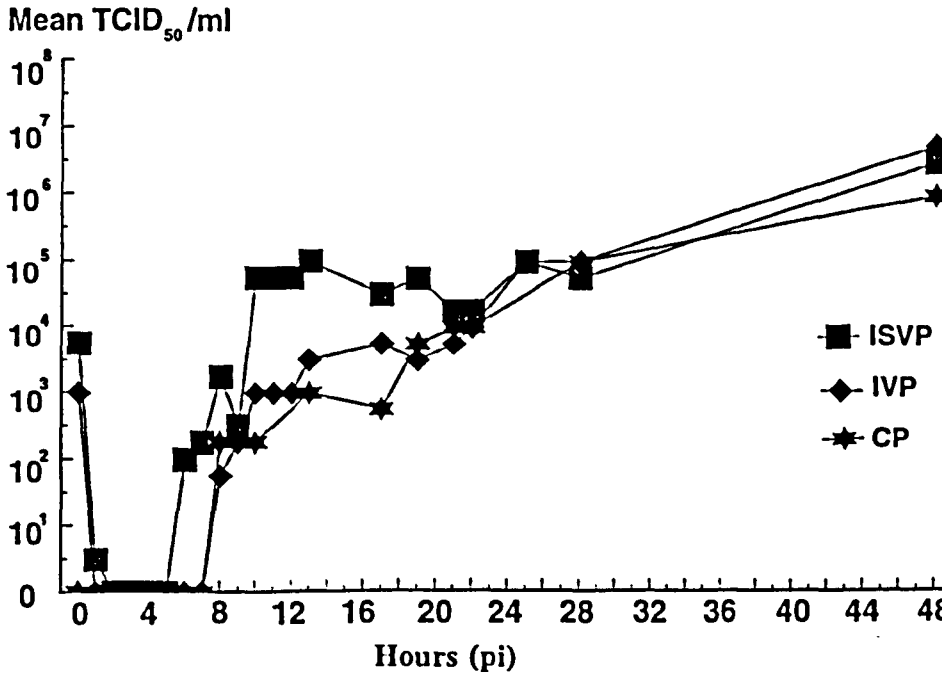

Fig. 2.2 Yield of progeny BTV in KC cells with IVP, ISVP and CP of BTV 1SA at 0-48 h postinfection. data are derived from three experiments. IVP: intact virus particle; ISVP: infectious subviral particle; CP: core particle. Data and standard error are shown in appendix 2.

gave highly reproducible results. Data are shown in appendix 1. In BHK cells infected with ISVP and IVP, virus replication showed a similar pattern. The virus was first detected in ISVP infected cells at 4 h pi, titre increased gradually and reached the peak level at about $10^{8.0}TCID_{50}$/ml. However in IVP infected cells, the virus could only be detected from 8 h pi, about 4 h later than in ISVP infected cells. The titre of the virus reached a peak level of about $10^{7.5}TCID_{50}$/ml at 28 h pi. The result of the infection was the death of the cells. No detectable virus was found either by titration or by IHC in CP infected BHK-21 cells.

The replication of BTV 1SA in KC cells infected with IVP, ISVP and CP is shown in Fig. 2.2. These results were from 3 experiments and were highly reproducible. Data are shown in appendix 2. In IVP and CP infected cells, the virus was first detected at 8 h pi and the titre then increased gradually to reach a peak level at 48 h pi. However, in ISVP infected KC cells, the virus was first detected at 6 h pi, about 2 h earlier than in IVP and CP infected cells. The virus grew rapidly to a level of about $10^{5.0}$ $TCID_{50}$/ml at 10 h pi and then increased slowly to the peak of $10^{6.5}$ $TCID_{50}$/ml at 48 h pi. The infection of KC cells was persistent and could last for months at around $10^{6.0}$ $TCID_{50}$/ml if the cells were well maintained.

A detectable virus reaction was found by IHC in infected BHK-21 cells as early as 6 h pi and in the infected KC cells at 8 h pi. After infection typical perinuclear inclusion bodies formed in BHK-21 cells (Fig. 2.4a) while no obvious morphological change was observed in KC cells. Virus positive reactions were only seen in a certain proportion (about 50%) of KC cells (Fig 2.3a). Fig. 2.3b shows uninfected KC cells.

2.3.2 Detection of NS3 from BHK-21 cell cultures infected with BTV

NS3 has previously been shown to be associated with release of progeny virus from infected cells (Hyatt *et al* 1991, 1993). Therefore in the present study a technique was devised to detect its presence in BHK-21 cells before commencing work with KC cells. In this study NS3 was successfully detected using a polyclonal antibody against BTV NS3

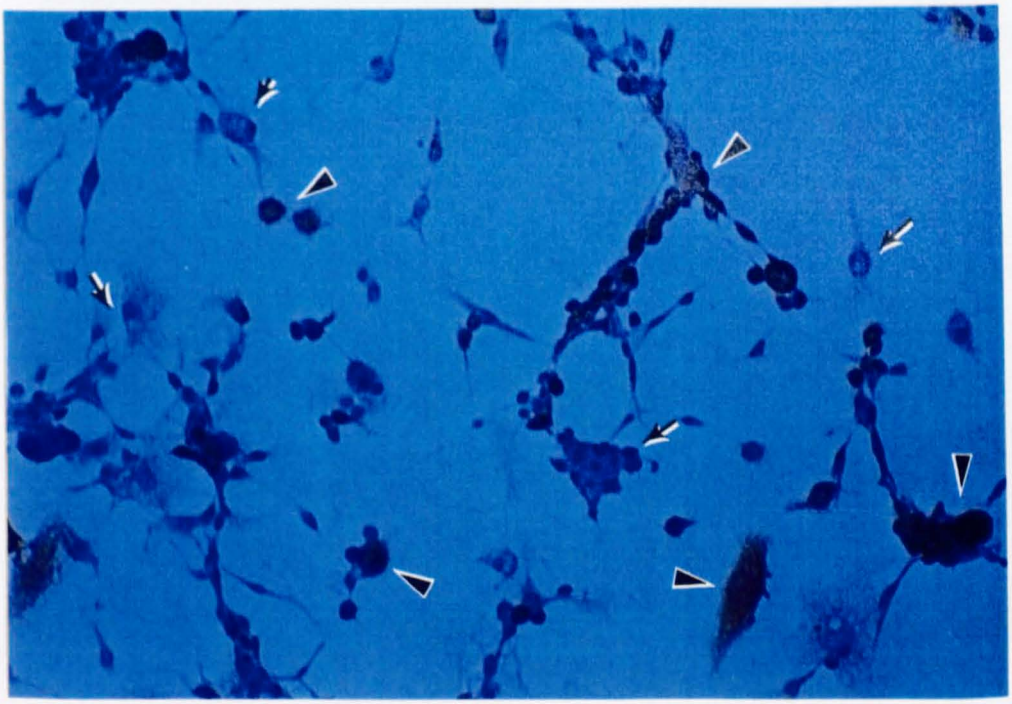

Fig 2.3a IHC detection of BTV infection in KC cells (48 hours pi). ► BTV positive cells; → BTV negative cells (counterstained with haematoxylin)

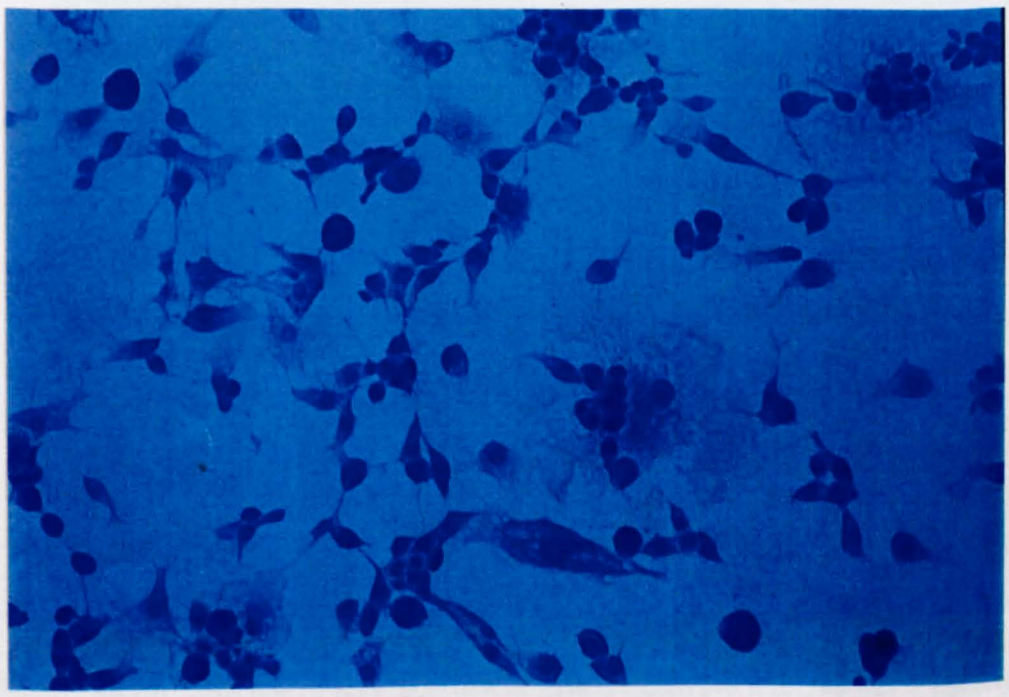

Fig. 2.3b IHC staining in uninfected KC cells (counterstained with haematoxylin)

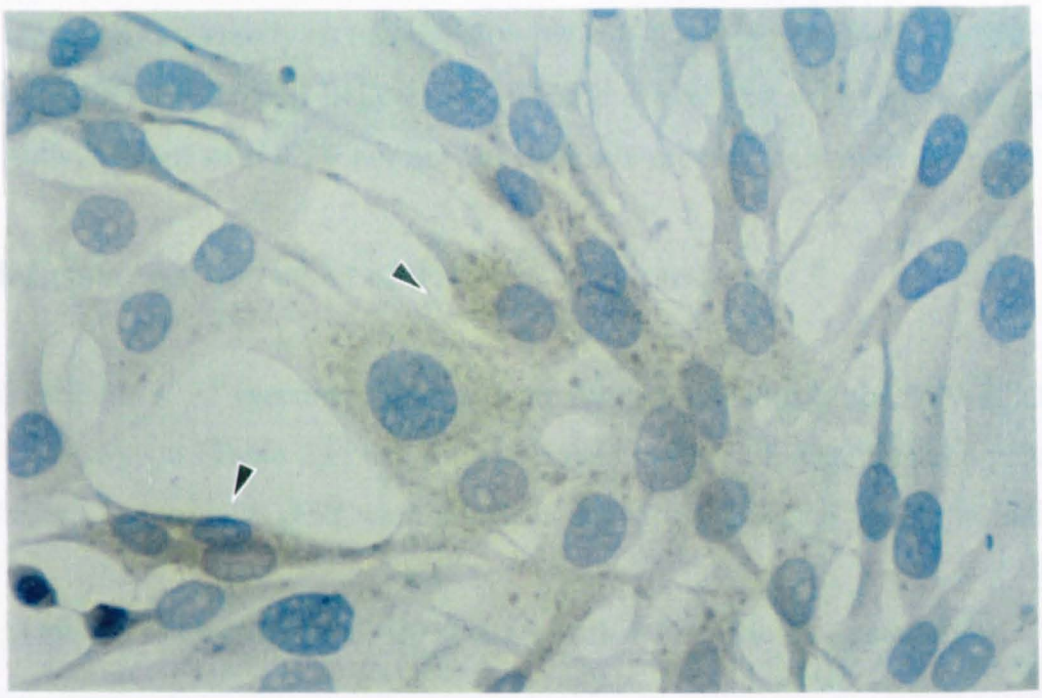

Fig. 2.4a IHC Detection of NS3 in BHK-21 cells infected with BTV 1SA (16 h pi)

► NS3 positive cell (counterstained with haematoxylin)

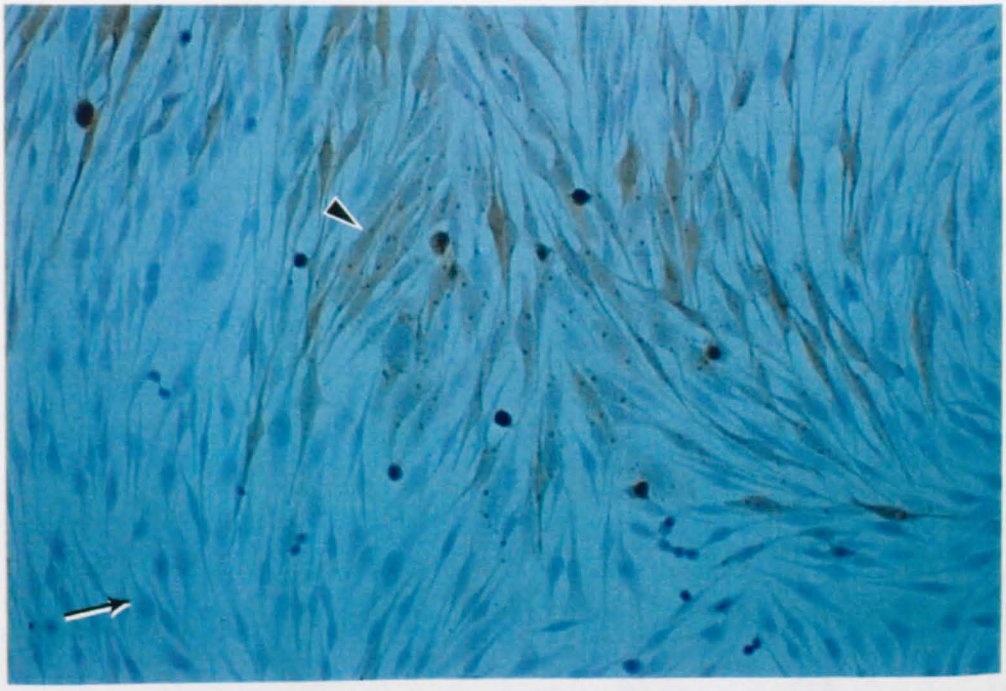

Fig. 2.4b IHC detection of BTV infection in BHK-21 cells (16 h pi). ► BTV positive cells;

→ BTV negative cells (counterstained with haematoxylin)

polypeptide, by using the IHC technique. Fig. 2.4 shows the expression of NS3 in BHK-21 cells infected with BTV 1SA. It was expressed at a very low level and was distributed in the cytoplasm apparently in association with viral inclusion bodies (VIB) (Fig 2.4a). Fig 2.4b shows BTV infected BHK-21 cells stained with MAbA3. The expression of NS3 in KC cells infected with BTV has not been studied and will be subject of future work.

2.3.3 Infection and replication of BTV 1SA in *C. variipennis*

A final total of 282 IT inoculated midges were titrated individually for BTV at intervals of up to 14 days pi (Table 2.1) and growth curves in Fig. 2.5. One hundred percent of ITinoculated midges with BTV were infected. The virus replicated rapidly in individual

Table 2.1 Infection of *C. variipennis* with BTV after IT inoculation

Time pi days	hours	No. of midges (inoculated)	No. of midges (BTV+)	Detection rate (%)
<1	1	10	10	100
	2	10	10	100
	4	10	10	100
	8	10	10	100
	12	10	10	100
	16	10	10	100
	20	10	10	100
1	24	10	10	100
2		20	20	100
3		18	18	100
4		24	24	100
5		21	21	100
6		21	21	100
7		19	19	100
8		20	20	100
9		18	18	100
10		19	19	100
14		22	22	100
Total		282	282	100

Table 2.2 Infection of *C. variipennis* with BTV after an infectious blood meal

Time pi days	hours	No. of midges (fed)	No. of midges (BTV positive)	detection rate (%)*
< 1				
	1	20	20	100.0
	2	20	20	100.0
	4	62	62	100.0
	8	67	67	100.0
	12	63	63	100.0
	16	66	66	100.0
	20	68	68	100.0
1	24	59	59	100.0
	28	87	69	79.3
	32	76	30	39.5
2		71	29	40.8
3		98	26	26.5
4		81	27	33.3
5		74	23	31.1
6		60	19	31.7
7		157	54	34.4
8		80	31	38.8
9		99	36	36.4
10		80	29	36.3
11		95	35	36.8
12		59	20	33.9
14		133	46	34.6
Total		1,675	---*	---*

* detection rates for initial 24 hours of 100% reflect presence of residual virus from the blood meal in the gut lumen. Rising detection rates from 3 days onwards reflect productive infection.

midges to a level about $10^{5.0}$ $TCID_{50}$ per insect in 3 days and apparently remained at the same level for the rest of the insect's life (Fig. 2.5, Appendix 3). There was no 'lag phase' in IT inoculated *Culicoides* as observed in orally infected ones. It was later confirmed that fully disseminated tissue involvement could be seen by IHC at this time.

Table 2.2 shows that a total of 1,675 midges fed with an infectious blood meal

weretitrated individually for BTV at intervals of up to 14 days pi. Initially virus was detected in 100% of the orally infected insects up to 24 hours pi. This then declined rapidly to 26.5% positive by day 3 pi as virus was eliminated from orally insusceptible individuals. Subsequently recovery of this infection rate to over 30% represents replication and dissemination of the virus in persistently infected insects. There was no significant difference between the highest and lowest infection rates during 7 to 14 days pi [χ^2=0.160, degree of freedom: df=1, P>0.05), suggesting that the infection rate was constant over this period. The infection rate of orally infected *Culicoides,* based on the titration results of the midges from 7 to 14 days pi, was 35.4% (270/763, SEP=0.017: i.e. SEP=1.7%). The replication of BTV in orally infected *Culicoides* varied with individuals. Detailed titration results showed that the titre of BTV fell from about $10^{3.0}$ to $10^{0.75}$TCID$_{50}$ per midge in some insects in the first 24 hours pi. This fall was significant (t=6.43, P<0.01 df=76). The virus then replicated gradually to reach a peak of about $10^{5.0}$TCID$_{50}$ per insect by 5 days pi in some individuals. This increase from $10^{0.75}$TCID$_{50}$/midge was also significant (t=3.03, df=78, P<0.01)(Fig. 2.5, 2.6, Appendix 4). The daily titres of BTV in different individuals varied from 0.32 to $10^{5.0}$TCID$_{50}$ per insect (Fig. 2.5, 2.6, Appendix 4). Once the midges were persistently infected, they remained so for life.

2.3.4 Characteristics of BTV infection and dissemination in *C. variipennis*

A total of more than 2,000 midges fed on an infectious blood meal were processed for IHC detection. It was not practical to embed individual midges in separate blocks since the infection rate was low and a series of sections was needed from each midge to provide a reasonable chance of detecting the virus. Therefore about 20-30 midges were embedded in each block and approximately 80-100 sections were obtained from each block. Because of these constraints it was impossible to quantitate the patterns of virus dissemination observed by IHC. Generally speaking, after ingestion of a viraemic blood meal, infections were sporadic and the following patterns were observed in persistently infected individuals: 1) virus was restricted to the anterior and posterior midgut, and the foregut-midgut junction; 2) virus replicated in the gut cells, disseminated into the haemocoel but could only be detected in a few sporadic fat body cells beyond the gut; 3) virus escaped from the gut cells into the haemocoel and replicated in secondary organs/tissues but at low

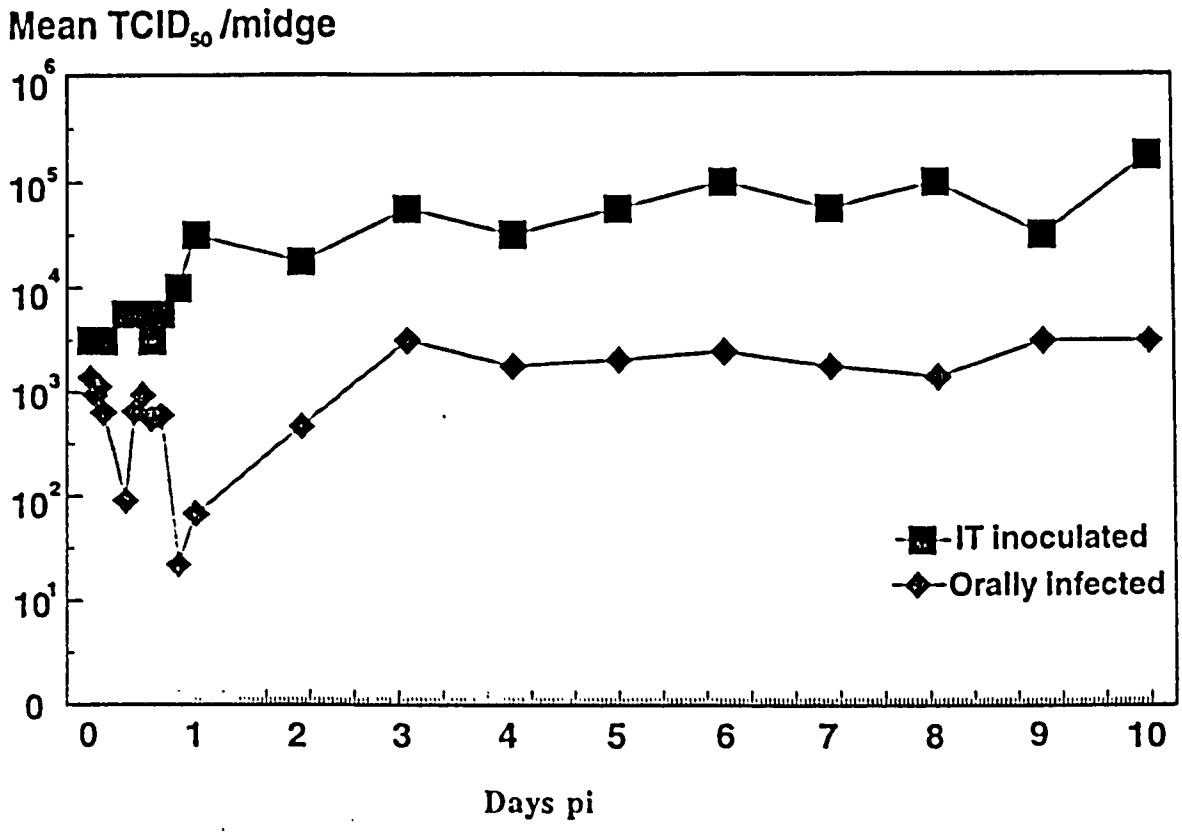

Fig. 2.5 Comparison of the replication of BTV 1SA in inoculated and orally infected *C. variipennis*. Mean titre each day is from the titres of 10-24 IT inoculated or 19-69 orally infected midges. Data are shown in Appendices 3 and 4.

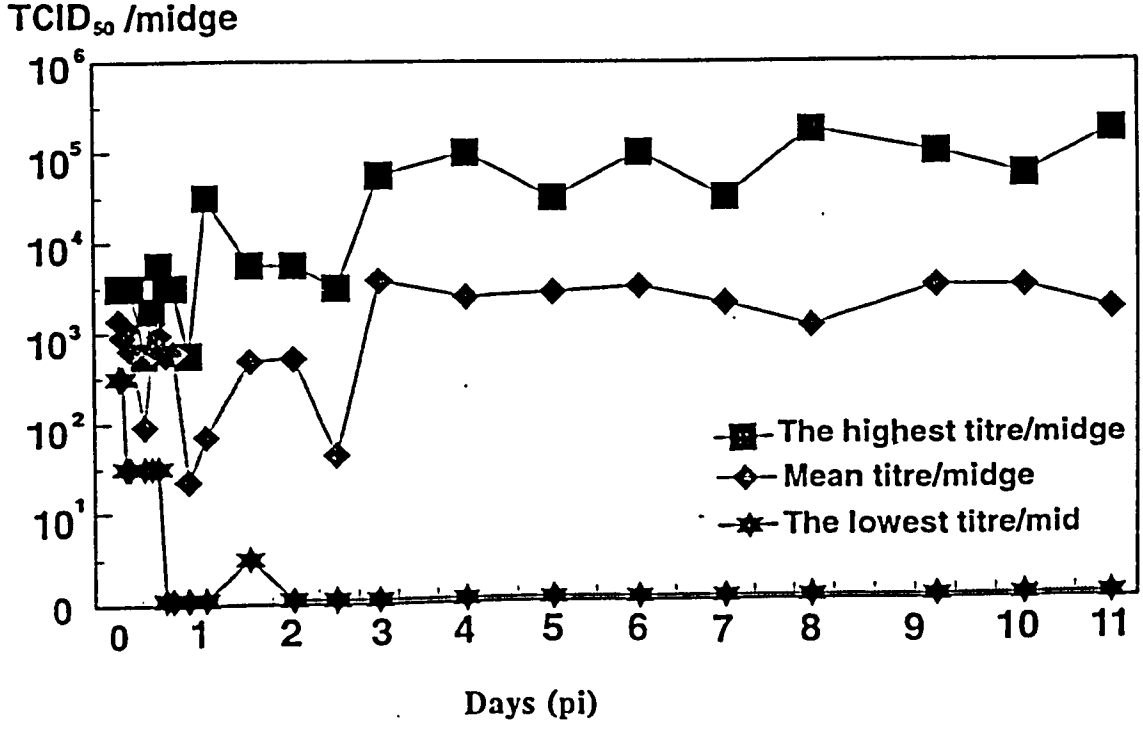

Fig. 2.6 The replication of BTV 1SA in orally infected *C. variipennis*. The titres each day are from the titration results of 19-69 BTV positive midges. Data are shown in Appendix 4.

levels; 4) a fully disseminated infection was observed and virus replicated in secondary organs/tissues at high level. Each of these patterns was seen in the sections from at least 60 blocks. These patterns were not temporal stages in virus dissemination, since each of them could be seen at any time interval from 3 to 14 days pi. Once virus escapes from the gut into the haemocoel, dissemination to other tissues is usually a rapid process. The virus was first observed by IHC in fat body cells at 48 h, in ganglia at 3 days and in the salivary glands at 5 days after ingestion of an infective blood meal. However it is difficult to determine the exact time when the salivary glands first became infected because they were not always contained in the sections. The virus infection patterns of gut cells in orally infected midges prior to 2 days pi are difficult to interpret because endogenous peroxidase in the red blood cells of the blood meal causes false positive reactions.

More than 500 IT inoculated midges were processed using IHC (in 33 blocks, 15-20 midges per block). All IT inoculated midges showed a fully disseminated infection and, as expected, virus spread to other tissues was more rapid than in orally infected insects. However after 5 days pi, there appeared to be no difference in the degree of dissemination between inoculated and some orally infected individuals.

2.3.5 Distribution of the virus in *C. variipennis*

In orally infected individuals, the virus was detected in gut cells (specifically the anteriorand posterior midgut epithelium, and also the cells of the foregut-midgut junction possibly involving cardial and posterior foregut cells) (Fig. 2.7), in fat body, cephalic and thoracic ganglia, salivary glands and ommatidia of the compound eyes. No virus was found in the hindgut cells, muscles, Malpighian tubules and oocytes/nurse cells of the ovaries. In the 500 IT inoculated individuals examined, the organs/tissues infected with the virus were the same except that infection of gut cells was never observed. No evidence of infection of the abdominal ganglia or heart was observed because these tissues were not seen in the sections.

In orally infected flies, anterior midgut, posterior midgut and foregut-midgut junction cells including cardial epithelium and possibly the posterior part of foregut were the most

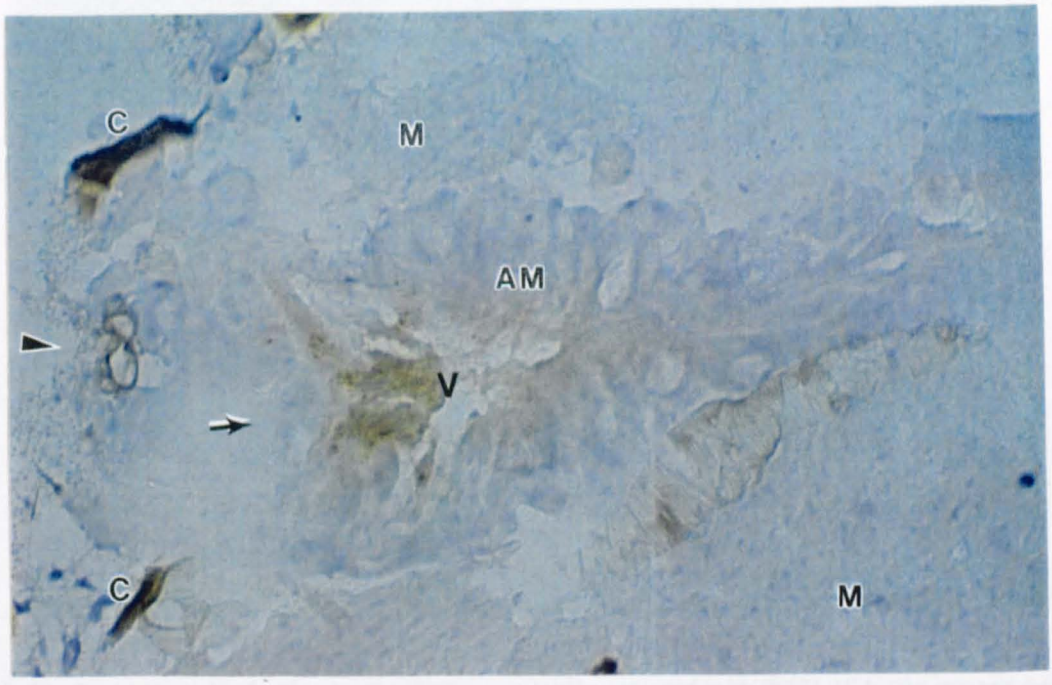

Fig. 2.7 Infection of the gut (in thorax) in *C. variipennis* orally infected with BTV 1SA (10 days pi). **AM** anterior midgut; **M** muscle; **V** BTV positive reaction; ► the anterior end of thorax; ➔ foregut-midgut junction; **C** cuticle (counterstained with haematoxylin)

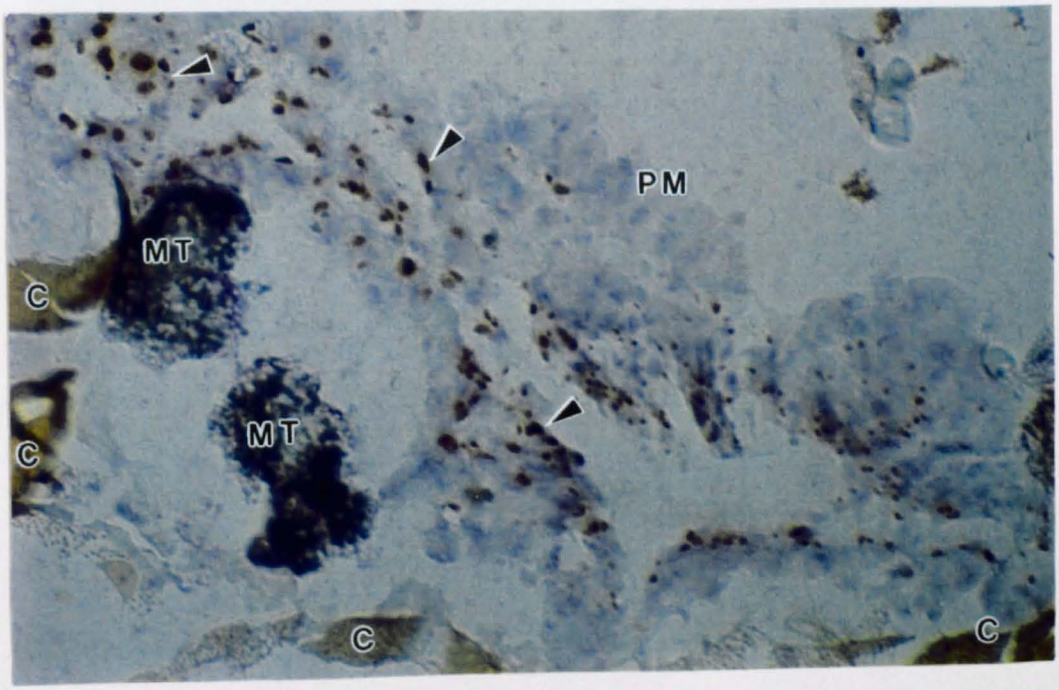

Fig. 2.8 Infection of the gut (in abdomen) in *C. variipennis* orally infected with BTV 1SA (10 days pi). **PM** posterior midgut; ► BTV positive reaction; **MT** Malpighian tubule; **C** cuticle (counterstained with haematoxylin)

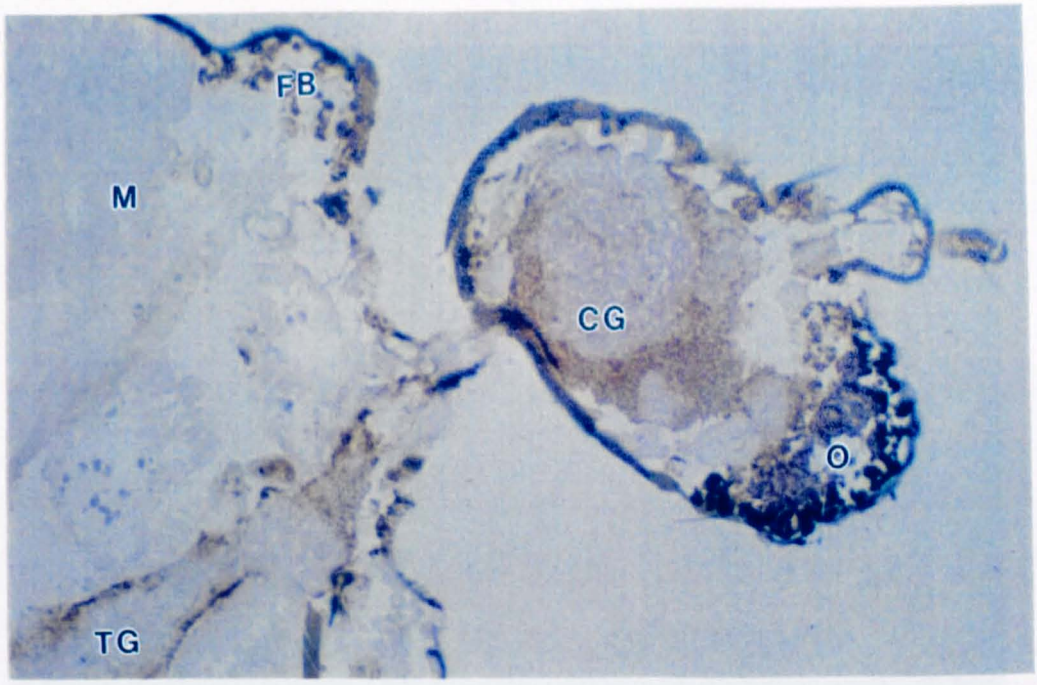

Fig. 2.9 Fully disseminated infection in *C. variipennis* orally infected with BTV 1SA (thorax and head, 7 days pi). BTV+ reaction in: **CG** cephalic ganglia; **TG** thoracic ganglia; **FB** fat body cells; **O** ommatidia of compound eyes; **M** muscle (BTV-) (counterstained with haematoxylin)

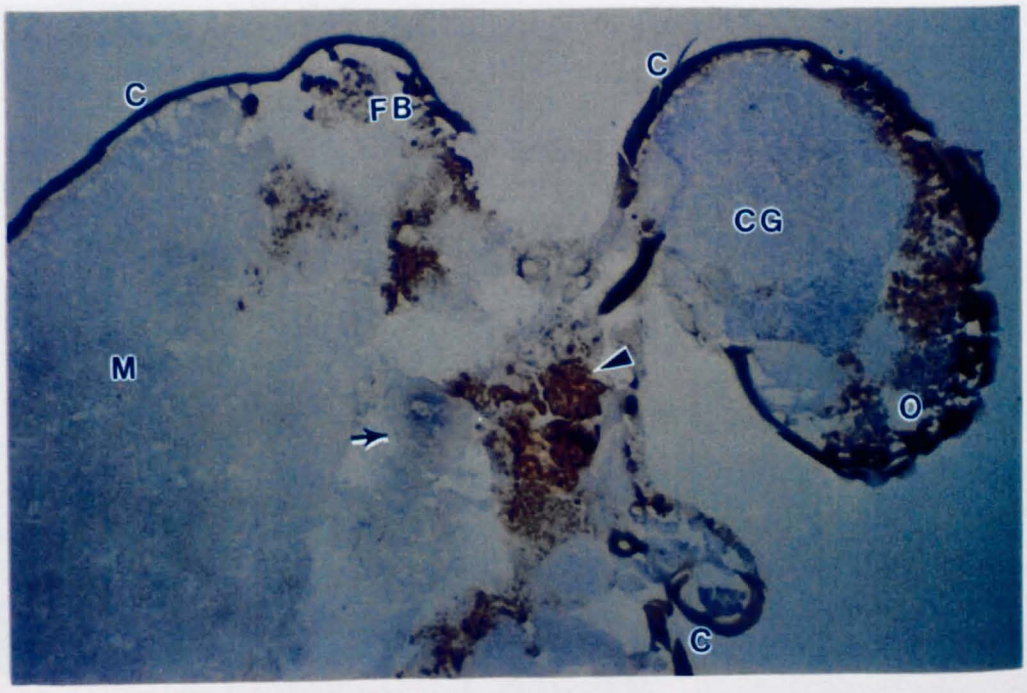

Fig. 2.10 Fully disseminated infection in *C. variipennis* orally infected with BTV 1SA (thorax and head, 7 days pi). BTV+ reaction in: **CG** cephalic ganglia; ➔ anterior midgut; **FB** fat body cells; ► salivary glands; **O** ommatidia of compound eyes; **M** muscle (BTV-); **C** cuticle (counterstained with haematoxylin)

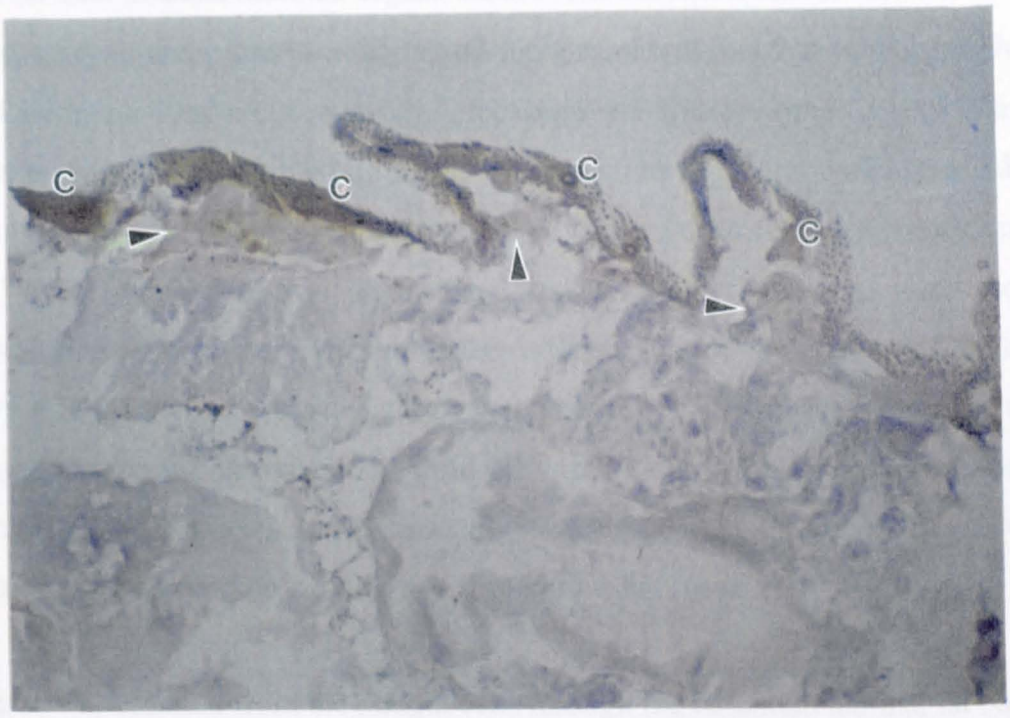

Fig. 2.11 Detection of BTV 1SA in a few cells in the haemocoel of orally infected *C. variipennis* (abdomen, 7 days pi). ► BTV+ fat body cells; **C** cuticle (counterstained with haematoxylin)

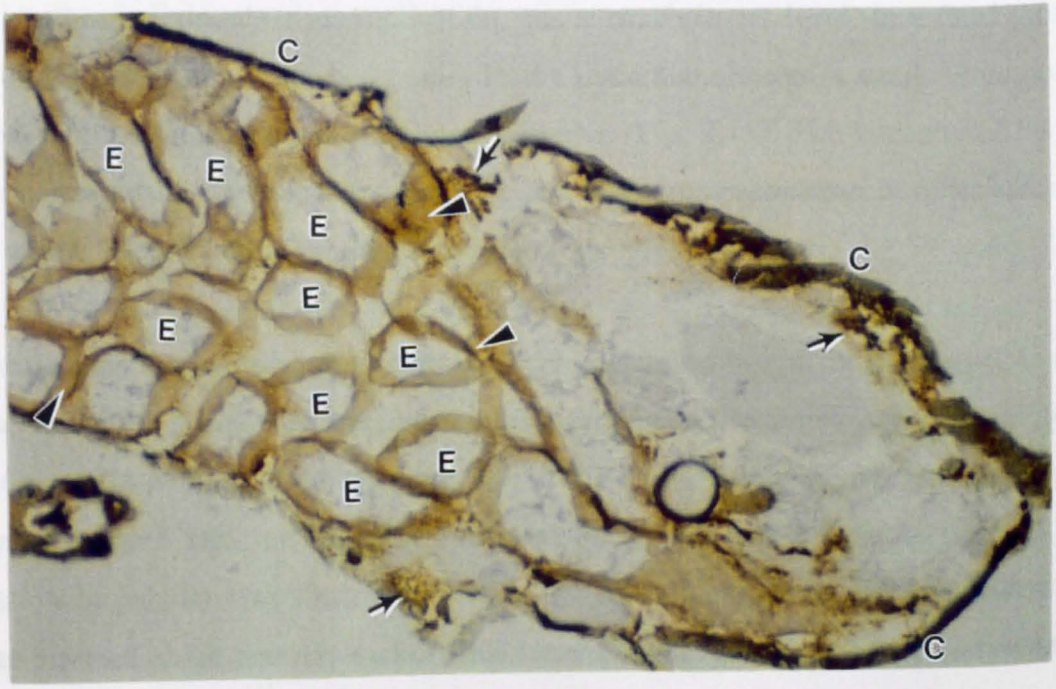

Fig. 2.12 Fully disseminated BTV infection in *C. variipennis* after an infectious blood meal (abdomen, 7 days pi). ⟶ BTV+ fat body cells; ► egg sheath; **E** egg cells; **C** cuticle (counterstained with haematoxylin)

commonly infected tissues. However virus replication in these tissues seemed to be at a lower level than in other infected organs/tissues. The infection rate of the cells of the gut including anterior and posterior midgut and foregut-midgut junction was higher than that of the disseminated infection rate, i.e., infection of midgut occurred in both individuals with and without a disseminated infection. The infection of the gut could be divided into two main types: 1) virus replication in gut cells ranging from very low to higher levels (Fig. 2.7) but virus spread throughout the cytoplasm. Sometimes there were also a few condensed virus-positive structures of different sizes found in the cytoplasm, which were probably VIBs. 2) virus restricted to endosome-like structures in the cytoplasm of gut cells, which were strongly stained, while the rest of the cytoplasm showed a negative reaction (Fig. 2.8). No association was found between the infection pattern in gut cells and the degree of dissemination of the virus throughout the insect.

Once virus was released from the midgut cells through the basement lamina, fat body cells in the haemocoel were invariably infected. Virus was first identified in the fat body by IHC at about 48 hr after an infective blood meal. In some individuals virus replicated at a high level in all of the fat bodies in the head, thorax and abdomen (Fig. 2.9, 2.10), while in other individuals virus replication was at much lower level. In a third group of insects only a few of the fat body cells in the abdomen showed a weak virus positive reaction, while all other tissues were virus negative (Fig. 2.11). The intensity of fat body infection was positively associated with the level of virus replication in other secondary target organs such as salivary glands and nerve ganglia.

Once dissemination had occurred, nerve ganglia seemed to be the most susceptible tissue to virus infection. Virus was detected in nerve ganglia in the thorax and head, including the antennae, in many individuals (Fig. 2.9, 2.10). The earliest infection of ganglia was detected by day 3 after an infective blood meal. It was found that the level of virus replication in ganglia was similar to that in the fat bodies but the fat body seemed to become infected about one day earlier than nerve ganglia. The infection of nerve ganglia in the abdomen has not been observed because these tissues were absent from the sections examined. Ommatidia of the compound eyes were also a frequently infected neural tissue (Fig. 2.9, 2.10).

The involvement of the salivary glands seemed to be associated with that of the ganglia and fat bodies. The stronger the virus positive reactions were in nerve ganglia and fat bodies, the more likely were the salivary glands to also be infected. The level of virus replication in the salivary glands also appeared to be positively related to that detected in the ganglia and fat bodies. However salivary glands usually became infected at the same time as the neural tissues while fat body was always infected at an earlier stage.

The egg sheath was non-specifically stained in the IHC test and a strong positive reaction was observed from both infected and negative control individuals. However oocytes/nurse cells were never seen to become infected with BTV in either IT inoculated or orally infected midges (Fig. 2.12).

2.4 DISCUSSION

Immunohistochemical techniques have been used previously in the study of vector insects infected with arboviruses (Leake and Johnson 1987; Romoser *et al* 1992), and in this context immunoperoxidase assays have a number of advantages. Firstly, sensitivity is high. Secondly the results can be examined repeatedly by light microscopy and the sections can be maintained permanently. Thirdly, good tissue/cell architecture can be obtained because the reaction conditions used during detection are mild.

The wax-embedding technique is commonly used in the study of arbovirus-insect interactions. However, compared with cryostat sectioning, the wax-based technique is time consuming and sometimes antigenicity can be decreased or lost during processing. Cryostat sectioning is ideal for IHC assays because the antigenicity of the virus and the structure of tissues are not damaged during processing. However, technically it is very difficult to produce insect sections with good structure because the hardness of the cuticle compared with other tissues frequently causes distortion. In this study the method of cryostat sectioning was modified to overcome inherent problems when sectioning insects. It was found that ideal sections of *Culicoides* can be obtained if the embedded insects are frozen at -70°C, instead of being quickly frozen by dipping in liquid nitrogen, and are cut at -24°± 1°C.

In this study, 100% of IT infected *C. variipennis* were shown to develop a fully disseminated BTV infection both by the IHC test and by viral titration; while the infection rate of orally infected midges was only 35.4%. Consequently, it is clear that the alimentary tract of *C. variipennis* presents the primary (major) barrier to virus infection, i.e. a mesenteronal infection barrier (MIB), which determines the susceptibility of *Culicoides* to BTV infection. Furthermore virus was shown to replicate to a range of levels in orally infected individuals (Fig. 2.7, 2.8, 2.9, 2.10, 2.11, 2.12) and the results by IHC showed that virus dissemination only occurred in a proportion of these individuals and the degree of dissemination also varied between individuals. It is therefore apparent that a mesenteronal escape barrier (MEB) also exists in some *C. variipennis*. These results support the hypotheses of gut barriers to arbovirus infection in biting midges based on previous studies of BTV-*Culicoides* and of arbovirus-mosquito interactions (P.S. Mellor, personal communication; Hardy *et al* 1983, 1988; Chandler *et al* 1985; Leake 1992; Romoser *et al* 1992; Kramer *et al* 1993). These earlier studies suggested that the MIB to arbovirus infection of insects operated at the level of the midgut and could be related to the presence or absence of specific viral receptors on the luminal surface of the midgut cells, or to the composition of digestive enzymes in the gut (Chamberlain and Sudia 1961; Hardy *et al* 1983; Leake 1992; Mellor 1990; Mertens *et al* 1993). The present results confirm the existence of a MIB in *Culicoides* midges but the nature of this barrier remains to be elucidated. Specific virus receptors may be involved in the mechanism, but further work is clearly necessary to confirm this supposition.

The results of the infection of BHK-21 and KC cells with IVP, ISVP and CP of BTV have shown that ISVPs initiate an infection earlier than IVPs in BHK-21 cells and earlier than either IVPs or CPs in KC cells (Fig. 2.1, 2.2). This may indicate a different route of entry of ISVPs into the cytoplasm. In addition, at a concentration equivalent to $10^{3.0}TCID_{50}$ of IVP/ml, CPs were unable to infect BHK-21 cells but the same particle concentration was able to infect KC cells and has a similar level of infectivity for them as does IVP. These results support the results of previous studies on mammalian cells (Mertens *et al* 1987) and on the oral infection of susceptible *Culicoides* species (Mertens *et al* 1993). These authors found that ISVPs had a similar infectivity for mammalian cells (BHK-21 cells) as do IVPs, whereas CPs had only a very limited infectivity. They also

found that all three types of BTV particle are orally infective to susceptible *Culicoides*, both IVPs and CPs showing similar levels of infectivity while ISVPs seemed to be 100-500 times more infectious than either IVPs or CPs. Therefore it is likely that the initiation of infection of BTV in mammalian cells is different from that in insect cells. Consequently specific virus receptors on the cell surfaces may also differ or it may be that entry of virus into insect cells involves an entirely different mechanism from those used to enter mammalian cells.

The KC cell line consists of a mixed population of cells from different tissues of *C. variipennis*. Only a certain proportion (about 50%) of these cells were shown to be susceptible to BTV infection in this study (Fig. 2.3). Similarly, some organs/tissues in intact *C. variipennis*, including the hindgut cells, muscles and Malpighian tubules, were also found to be insusceptible.

It was found that, after ingestion of an infectious blood meal by susceptible *Culicoides*, the gut epithelium, including anterior and posterior midgut and the foregut-midgut junction were the first cells to be infected, similar to the findings of Romoser *et al* (1992) and Leake (1992) when working with mosquitoes. In addition, the anterior midgut epithelium and the cells at the foregut-midgut junction of *C. variipennis* became infected as frequently and as early as the posterior midgut, and showed the same types of infection as did the posterior midgut. The high frequency of infection of the cells at the foregut-midgut junction is not surprising because all of the virus in an infective blood meal passes through a very narrow opening at this junction and the susceptible epithelium in this region has extensive exposure to the virus. Therefore, although the posterior midgut of *Culicoides* is considered the main route of infection for BTV, the anterior midgut and cardia, plus parts of the posterior foregut at the foregut-midgut junction, probably share this property. In other words, the MIB and MEB to BTV probably also exist in this part of the alimentary tract in addition to the posterior midgut. Also, considering the proximity of the foregut-midgut junction to the proboscis of the insect, if BTV were to be released back into the gut tract from the luminal surface of this region of the gut then this might supplement virus from salivary glands in being available for transmission to susceptible animals during biting. Furthermore, Webb (1990), working with *Stomoxys calcitrans*

(Linnaeus 1758), observed that, after being ingested with a blood meal, virus could be pushed back to the anterior part of the alimentary tract by regurgitation. Should this reflex also occur in *Culicoides* species then this would enhance the likelihood that virus released back into any part of the gut lumen would be available for transmission. In addition, the natural digestive enzymes present in the *Culicoides* gut would then partially digest the intact virus particles during their time in the gut lumen to produce more infective ISVPs and so further enhance the likelihood of gut infection.

The initiation of a *Culicoides* gut cell infection with BTV apparently occurs rapidly and infected cells were first observed 48 h after an infective blood meal. Two main types of gut infection were observed (Fig. 2.7, 2.8). It is possible that the first type of low level infection is merely an early stage of the higher level infection. However, both the low and high level types of gut infection occurred with or without a disseminated infection. Furthermore both types of gut infection were observed in different individuals at the same time after infection, and over periods extending to more than 10 days after an infective blood meal. Based on the gut infection patterns that have been observed so far in this study, the MEB seems to involve at least two factors: 1) the physical barrier effect of the basement lamina of the gut wall; 2) the endocytosis-like function of gut epithelia (Fig. 2.8), which could stop the spread of BTV to the haemocoel from gut cells. The detailed nature of these phenomena remains to be elucidated.

Like arbovirus infections of mosquitoes (Leake and Johnson 1987; Faran *et al* 1988; Romoser *et al* 1992), in the present study the dissemination of BTV in *C. variipennis* has been found to be a rapid process, subsequent to virus passing the gut barriers. The virus was first found by IHC to have passed through the gut cells and entered the fat body within 48 h pi. In a fully disseminated infection, once the fat body had been infected then other secondary target organs/tissues including ganglia, salivary glands and ommatidia of the compound eyes were also usually involved, and the greater the amount of virus in the fat body, the more that was present in the other tissues. This indicates that fat body, which is a relatively massive tissue in *Culicoides*, might be an important factor affecting the infection of other secondary organs/tissues beyond the gut. However, it is also possible that fat body might serve as a barrier to limit virus dissemination through the haemocoel.

In the present study it has been observed that on some occasions only a few sporadic fat body cells in the abdomen showed virus positive reactions and at relatively low level, while no detectable virus was found elsewhere beyond the gut (Fig. 2.11). This may represent a 'self-clearance function' of the *Culicoides* 'immune system', which could thereby play a major role as a barrier to BTV dissemination to the salivary glands and other secondary organs/tissues. The same phenomenon has not been observed in IT inoculated *Culicoides*. This may be because inoculation provides concentrations of virus at a level above the threshold at which the fat body could clear the system; while in some orally infected individuals virus is only released into the haemocoel at concentrations below this level. The infection of fat body has been described in other arbovirus-vector insect studies though its possible significance in modulating virus dissemination has not previously been suggested (Beaty and Thompson 1978; Kuberski 1979; Scott *et al* 1984; Leake and Johnson 1987; Romoser *et al* 1992; Weaver *et al* 1992).

The ganglia in the thorax and head are some of the most susceptible secondary tissues to BTV infection in *C. variipennis* and virus was first detected in the thoracic ganglia 3 days after an infective blood meal. The level of virus replication in the ganglia seemed coincident with that of the fat body and salivary glands but the significance of this relationship, if any, is not known.

Infection of the salivary glands is a prerequisite for biological transmission of arboviruses to vertebrate animals by vector insects. Previous studies have shown that infection of the salivary glands is a rapid process in some mosquitoes (Scott *et al* 1984; Romoser *et al* 1992). These workers showed that Rift Valley fever virus is found in the salivary glands of *Cx pipiens* (Linnaeus 1758) as early as 48 h after an infective blood meal. In the present study, detectable BTV infection of the salivary glands of *C. variipennis* was first found on day 5 after an infective blood meal. The infection of salivary glands seems to be associated with the infection of nerve ganglia and the fat body. The replication level of BTV in salivary glands was similar to that in neural tissues and the fat body (as indicated by the extent and intensity of virus specific staining using IHC). Studies of arbovirus-mosquito interactions have suggested that in IT inoculated specimens neural cells become infected before the salivary glands and that the salivary glands subsequently

become infected via the nerve trunks from infected cerebral ganglia (Miles *et at* 1973; Leake and Johnson 1987). In addition, the latter authors indicated that fat body may also become infected before the salivary glands. Since the salivary glands are surrounded by fat body cells, it is possible that salivary gland infection could originate from these surrounding fat body cells. On the other hand, as mentioned earlier, the 'self-clearance' phenomenon postulated for some orally infected *Culicoides* in the present work, implies that the fat body might act as a barrier to the infection of salivary glands with BTV. The mechanisms involved in this are unknown and may possibly be a part of the immune response of *Culicoides* to BTV infection. No evidence has been obtained from this study to describe the initiation of infection of the salivary glands because of the low infection rate.

Vertical transmission of arboviruses in mosquitoes and sandflies has been reported and has been demonstrated experimentally and in nature for several vector-virus combinations (Beaty *et al* 1980; Leake and Johnson 1987; Leake 1992; Turell *et al* 1982; Turell 1984; Comer *et al* 1990; Bosio *et al* 1992; Baquar *et al* 1993; Kramer *et al* 1993; Fulhorst *et al* 1994). However other studies dealing with *Culicoides*-borne viruses have indicated that vertical transmission does not appear to occur with BTV in *C. variipennis*, and BTV has not been detected in progeny *C. variipennis* of parental midges infected with the virus (Jones and Foster 1971b; Nunamaker *et al* 1990; P.S. Mellor, personal communication). In the present study, oocytes/nurse cells of susceptible *Culicoides* were never found to become infected with BTV in either IT inoculated or orally infected midges, even in individuals with fully disseminated infections (Fig. 2.12). This supports the assertion that vertical transmission of BTV does not occur in *C. variipennis* and suggests the presence of a transovarial transmission barrier which prevents access of the virus to the egg cells.

SEP*: Standard error of proportion. With a large number of samples, the distribution of the proportion would be approximately normal and centred around the true proportion (the proportion of the population). SEP which can be calculated from a single proportion is used to show how close the observed proportion is to the true proportion. Generally speaking, the bigger the sample size and the smaller SEP, the more certain we can be about that the observed proportion is close to the true proportion.

CHAPTER 3
QUANTIFICATION OF BLUETONGUE VIRUS RELEASE FROM THE SALIVARY GLANDS OF *C. VARIIPENNIS*

3.1 INTRODUCTION

Salivary gland barriers, including the salivary gland infection barrier (SGIB) and the salivary gland escape barrier (SGEB), are believed to be the final barriers to arbovirus transmission by vector insects (Jones and Foster 1966; Hardy *et al* 1983; Hardy 1988; Mellor 1990; Leake 1992). However, the nature of these barriers in mosquitoes is unknown whilst even their very existence in *Culicoides* has not been proven.

This study compared the infection and transmission rates of BTV in intrathoracically (IT) inoculated, and orally infected midges of a susceptible and a refractory colony of *C. variipennis* using virus titration and *in vitro* transmission assays. The purpose of this study was to investigate the role of the salivary glands of the midge as a barrier to the transmission of BTV.

3.2 MATERIALS AND METHODS

3.2.1 Virus and tissue culture cells

BTV 1SA and BHK-21 cells used in this experiment were as described in Chapter 2.

BHK cells were used to titrate BTV in infected *C. variipennis*, in saliva secreted by infected midges and in the blood samples fed upon by infected midges. The cells were grown at 37^{0}C in modified Eagle's medium containing 5% normal foetal calf serum, 2 mM glutamine and antibiotics (100 IU/ml penicillin and 0.1 mg/ml streptomycin) and were maintained in Eagle's medium with 2% normal foetal calf serum, 2 mM glutamine and antibiotics.

3.2.2 Infection of *C. variipennis* with BTV 1SA

Both the BTV susceptible and refractory colonies of *C. variipennis* used in this study were maintained in the insectaries of Pirbright Laboratory, Institute for Animal Health, UK.The susceptible colony was originally imported from the USA in 1967 (see Chapter 2). The refractory colony of *C. variipennis* was derived by selection from the susceptible colony and has been shown to be unable to transmit BTV to susceptible host animals (P.S. Mellor personal communication). Adult female midges, 2-3 days old, were infected with BTV 1SA by IT inoculation or by feeding upon viraemic blood and were maintained at 24±1^{0}C provided with 10% sucrose as described in Chapter 2. The infected individuals were collected for *in vitro* transmission tests (see below) by using micro-membrane feeding and salivation tests from day 3 on, after infection. Uninfected female midges inoculated with Eagle's medium or fed with a similar blood meal but without BTV were used as negative controls.

3.2.3 *In vitro* transmission

3.2.3.1 Micro-membrane feeding test

Sterilised microtubes with parafilm bottoms were used in the feeding test. Each tube was filled with 0.1 ml of a mixture of warm (37^0-40^{0}C) sheep blood plus Eagle's medium in a ratio of 1:5 respectively and was placed on the top of a waxed pill box cage containing a single midge. Feeding and engorgement were then observed over a period of 10-30 minutes. Engorged individuals only, were collected and were stored individually at -70^{0}C in Eppendorf tubes until assayed for BTV 1SA. The blood samples fed upon by the midges were also collected subsequent to feeding. Each sample was added to 0.9 ml of Eagle's medium and was kept at -70^{0}C until assayed. Both midges and the relevant blood meal samples were labelled individually to ensure that each midge was linked to its own blood meal sample.

3.2.3.2 Salivation test

Induction of salivation was carried out following a method used previously (Boorman 1987). A garden insecticide, malathion (Murphy Chemical Co., Wheathamstead, Hertforshire), was used to initiate salivation. The midges were anaesthetized with carbon dioxide and were then stuck ventral-side up on a petri dish with double-sided sticky tape. The salivation test was carried out under a dissecting microscope. A glass capillary tube was used to apply approximately 0.5 µl of malathion solution (0.01% in absolute acetone) topically onto the abdomen of each fly. One to two minutes after the application of the malathion, globules of saliva were observed and saliva samples were collected from individual midges via capillary action from the distal end of the proboscis by using extra fine glass capillary tubes (Fig 3.1). The part of each capillary containing the saliva was then broken off, put into 1 ml Eagle's medium and stored at -70°C until assayed for BTV 1SA later. The midges were also collected individually and stored at -70°C in Eppendorf tubes for virus detection. The midges and their saliva samples were labelled as described above.

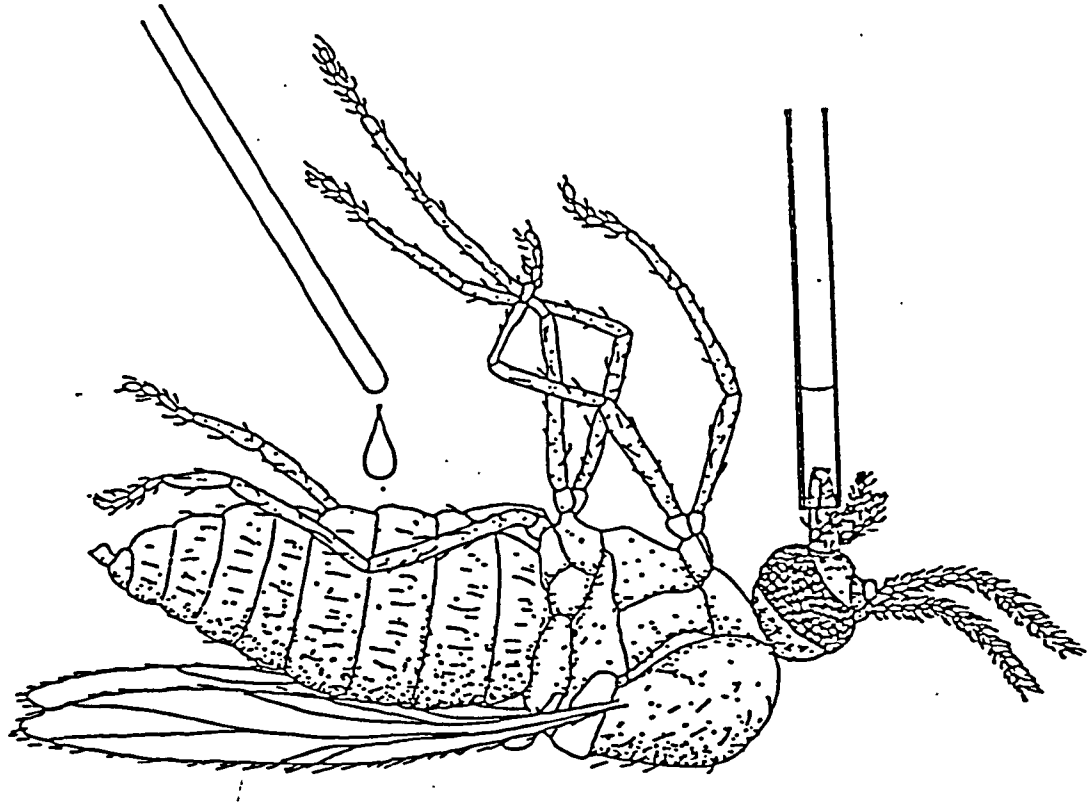

Fig. 3.1 Salivation test on C. variipennis

3.2.4 Titration of BTV

For titration of BTV, midges were ground individually in Eppendorf tubes using a motor-driven plastic pestle and each was then suspended in 1.0 ml of Eagle's medium. The saliva samples, the blood meal samples and the suspensions of the midges were each diluted tenfold serially, with Eagle's medium. The samples were then inoculated on to 96-well microplates with confluent monolayers of BHK-21 cell cultures. The inoculated plates were incubated at 37^{0}C for 3-4 days in maintenance medium (using 10 replicates for the saliva and blood meal samples and 4 replicates for the midges, 0.1 ml/well). The development of cytopathic effect (CPE) was used as a positive indicator of the presence of the virus. The titre of virus, 50% tissue culture infection dose ($TCID_{50}$) per ml, was determined using the method of Karber (quoted by Whitaker 1972, see Chapter 2).

Statistical analyses, including calculation of mean, standard error of mean (SEM), standard error of proportion (SEP, see Page 50), t-test of means and χ^2-test were carried out using conventional methods (Rowntree 1991; Clarke and Cooke 1992).

3.3 RESULTS

3.3.1 Experimental infection rate

Both infection rates and transmission rates were calculated, based on the data collected at day 14 pi.

The infection rate was 100% in both IT inoculated susceptible (52/52) and refractory (27/27) *C. variipennis* (Table 3.1). However, only 35.4% (270/763, SEP=0.017, i.e. SEP=1.7%) of susceptible midges and 31.3% (62/198, SEP=0.033, i.e. SEP=3.3%) of refractory midges, orally ingesting virus, were persistently infected. These two proportion do not significantly differ [χ^2=1.154, degrees of freedom(df)=1, P>0.05] (Table 3.1). No BTV replication was observed in any negative control midges.

Table 3.1 Comparison of BTV infection rates and transmission rates of susceptible and refractory colonies of *C. variipennis*

The routes of infection	*C. variipennis*	Infection rate (%)	Transmission rate (%)
IT inoculation	Susceptible colony	100 (52/52)*	100(52/52)***
	Refractory colony	100 (27/27)*	100(27/27)***
Oral infection	Susceptible colony	35.4 (270/763)**	3.8 (4/105)***
	Refractory colony	31.3 (62/198)**	0 (0/62)***

* No. of persistently infected midges/No. of IT inoculated midges with BTV
** No of persistently infected midges/No. of midges fed with infectious blood meal
*** No. of midges transmitting BTV through saliva/No. of midges tested

3.3.2 *In vitro* transmission

Virus was detected in 100% of the saliva samples taken with capillary tubes from IT inoculated individuals, comprising 52 midges from the susceptible colony and 27 midges from the refractory colony (Table 3.1). However, only 35.4% of susceptible midges and 31.3% of refractory insects were persistently infected after ingestion of an infectious blood meal (Table 3.1). Virus was detected in 3.8% (4/105) of samples of saliva from the orally infected susceptible midges, of which 33 were known to be persistently infected, i.e., 12.1% (4/33) of known orally infected individuals released BTV through their saliva. No saliva samples from the orally infected refractory insects (0/62) (Table 3.1) and the negative controls were shown to be BTV positive. These proportions (4/105 and 0/62) are significantly different (χ^2= 7.845, df=1, P<0.01).

Only midges of the susceptible strain were tested for transmission by using the micro-membrane feeding method. Slightly lower transmission rates were observed using this test than using the salivation method. BTV was detected from 92.3% (24/26) and 10.8% (7/65) of the blood meal samples fed upon by IT inoculated midges and virus positive orally infected midges, respectively. These levels are significantly different (χ^2=54.97, df=1, P<0.01).

The titre of virus detected in saliva and blood meal samples from individual flies varied significantly. Individual BTV positive saliva samples collected by the salivation test contained virus ranging from 1.78-7.97 $TCID_{50}$, (mean±SEM=3.27±0.03, n=69) while single midges released 0.32-1.78 $TCID_{50}$ of BTV (mean±SEM=0.85±0.09, n=37) into a blood meal during engorgement. The earliest time of transmission, measured by both salivation and micro-membrane feeding test, occurred at 4 and 7 days pi, respectively in IT inoculated (10/13) and orally infected midges (2/68) maintained at 24±1^{0}C.

The results also showed a threshold relationship between the replication of BTV in *C. variipennis* and the ability to transmit the virus through saliva. Only those infected midges, containing BTV at a level of $10^{3.0}TCID_{50}$ per midge or higher, were able to transmit virus via saliva (13/144). All infected midges, containing less than $10^{3.0}TCID_{50}$ of BTV, consistently failed to transmit detectable virus via their saliva (131/144). It should be pointed out that the limit of the detection method used (BHK-21 cells) was 0.32 $TCID_{50}$/sample of BTV.

3.4 DISCUSSION

This study showed that all *C. variipennis*, whether from susceptible or refractory strains, when infected with BTV by IT inoculation, were able to support virus replication and were able to transmit virus in their saliva. However, orally infected susceptible and refractory colonies of *C. variipennis* both had much lower infection rates (35.4% and 31.3% respectively). Only 12.1% of orally infected susceptible midges transmitted the virus while none of the orally infected refractory ones released the virus through their saliva. This study also showed a threshold relationship between the replication level of BTV in the midge and ability to transmit the virus through saliva. Individuals with BTV replicating to a level lower than $10^{3.0}TCID_{50}$ per midge were unable to transmit the virus. A similar result was also obtained in a previous study (P.S. Mellor and M. Jennings, personal communication). These results suggest that the salivary glands of *C. variipennis* are inevitably infected with BTV if the virus succeeds in gaining entry to the haemocoel and replicates above a certain level (ie. $\geq 10^{3.0}TCID_{50}$/midge). Furthermore, midges are always able to transmit BTV through their saliva as long as their salivary glands are

infected. These data therefore support the contention that in *C. variipennis* the salivary glands themselves present neither a SGIB nor a SGEB to BTV. These results also confirm that the main barriers to the oral transmission of BTV by *C. variipennis* exist at the level of the gut of the insect.

However, in the present study it has also been observed that in some orally infected midges small amounts of BTV are sometimes 'trapped' in fat body cells after release of the virus from the gut into the haemocoel (see Chapter 2). This 'trapping' prevents further virus dissemination in these individuals. The same phenomenon of 'virus trapping' by fat body cells did not occur in IT inoculated midges probably because in this study IT inoculation always resulted in relatively large amounts of the virus being introduced directly into the haemocoel. It may be therefore that in circumstances when only small amounts of virus enter the haemocoel, the fat body cells can operate as a 'self-clearing mechanism' acting as a part of a *Culicoides* 'immune system'. This may prevent infection of the salivary glands and thereby preclude transmission. If virus enters the haemocoel at a level above the threshold at which the 'immune system' is able to inactivate it, then the barrier is 'swamped', the fat body is heavily infected and infection of the salivary glands proceeds normally and transmission can occur. In this respect, it is known that mosquitoes have efficient cellular and humoral 'immune systems' which defend the insect against bacteria and parasites (Lackie 1988, Hultmark 1993). However there is only very limited information on antiviral activity in insects infected with arboviruses (Chadwick and Dunphy 1986). Although the existence of SGIBs and SGEBs to viruses has been confirmed by studies on the interaction of arboviruses and mosquitoes, the essential nature of these barriers remains unknown (Hardy *et al* 1983; Leake 1992). According to these authors, the SGIB of mosquitoes seems to operate at some point(s) before infection of the salivary glands, though not necessarily at the surface of the salivary glands themselves but subsequent to virus release from the gut. If this is the case then this barrier might more properly be termed a dissemination barrier rather than a SGIB. Nevertheless, the threshold relationship between the level of viral replication in the haemocoel and the likelihood of infection of the salivary glands, as described here for *Culicoides*, also occurs in mosquitoes (Hardy *et al* 1983; Leake 1992).

In the present study, the transmission of BTV by *C. variipennis* occurred as early as 4 days pi in IT inoculated midges but not until 7 days pi in orally infected ones. These times are earlier than the results shown in previous studies (Jones and Foster 1966; P.S. Mellor, personal communication). Such detailed and reliable data are essential for accurate epizootiological studies, modelling and prediction of disease spread.

The amount of the virus transmitted in the saliva of *C. variipennis* per bite was very low, 1.78-7.97 $TCID_{50}$ by the salivation test and 0.32-1.78 $TCID_{50}$ when feeding through a membrane. However, earlier studies have shown that despite this, the bite of a single *C. variipennis* is sufficient to cause infection and disease in susceptible ruminant animals (Foster *et al* 1968).

Malathion is an organic ester of thiophosphoric acid and a powerful acetylcholinesterase inhibitor. Inhibition of acetylcholinesterase causes accumulation of acetylcholine, a neurotransmitter at cholinergic synapses in central, sympathetic and especially parasympathetic nervous systems. The accumulation of acetylcholine at the synaptic junction of the parasympathetic nervous system cause a series of strong physical reactions including oversecretion of the glands in the head, neck and thorax (Blood and Studdert 1988). The salivation test used in this study is an efficient and sensitive method for detecting the presence and the quantity of BTV in the saliva of *C. variipennis*. With care using the technique described in this chapter, 100% of midges could be encouraged to salivate. Therefore, it is an ideal technique to study the transmission of BTV and probably other arboviruses by vector *Culicoides*. The microtube feeding method is also a useful technique. Compared with the salivation test, this method was less sensitive but it does reflect a more natural way of assessing virus transmission by biting and may provide a more reliable estimate of the amount of virus actually transmitted through saliva when biting in the field. The reduced amounts of virus detected per bite by the microtube feeding method as compared to the salivation test is probably related to the smaller volumes of saliva that midges secrete when feeding naturally, rather than when stressed by the application of such pharmacologically active compounds as malathion.

In conclusion, this study has showed that the salivary glands of *C. variipennis* do not present SGIBs and SGEBs to BTV. However the study has also shown that factors in the haemocoel of *C. variipennis* operate as part of an 'immune system' and may play an important role in limiting dissemination of virus to the salivary glands. The nature of this 'immune' mechanism remain to be elucidated.

CHAPTER 4
ULTRASTRUCTURAL STUDIES ON MAMMALIAN CELLS (BHK-21), *CULICOIDES* CELLS (KC), THE GUT, NERVE TISSUE AND THE SALIVARY GLANDS OF *C. VARIIPENNIS* INFECTED WITH BLUETONGUE VIRUS

4.1 INTRODUCTION

Transmission electron microscopy (TEM) was used in this study to investigate ultrastructural aspects of BTV 1SA infection of BHK-21 cells, KC cells, the gut, nervous tissue and the salivary glands of *C. variipennis*. The objectives were to compare BTV infection in mammalian and *Culicoides* cells, and to elucidate those factors in the gut, nerve tissue and the salivary glands of *Culicoides,* which may affect dissemination or transmission of the virus by the midges.

4.2 MATERIALS AND METHODS

4.2.1 Virus, tissue culture and medium

BTV 1SA, BHK-21 cells, KC cells and the media used in this study are as described in Chapter 2. The cells were grown in 175 cm^2 plastic flasks. When the monolayers were almost (about 70%) confluent, the medium was discarded, the cells were rinsed three times with PBS and each flask was infected with 2 ml of virus suspension containing 10^{2-3} $TCID_{50}$/ml BTV 1SA mixed with 8 ml of fresh Eagle's medium (BHK-21 cells) or Schneider's media (KC cells). The cells were incubated at 37^0C (BHK-21 cells) or at room temperature (RT)(KC cells) for 30 minutes. The medium was then removed and replaced by 50 ml Eagle's maintenance medium containing 2% foetal calf serum (FCS) (BHK-21 cells) or Schneider's medium with 5% FCS (KC cells). The cells were then incubated at 37^0C (BHK-21 cells) or at RT (KC cells). The cells were rinsed in PBS (pH 7.3) when a cytopathic effect (CPE) occurred (BHK-21 cells) or at various times as required (BHK-21 and KC cells).

4.2.2 Antibodies

For immunogold EM, the following antibodies and immunogold conjugates were used: a polyclonal antibody against BTV 1SA (PAbV), and a monoclonal antibody against the core protein VP7 of the virus (MAbA3) [provided by Dr. J. Anderson and Mrs. J. Thevasagayam of the IAH Pirbright Laboratory (Anderson 1984)]; two polyclonal antibodies against BTV 1SA non-structural protein NS3 polypeptide 10C (amino acids 37-46 of NS3) (PAb10C), and a monoclonal antibody against NS1 (MAbNS1) (provided by Dr. A Wade-Evans also of the Pirbright laboratory). Gold-labelled goat antibodies against rabbit (GAR) and against mouse immunoglobulin (GAM) were obtained from British BioCell International Ltd (BioCell).

4.2.3 Infection of *C. variipennis* and preparation of gut and salivary gland samples

Midges were infected with BTV 1SA by intrathoracic (IT) inoculation or orally, and then incubated at 22±2^{0}C as previously described (see Chapter 2). The infected midges were dissected at 7 days (IT inoculated) or 10 days postinfection (pi) (orally infected). Dissection was carried out in cold PBS under a dissecting microscope. The salivary glands of IT inoculated midges and the guts (whole midgut and foregut-midgut junction) of orally infected insects were dissected out. The salivary glands were obtained by pulling off the heads of the midges. Both salivary glands (connected to the heads) and gut samples were transferred into cold 3% glutaraldehyde in cacodylate (0.1M) immediately after being dissected. Two pieces of thoracic ganglia tissue were obtained when dissecting the salivary glands.

4.2.4 Pre-embedding immunogold labelling of the cell cultures infected with BTV 1SA

For pre-embedding immunogold staining, infected BHK-21 cells showing CPE and KC cells at 24 hours pi, were washed at least three times with PBS and then incubated in blocking buffer (5% normal adult bovine serum in PBS, pH 7.3) at RT for 30 minutes. After incubation with one of the specific antibodies diluted in PBS (MAbA3 1:20; PAbV

and PAb10C 1:1,000) at RT for 2 hours, the cells were washed with PBS at least 3 times at RT and were then incubated for 30 minutes at RT in different immunogold conjugates diluted 1:30 in PBS. After being extensively washed with PBS, the cells were fixed in 3% glutaraldehyde in cacodylate (0.1M).

4.2.5 Transmission electron microscopy

Transmission electron microscopy was carried out by following conventional methods (Glauert 1975; Bancroft 1990).

Cells for routine examination and cells that had been immunogold labelled pre-embedding, were fixed *in situ* in 10 ml cold (4^0C) 3% glutaraldehyde in cacodylate buffer (0.1M, pH 7.4) for at least 2 hours at 4^0C. After fixation, the cells were detached from the flasks by gently scraping with a policeman and decanted into 15 ml centrifuge tubes and were spun at 3000 rpm. Spun cells were then washed in cacodylate buffer (0.1M, pH 7.4) twice, 30 minutes each. The cells were either stored in buffer at 4^0C until required or were post fixed immediately in 1% osmium tetroxide in cacodylate buffer (0.1M, pH 7.4) for an hour. After postfixation, the cells were washed in distilled water 5 times over a period of 1 hour before being further fixed in 1% uranyl acetate (UA) (This step was omitted for cells that had been immunogold labelled as UA enhances membrane contrast and makes it difficult to locate the gold particles). Cells were then rinsed in distilled water and were centrifuged at 4,000 g. The cell pellets were embedded in 1% agar. When the agar had solidified, the embedded cell pellets were sliced to 1 mm^3 and further processed as described below in glass Bijou bottles with constant agitation. The salivary gland, nerve tissue and gut samples were also fixed as described above.

The samples were dehydrated through a graded series of methanol at RT for 15 minutes each in 25%, 50% and 75% methanol, followed by 2 changes of absolute methanol, 30 minutes each. Methanol was replaced with propylene oxide for 2 intermediate washes of 15 minutes each. The samples were then infiltrated with Araldite resin (TAAB Reading) for at least 1 hour with continuous agitation at RT and then further infiltrated with fresh resin for 30 minutes at 60^0C. Several pieces of gut or salivary gland or a single block of

cells were then placed into individual BEEM capsules containing fresh resin and were polymerised at 60^{0}C for 48 hours.

Cells for post-embedding immunogold labelling were treated differently since chemical fixation adversely affects antigenicity, in particular the use of glutaraldehyde and osmium tetroxide. However, to preserve an element of ultrastructural integrity of the cells, it is necessary to incorporate some glutaraldehyde into the fixation schedule. To establish an acceptable compromise between preservation of tissue structure and preservation of antigenicity, fixatives containing both glutaraldehyde and paraformaldehyde were used. Different concentrations of fixatives were tried for different lengths of time.

Cells were fixed in a mixture of either 2% paraformaldehyde and 0.5% glutaraldehyde in phosphate buffer (0.1M, pH 7.4)) or 2% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer (0.1M, pH 7.4) for either 10 minutes, 20 minutes or 2 hours. The cells were then washed twice in phosphate buffer (0.1M, pH 7.4), 1 hour each wash. Occasionally the cells were washed overnight (it is important to remove all traces of glutaraldehyde to avoid non-specific staining). The cells were dehydrated through a series of graded ethanol as described above when using methanol. Pelleted cells were then transferred directly from absolute ethanol into London Resin White Resin (LR White) and kept overnight at 4^{0}C with continuous agitation. The samples were transferred to fresh resin in gelatin capsules and polymerised at 60^{0}C for 22 hours.

Ultrathin sections of all the embedded blocks were cut at a thickness of approximately 80 nm on a Reichert Ultracut E ultramicrotome. Sections were collected on nickel grids with a 300 mesh (3.05 mm in diameter). The mounted sections for routine examination were stained with 2% UA in 50% ethanol for 10 minutes followed by freshly prepared lead citrate solution (0.04g lead citrate dissolved in 1 ml 1N NaOH and made up to 10 ml with distilled water) for a further 10 minutes. The sections that had been embedded in LR White resin were stained after immunogold labelling with 1% UA for 1 minute followed by the lead citrate solution also for 1 minute. All grids were washed with 30% ethanol and then with fresh distilled water, and were air dried at RT. The specimens were viewed under a Phillips 300 transmission electron microscope at an accelerating voltage of 80 kV.

4.2.6 Immunogold labelling of ultrathin sections

The whole process was carried out at RT (Bancroft 1990). Firstly, the sections were blocked in 5% normal goat serum in 0.1M phosphate buffer (pH 7.4) for 15 minutes before being incubated with the antibodies detailed above (undiluted) respectively for 2 hours. After being jet washed with phosphate buffer for 1 minute, the sections were then labelled with the relevant immunogold conjugates, either GAR or GAM (1:30 in phosphate buffer) for 15 minutes. Finally they were jet washed with phosphate buffer for 1 minute, rinsed with distilled water and then air dried. The sections were stained with 1% uranyl acetate (in distilled water) and lead citrate as mentioned above before being viewed under a Phillips 300 TEM.

4.3 RESULTS

4.3.1 Characteristics of BTV replication in BHK-21 cells

In infected BHK-21 cells, virus inclusion body (VIB) matrices were observed in the cytoplasm at 6-24 hours pi (Fig.4.1 and 4.2). VIBs, without limiting membranes, were randomly distributed in the cytoplasm and their sizes varied from 500x340 nm to 600x480 nm (length x width). VIBs contained numerous newly synthesised nascent core particles and nascent virus-like particles, while double-shelled virus particles were only seen in the cytoplasm beyond the VIBs. The diameter of nascent core particles within the VIB matrices ranged from 27 to 39 nm (mean±SEM=35.8±0.38, n=98) and the nascent virus-like particles at the periphery of VIBs were from 46 to 60 nm in diameter (mean±SEM=57.2±0.33, n=117). The more mature the virus particles were, the further they were away from the middle of VIB matrix. The sizes of the double-shelled virus particles in the cytoplasm were from 62 to 67 nm in diameter (mean±SEM=65.6±0.14, n=115).

Two types of virus release, involving only double-shelled, mature virus particles, were observed from BTV infected cells. These particles were released either by membrane budding or by extrusion from the cell surface (Fig. 4.3, 4.4, 4.5 and 4.6). Viruses released

via budding were observed to acquire an 'envelope' from the cell membrane. Virus particles exiting through the cell membrane did not appear to acquire an envelope. However these virus particles were surrounded by cell surface debris after being released. The cell membrane was not obviously damaged after virus release (Fig 4.3 and 4.5). Virus particles were released either individually or in aggregates by both budding and extrusion.

The formation of large amounts of microtubules, from 58 to 65 nm in width, in the cytoplasm, was a characteristic phenomenon of the infected BHK-21 cells (Fig. 4.2, 4.4, and 4.7). These tubules were noticeably co-located with mature virus particles. Double-shelled virus particles were observed lining up on the tubules and the distances between the particles was remarkably regular (Fig 4.2 and 4.7). Furthermore, the width of the

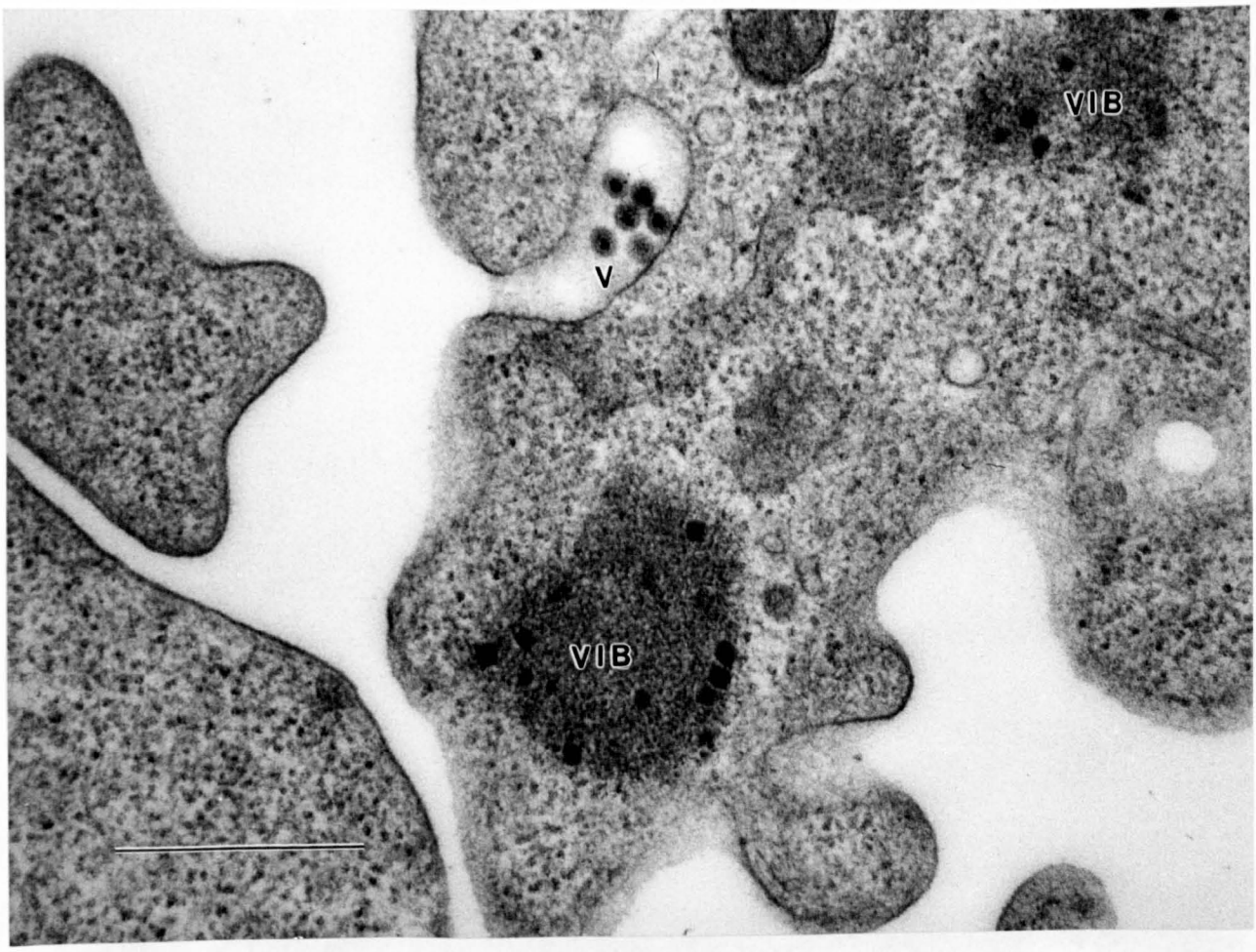

Fig. 4.1 Electron micrograph of BTV infection of BHK-21 cells (20 hours pi). **VIB**: virus inclusion body; V: progeny virus particles; bar = 500 nm

tubules was similar to the diameter of the mature virus particles. The tubules also seemed associated with the VIBs and usually occurred in their vicinity.

Reinfection of BHK-21 cells was also seen and uptake of progeny virus particles through endocytosis was observed by infected cells (Fig.4.1). The virus particles reinfecting cells were double-shelled and the cell surfaces in the reinfection sites were clean and smooth, with no cell debris around them; the particles reinfecting cells were therefore easily distinguishable from virus being releasing from the cell surface. Endosomes containing virus-like particles and vesicles containing uncoated virus particles were also seen in the cytoplasm, near to the surface of the infected cells (Fig. 4.8). Many vesicles formed near the cell surfaces (Fig. 4.8).

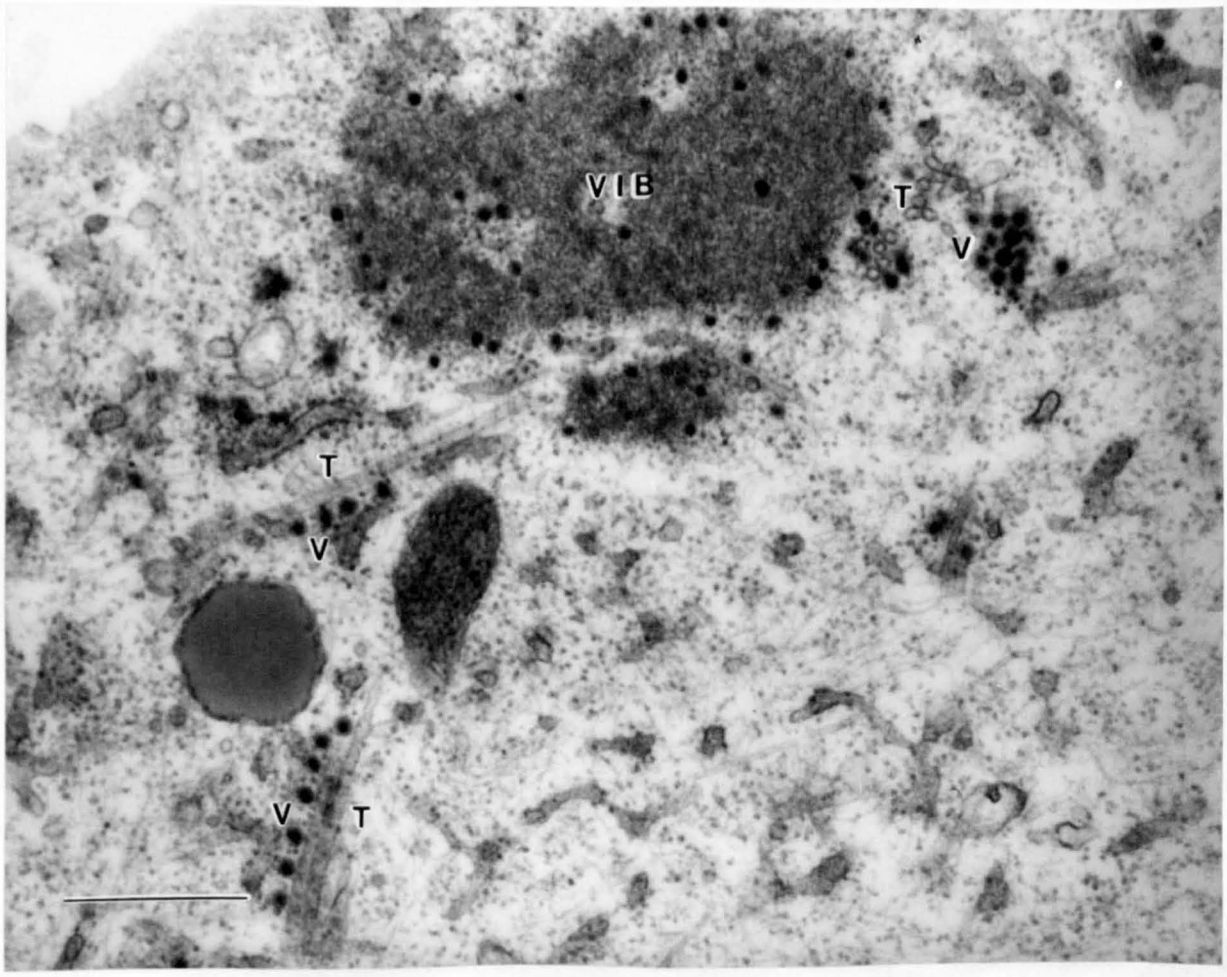

Fig. 4.2 Electron micrograph of BTV infection of BHK-21 cells (20 hours pi). **VIB**: virus inclusion body; **V**: progeny virus particles; **T**: tubules; bar = 500 nm

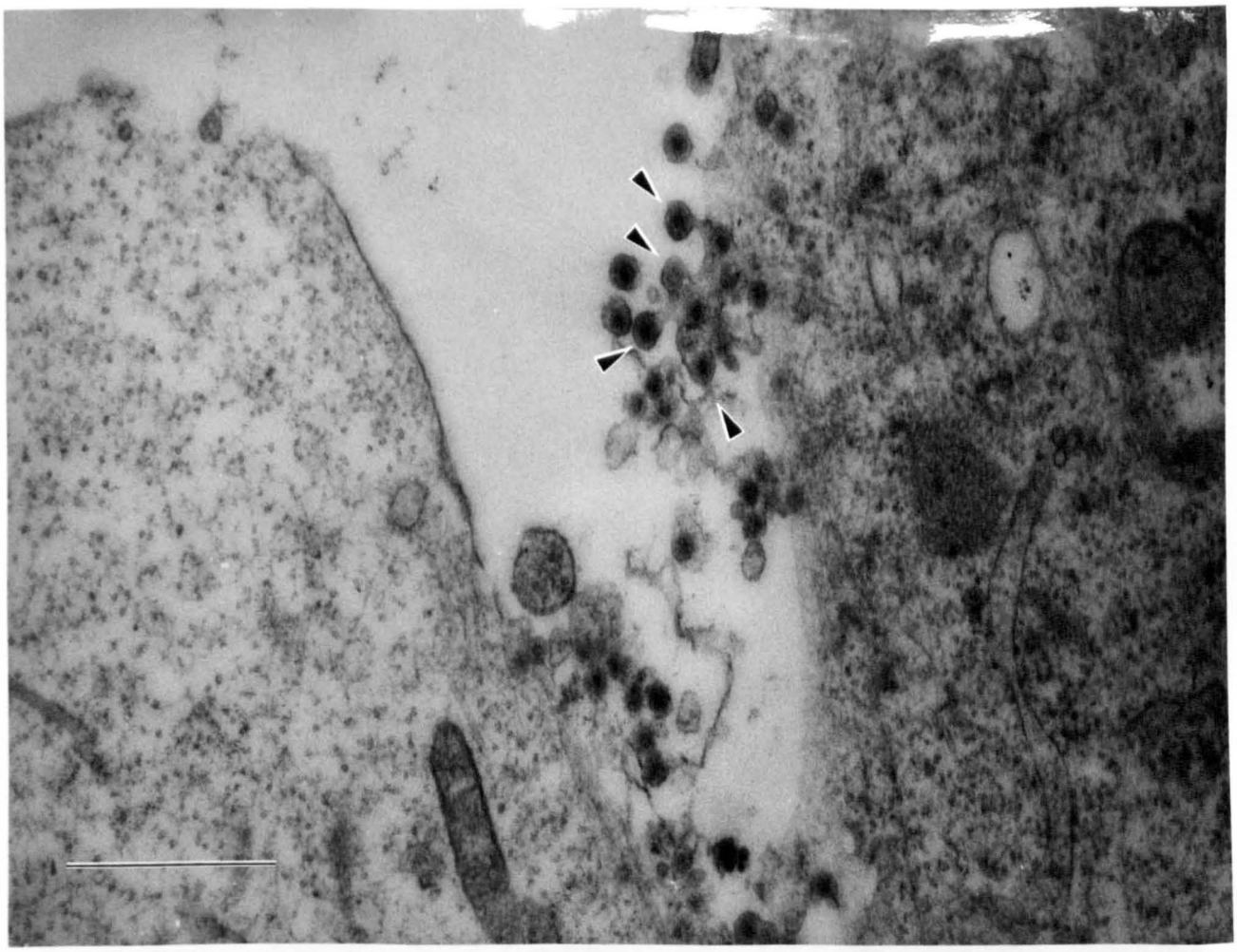

Fig. 4.3 Electron micrograph of BTV release by 'budding' from an infected BHK-21 cell (20 hours pi). arrows: release of BTV particles associated with cell plasma membrane; bar=500 nm

4.3.2 BTV replication in KC cells

KC cells infected with BTV were observed using TEM at 6, 20, 48 hours and 21 days pi. The characteristics of BTV replication in KC cells were obviously different from those in BHK-21 cells and are summarized in Table 4.1. VIBs were only observed in the cytoplasm of 13.5% (27/200) of KC cells in persistently infected cultures unlike BHK-21 cell cultures where approximately 70% of cells showed these structures 20 hours pi. Furthermore when VIBs were seen in KC cells, nascent core particles and nascent virus-like particles were absent from 41% (11/27) of them (Fig 4.9a). Mature virus particles were seen infrequently in infected KC cell cultures. Only 8% (16/200) of KC cells in infected cultures possessed VIBs containing nascent core particles and virus-like particles (59%, 16/27) and these were at a much lower density than those in infected BHK-21 cells

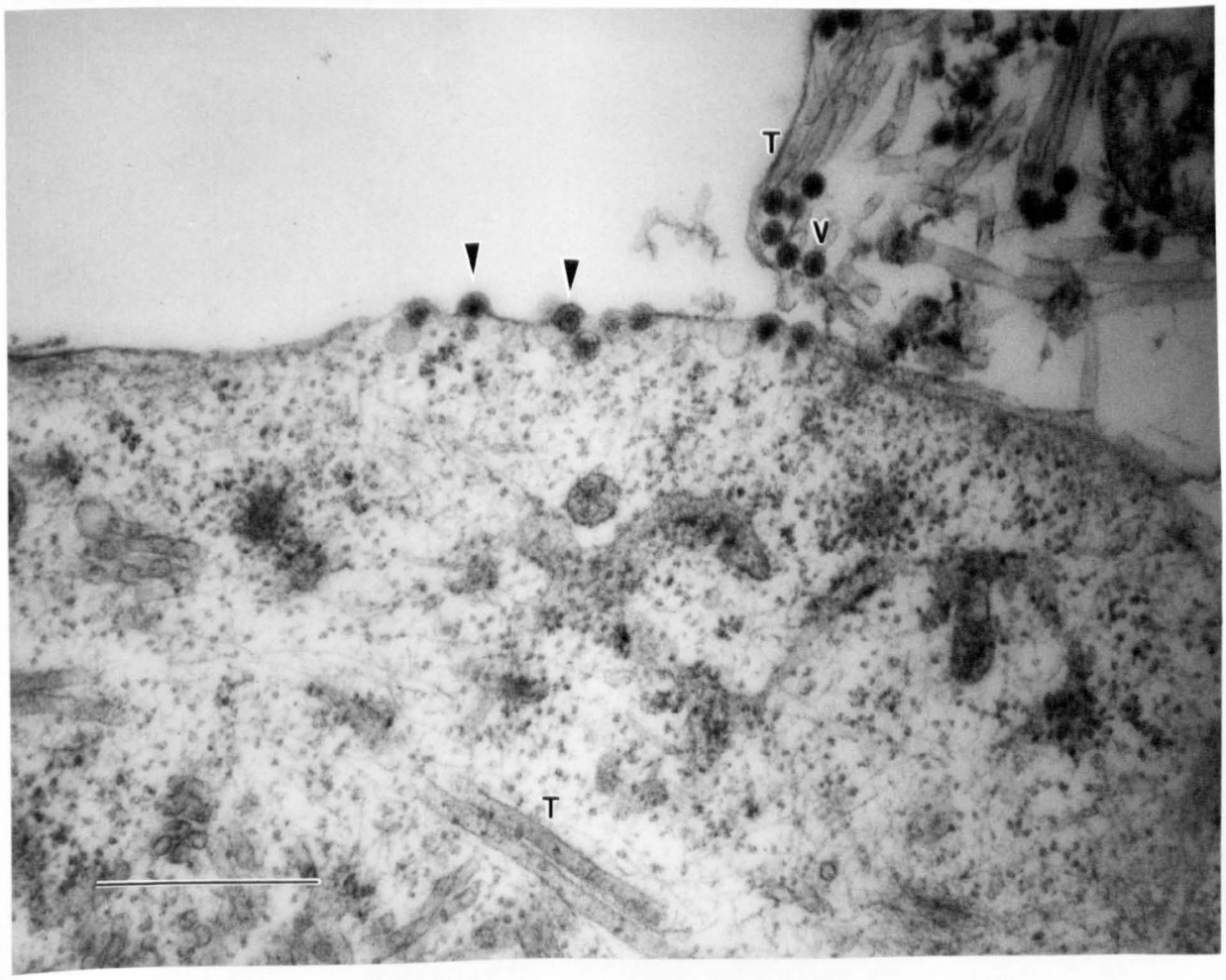

Fig. 4.4 Release of BTV from BHK-21 cells by 'budding' (20 hours pi). arrow: virus 'budding' from cell surface; **V**: double-shelled virus particles; **T**: tubules; bar=500 nm

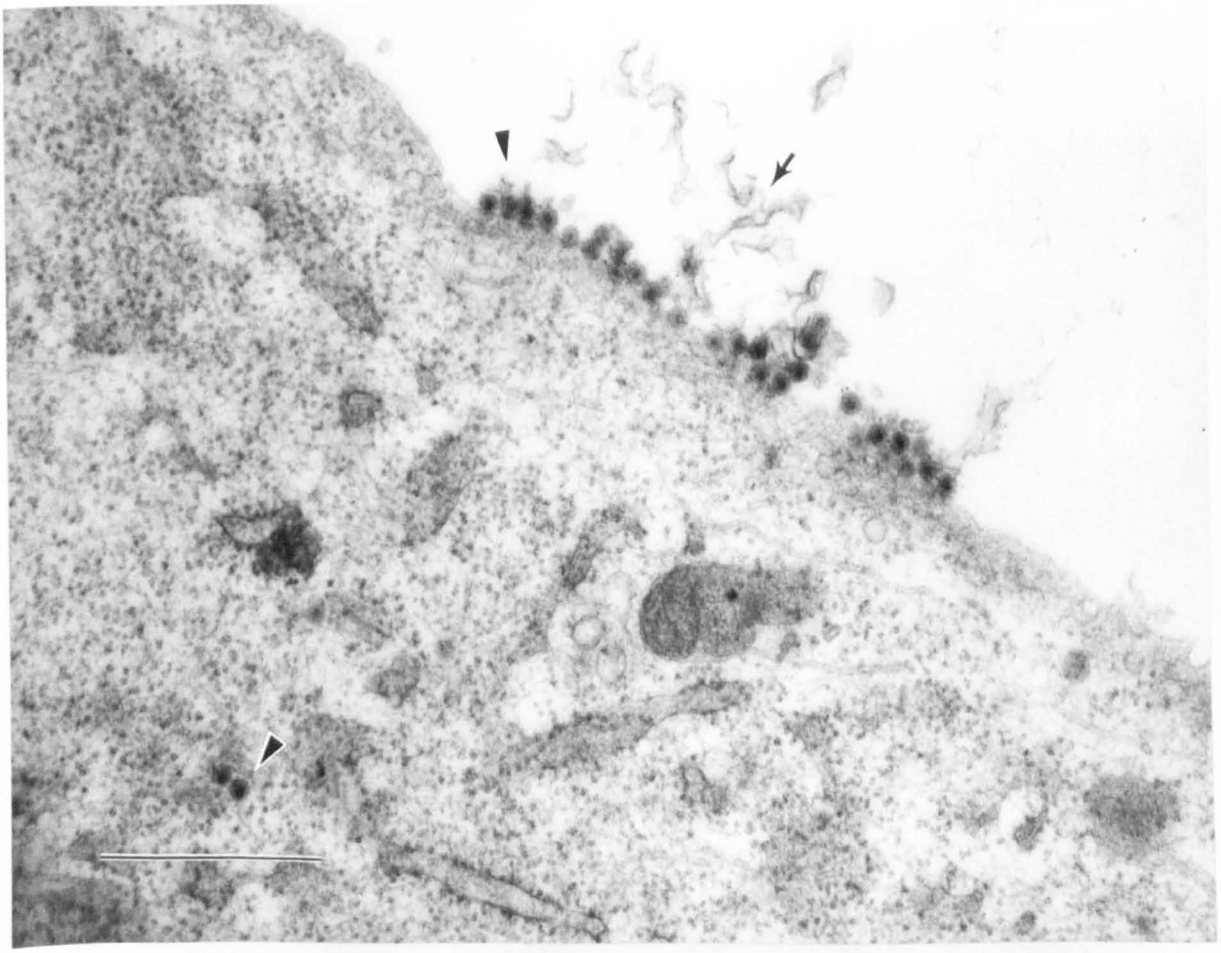

Fig. 4.5 Release of BTV from BHK-21 cells (20 hours pi) by extrusion without acquiring an envelope from the cell plasma membrane. ► double-shelled virus particles; → cell debris; bar=500 nm

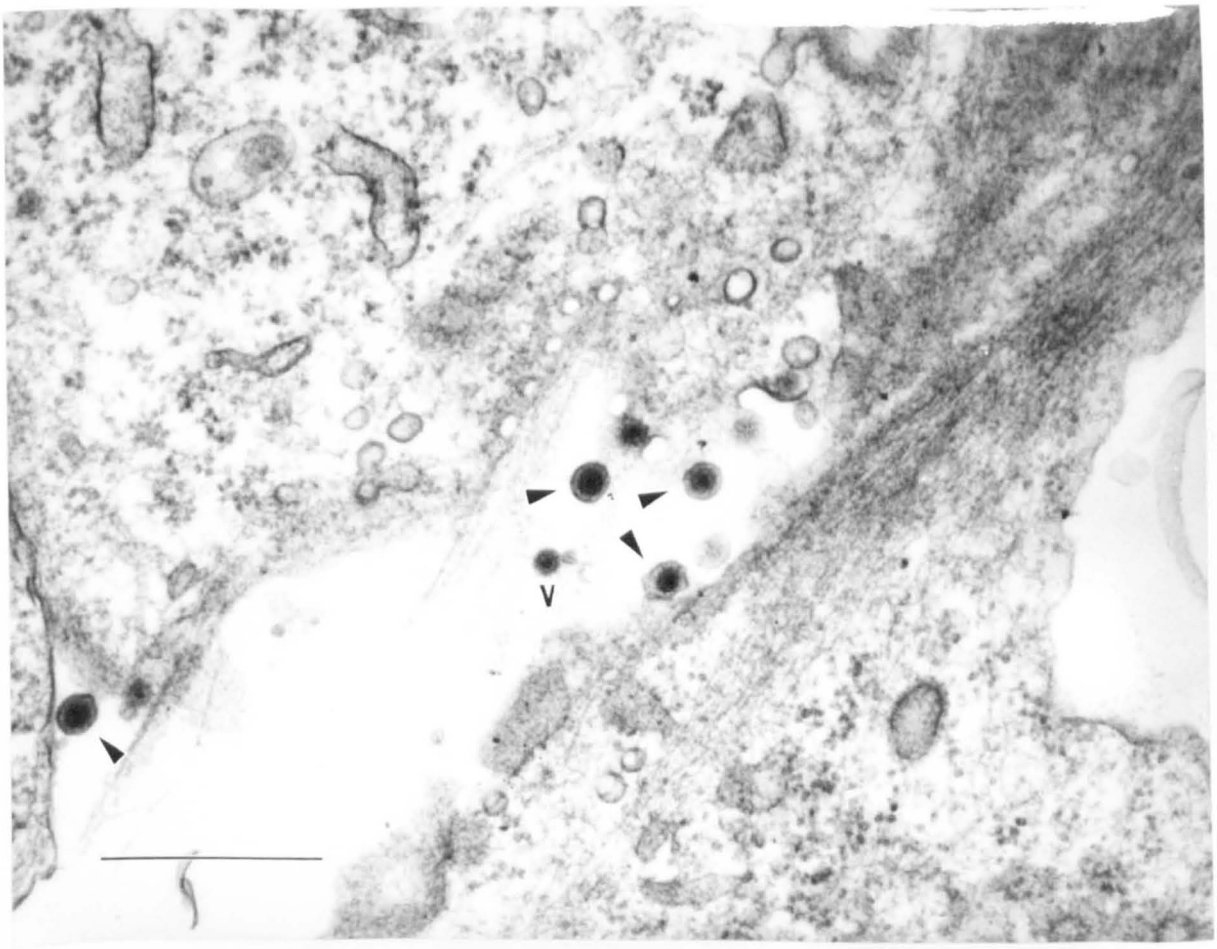

Fig. 4.6 BTV infection of BHK-21 cells (20 hours pi). arrows: progeny virus enveloped with cell plasma membrane; **V**: unenveloped progeny virus particle; bar=500 nm

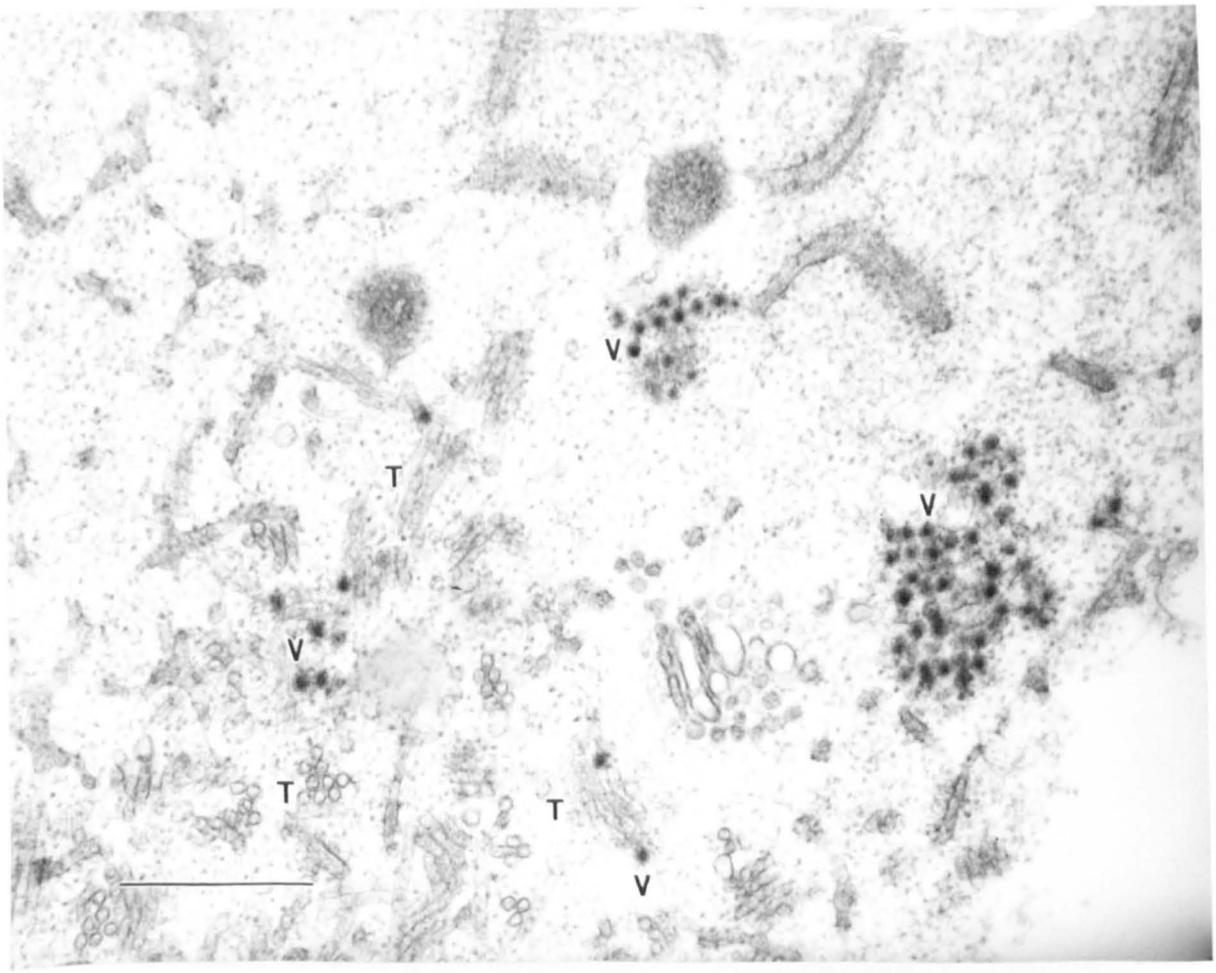

Fig. 4.7 BTV infection of BHK-21 cells (20 hours pi). **T**: tubules; **V**: double-shelled progeny virus particles; bar=500 nm

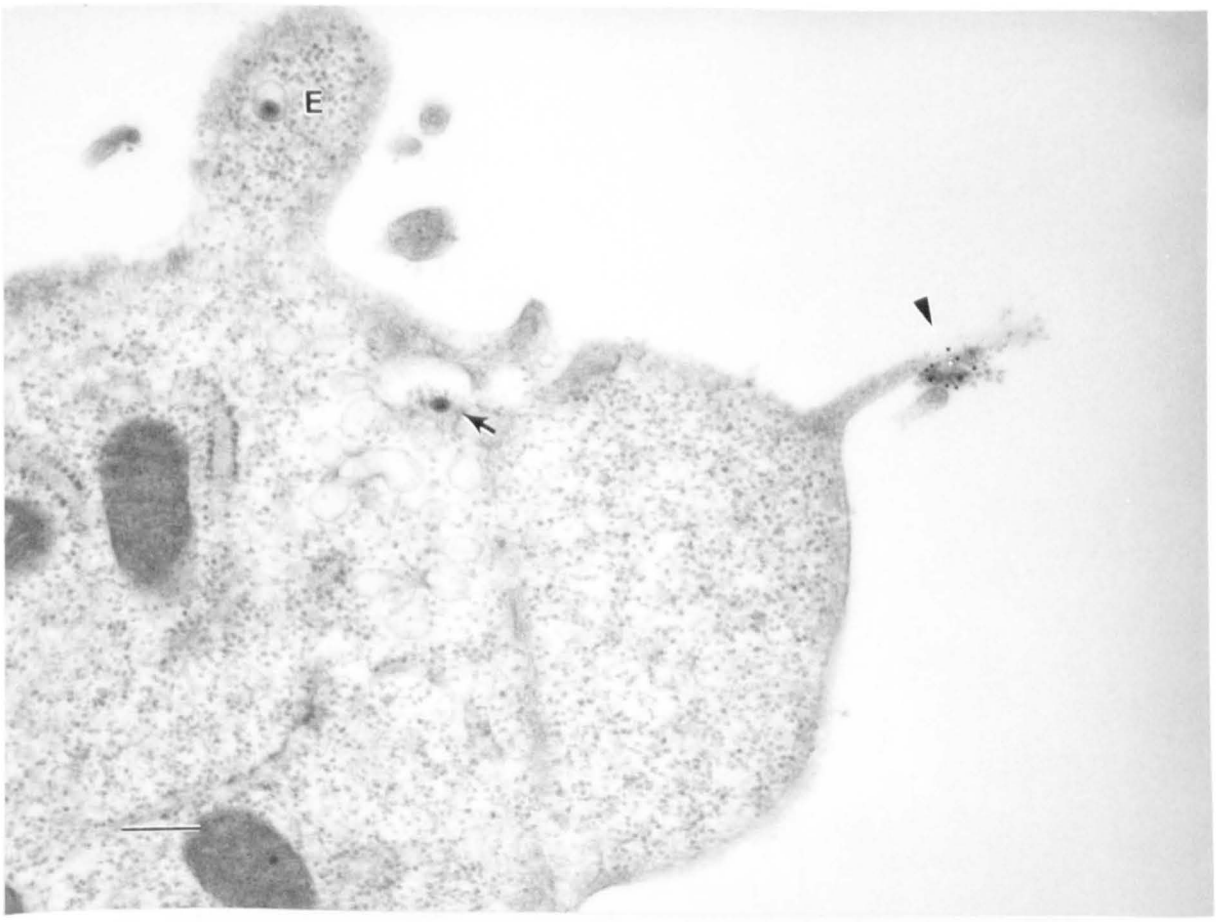

Fig. 4.8 Pre-embedding immunogold staining of infected BHK-21 cells (20 hours pi) incubated with polyclonal antibody against BTV 1SA. ► virus particles labelled with gold conjugate being released from cell; E: endosome containing a BTV particle reinfecting the cell; → a BTV containing endosome fusing with lysosomes; bar=200 nm.

(Fig. 4.9b). Mature virus particles were also seen in the cytoplasm close to VIBs in these KC cells (Fig. 4.10a, 4.10b and 4.11). The sizes of VIBs in newly infected KC cells (6 hours pi) were similar to those in BHK-21 cells (Fig. 4.12), about 500x400 nm (length x width), while the VIBs in persistently infected KC cells were much bigger than those in BHK-21 cells, ranging from 1,570x1,450 to 1,750x1,600 nm (length x width)(Fig. 4.10a, 4.10b and 4.11). Most of the VIB matrices were not uniform but were highly characteristic in appearance with distinctive fractures (Fig. 4.10.a and 4.11). The release of progeny virus particles was observed only by membrane budding from newly infected KC cells (6 hours pi)(Fig 4.12). Virus release was not seen from persistently infected KC cells. Large amounts of tubules were also seen in the cytoplasm of infected cells (Fig. 4.13a, 4.13b, 4.13c). The tubules were often seen to be associated with mature progeny virus particles in cytoplasm of infected KC cells (Fig. 4.13b, 4.13c). Reinfection of infected KC cells occurred as evidenced by the the presence of uncoated virus particles in lysosomes in some persistently infected KC cells (Fig.4.10b). Fig 4.14 shows the ultrastructure of uninfected KC cells.

Table 4.1 Ultrastructural observation of persistent infection of KC cells with BTV 1SA (3 weeks pi)

Characteristics of BTV infection	No. of cells	%
V + VIBs	15	7.5
T	15	7.5
VIB	10	5.0
V + T	3	1.5
V + VIBs + T	1	0.5
VIBs + T	1	0.5
None	155	77.5
Total	200	100

V: progeny virus particles
VIBs: Viral inclusion bodies
T: Tubules

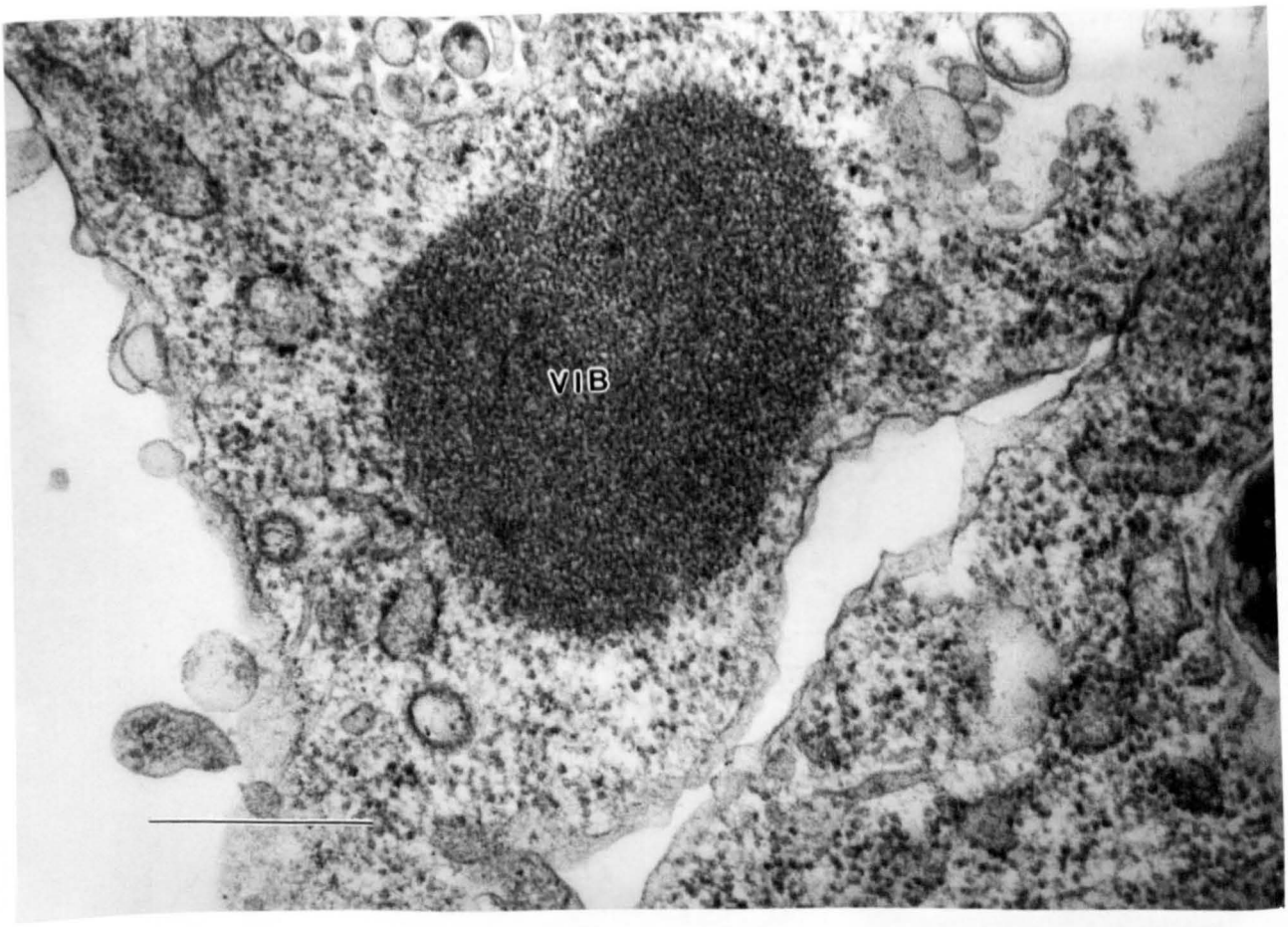

Fig. 4.9a BTV infection of KC cells (21 days pi). **VIB**: virus inclusion body; bar=500 nm

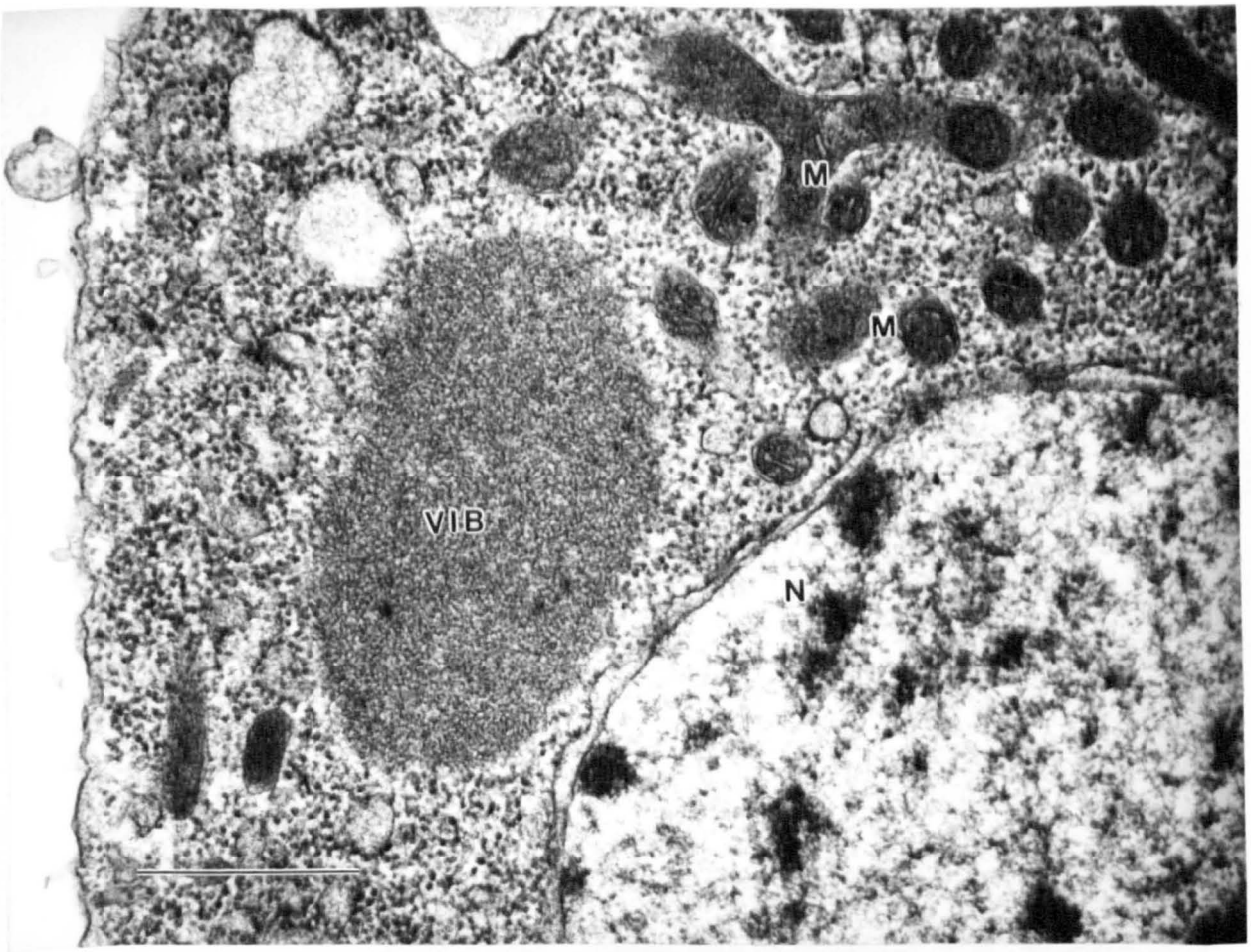

Fig. 4.9b BTV infection of KC cells (21 days pi). **VIB**: virus inclusion body; **N**: nucleus; **M**: mitochondria; bar=500 nm

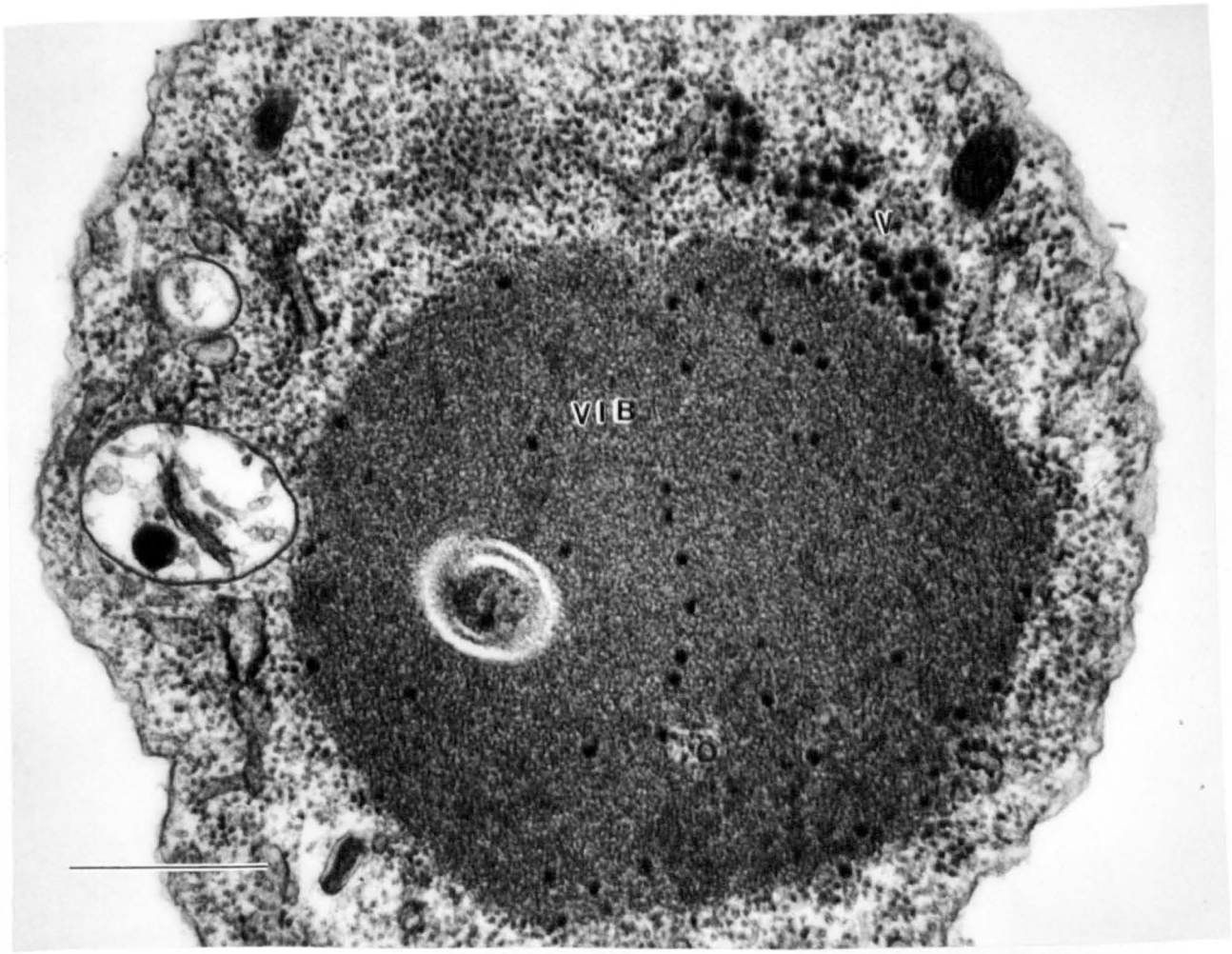

Fig. 4.10a BTV infection of KC cells (21 days pi). **VIB**: virus inclusion body; **V**: double-shelled progeny virus particles; bar=500 nm

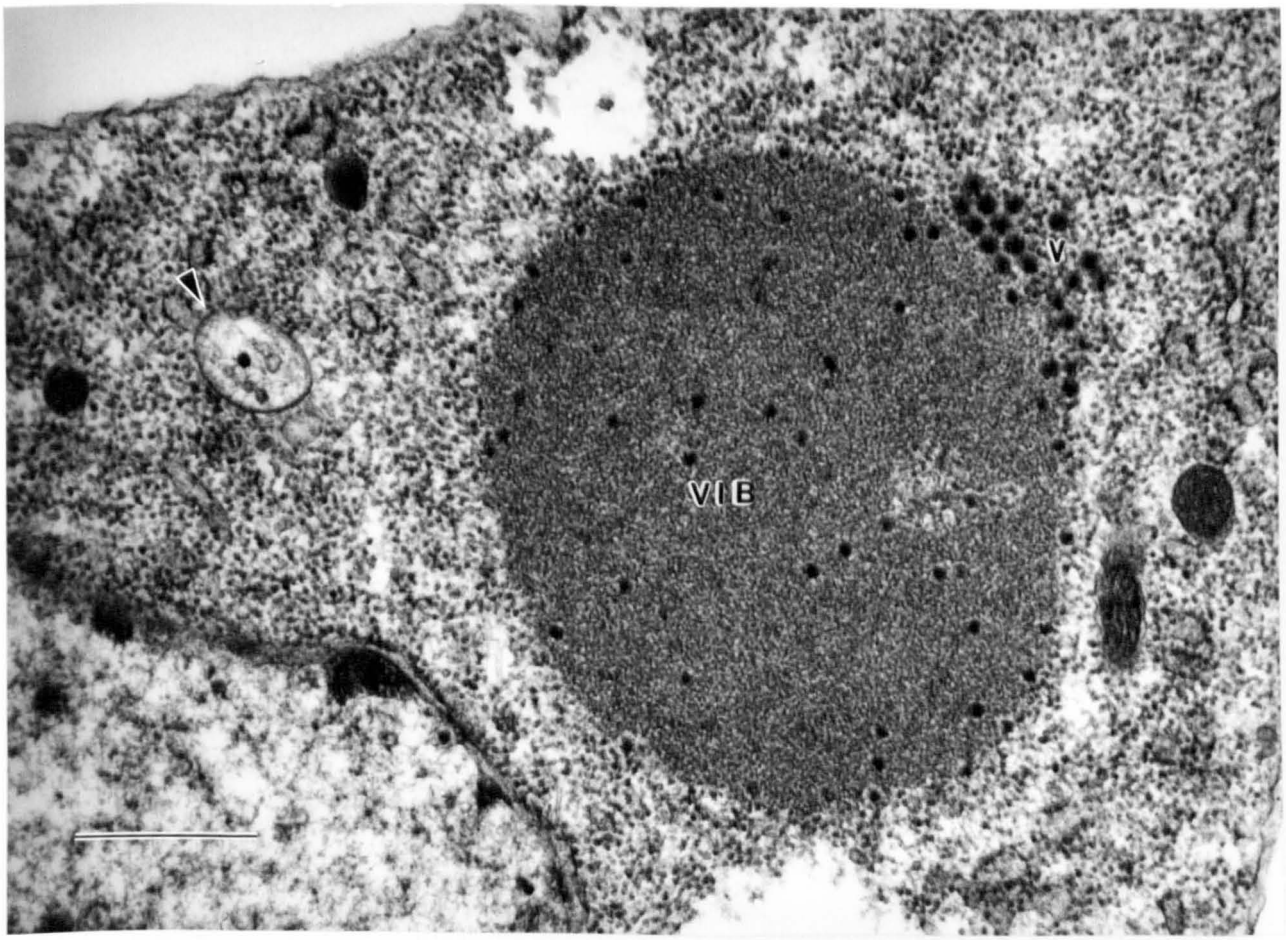

Fig. 4.10b BTV infection of KC cell (21 days pi). **VIB**: virus inclusion body; arrow: endosome containing an uncoated virus particle; **V**: progeny virus particles; bar=500 nm

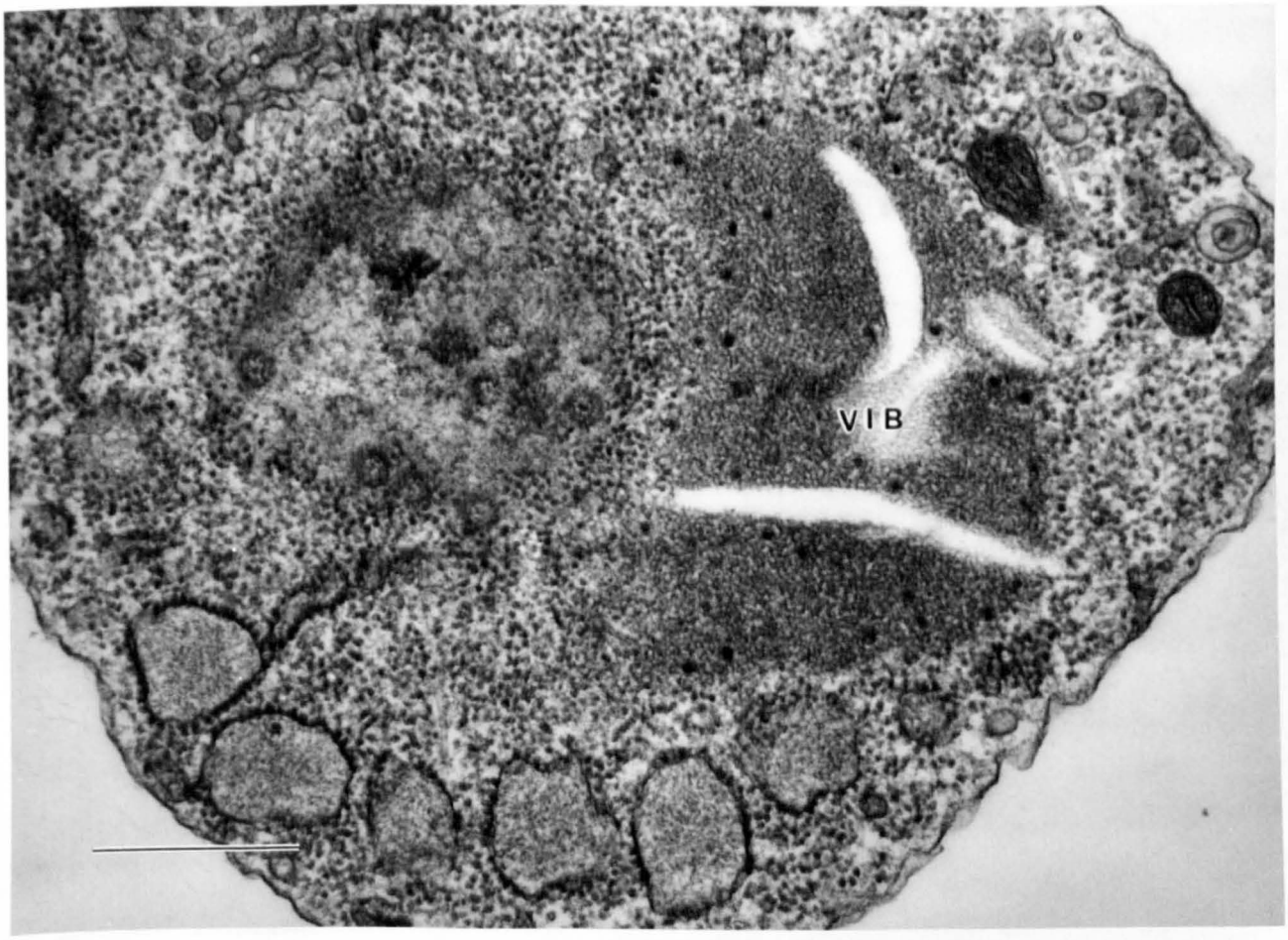

Fig. 4.11 BTV infection of KC cells (21 days pi). **VIB**: virus inclusion body; bar=500 nm

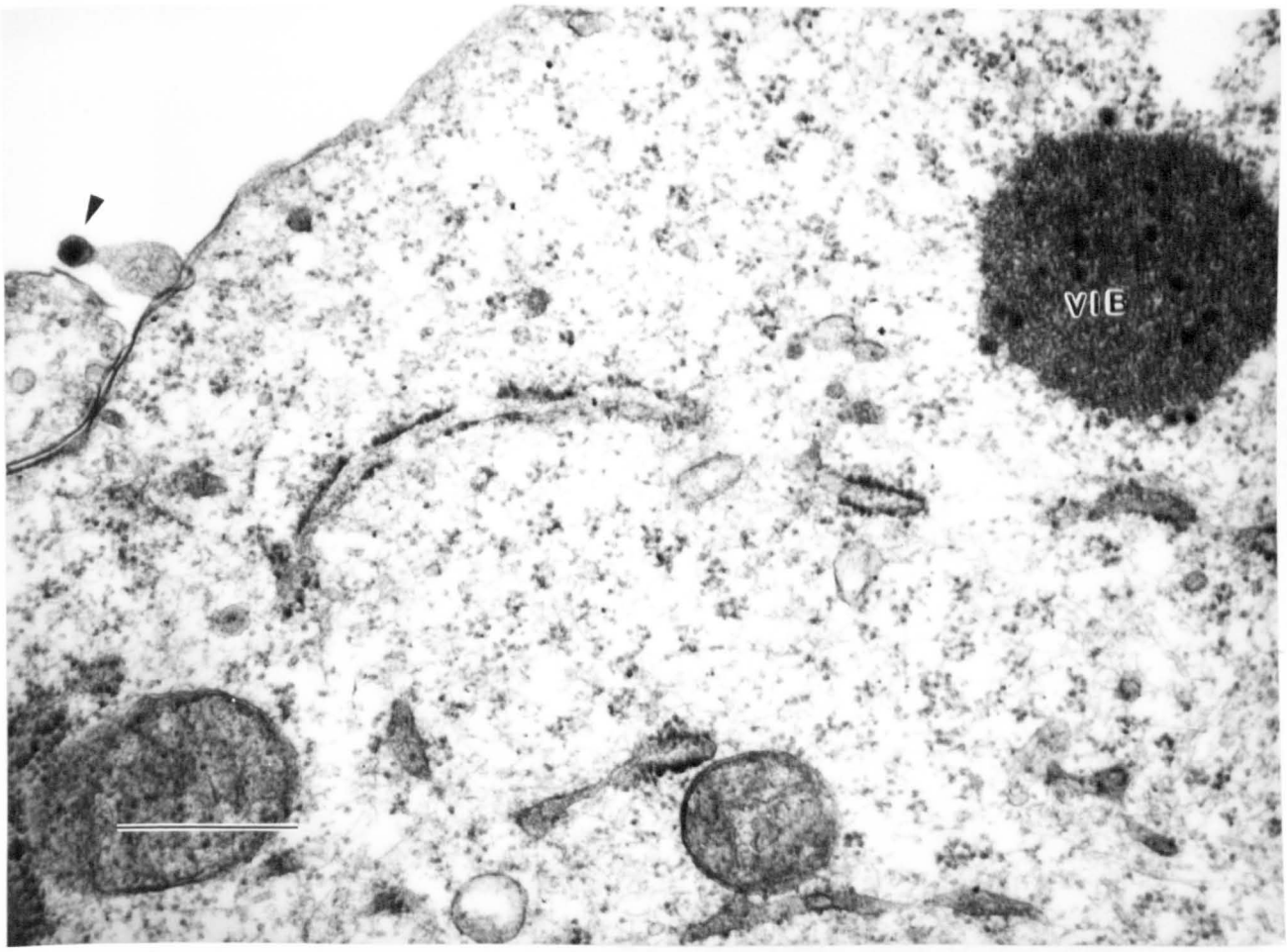

Fig. 4.12 BTV infection of KC cells (6 hours pi). **VIB**: virus inclusion body; arrow: a BTV particle being released within an extension of the cell plasma membrane; bar=500 nm

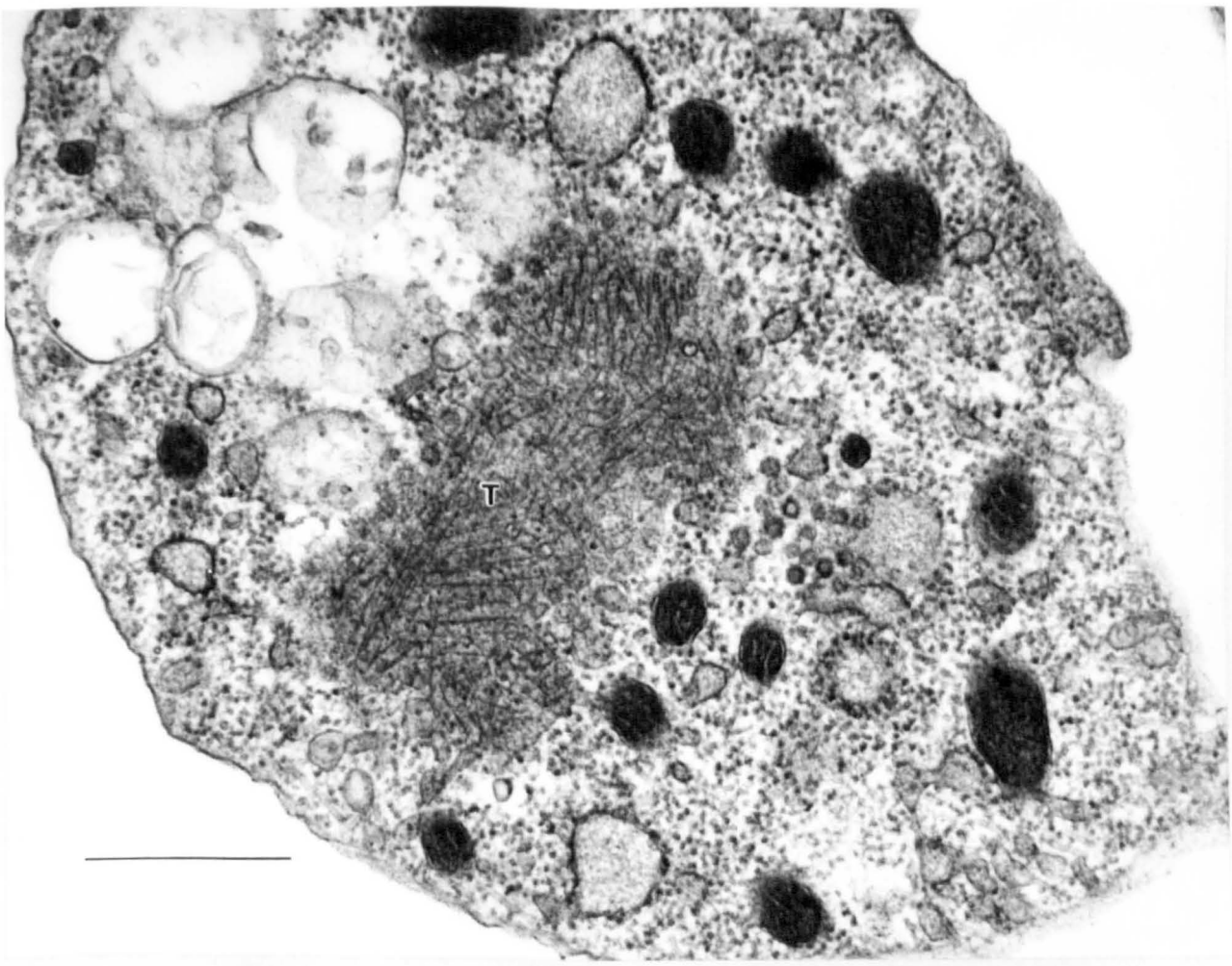

Fig. 4.13a Tubules in BTV infected KC cells (21 days pi). **T**: tubules; **M**: mitichondria; bar=500 nm

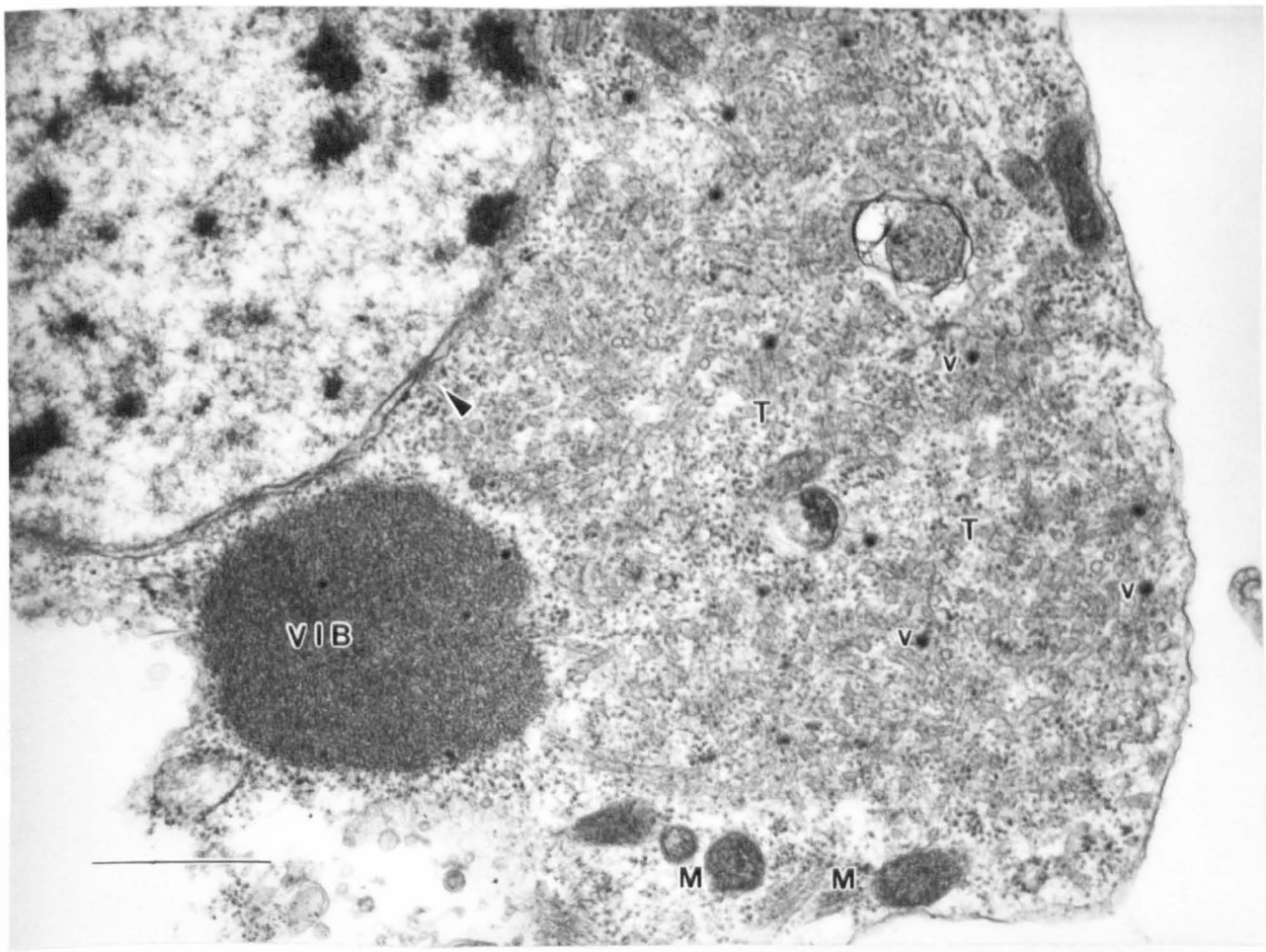

Fig. 4.13b Tubules in BTV infected KC cells (21 days pi). **T**: tubules; **V**: nascent progeny virus particles; **VIB**: virus inclusion body; ► nucleus; **M**: mitochondria; bar=500 nm

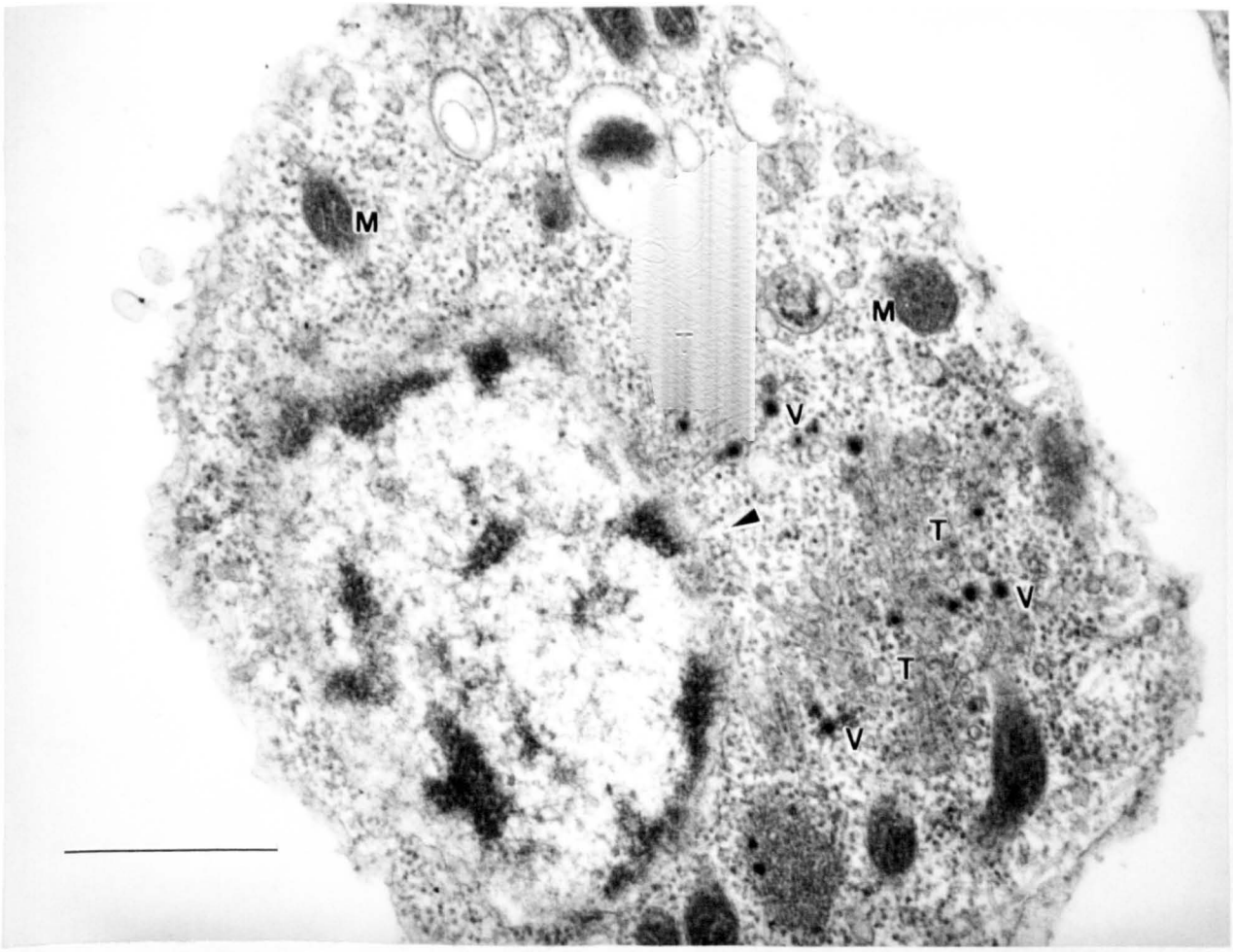

Fig. 4.13c Tubules in BTV infected KC cells (21 days pi). **T**: tubules; **V**: nascent progeny virus particles; ► nucleus; **M**: mitochondria; bar=500 nm

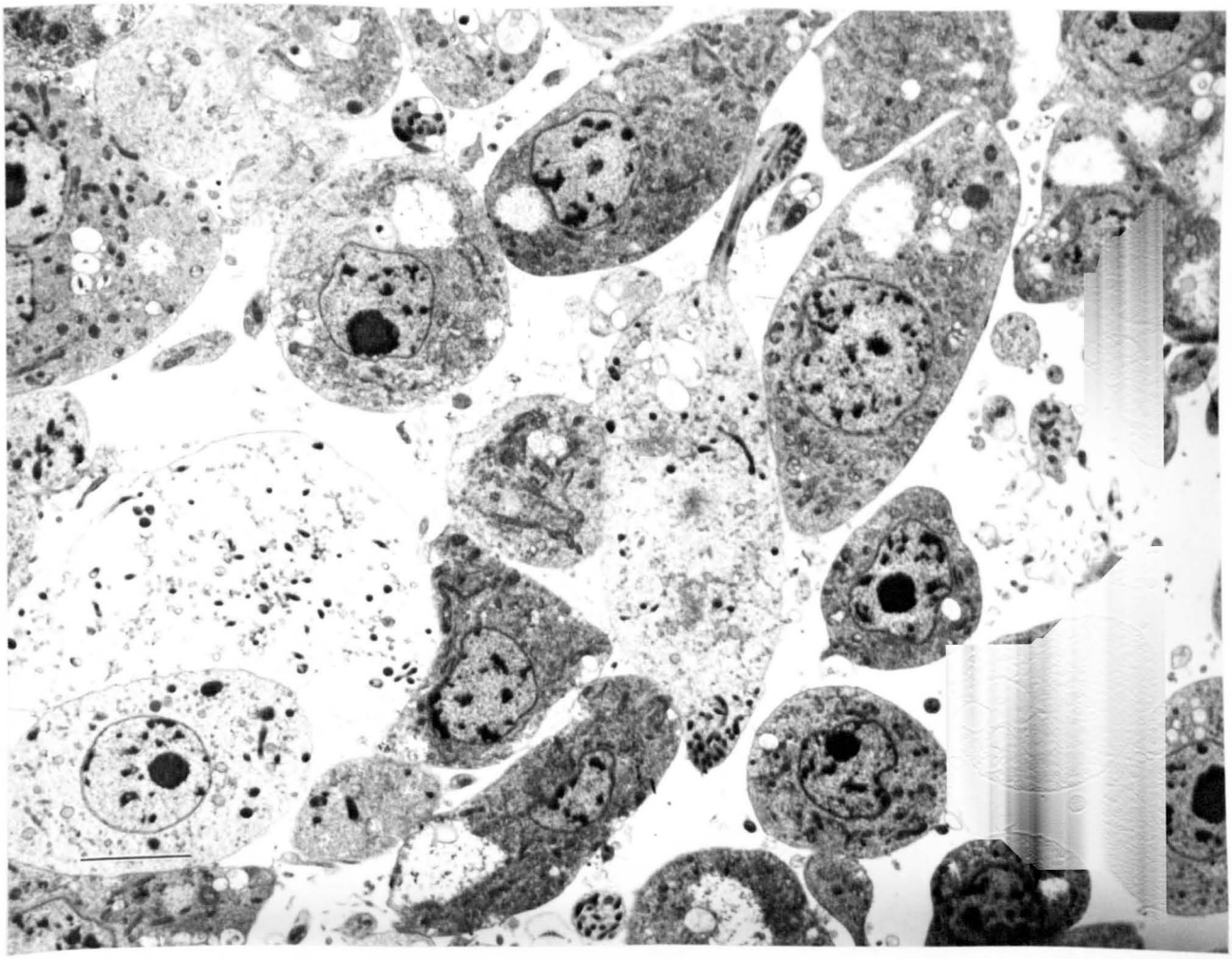

Fig. 4.14 Ultrastructure of uninfected KC cells. bar=2μm

4.3.3 Infection of the salivary glands of *Culicoides*

To ensure a high frequency of infection, only salivary glands from IT infected midges were analyzed by TEM. The salivary glands in *Culicoides* are composed of two clusters of lobes arranged bilaterally. Each cluster consists of a major lobe and 2-3 minor lobes. Each lobe contains a single layer of acinar cells surrounding the secretory tubes, the ducts. Because of the difficulties of dissection, only the major lobe was sampled for virus detection with TEM. The salivary glands from 11 insects were examined. BTV infection in the salivary glands of *Culicoides* occurred typically in the sequence of virus movement from the basement membrane → acinar cell → lumen of the ducts (Fig 4.15). In infected salivary glands, virus replication was similar in some ways to that seen in BHK-21 cells and VIB matrices were observed in the cytoplasm of most of the serous acinar cells (Fig. 4.16 and 4.17). However, in general, the sizes of these VIBs were much bigger [from 3,065x1,925 to 5,893x3,214 nm (length x width)] than those seen in infected BHK-21 cells and KC cells. Most of the VIBs were irregular in shape with distinctive fractures within the matrices as seen in KC cells (Fig 4.16 and 4.17). The cytoplasm, sometimes contained tubules and double-shelled virus particles, and was frequently observed intruding between different parts of the VIB matrix (Fig. 4.16 and 4.17). Nascent cores and virus-like particles were observed in VIBs, while double-shelled virus particles were only seen in the cytoplasm (Fig. 4.16). The mature progeny virus particles were released into the terminal serous alveoli (acini), only by membrane budding (Fig.4.18 and 4.19). No virus release was observed by extrusion through the cell membrane (without acquiring an envelope), which sometimes occurred in infected BHK cells. Virus release was not observed through the basement membrane of the salivary glands back into the haemocoel. Once in the acini, it is likely that the virus particles are transported via intermediate ducts into the lumen of the major secretory ducts where they accumulate (Fig. 4.20).

Large numbers of microtubules formed and were widely located in the cytoplasm of infected serous acinar cells, including the areas around the VIBs (Fig. 4.16). These tubules, as in infected BHK-21 cells, seemed to be associated with the mature progeny virus particles. The width of the tubules (58-65 nm) as in infected BHK-21 cells were similar to the diameter of mature virus particles.

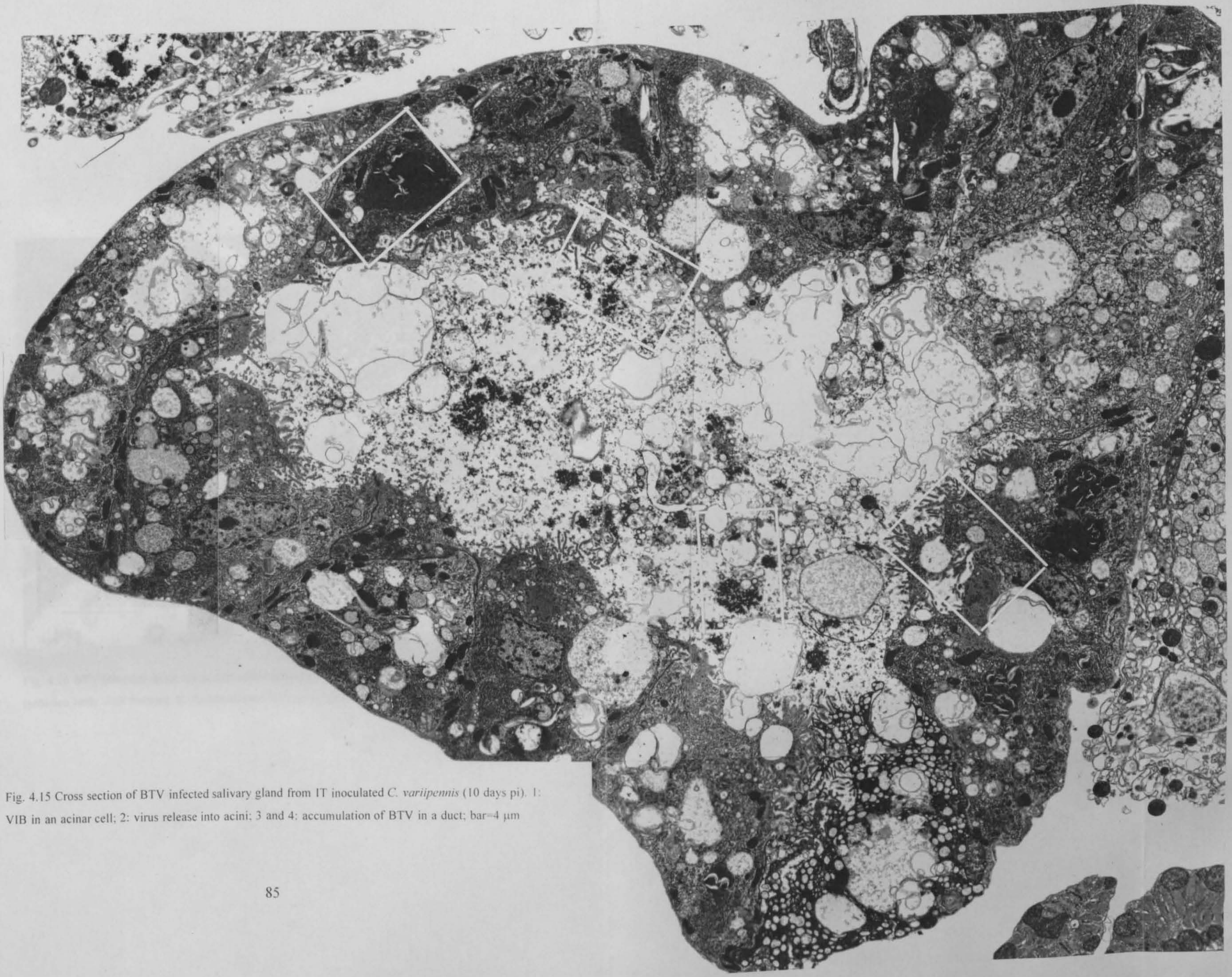

Fig. 4.15 Cross section of BTV infected salivary gland from IT inoculated *C. variipennis* (10 days pi). 1: VIB in an acinar cell; 2: virus release into acini; 3 and 4: accumulation of BTV in a duct; bar=4 μm

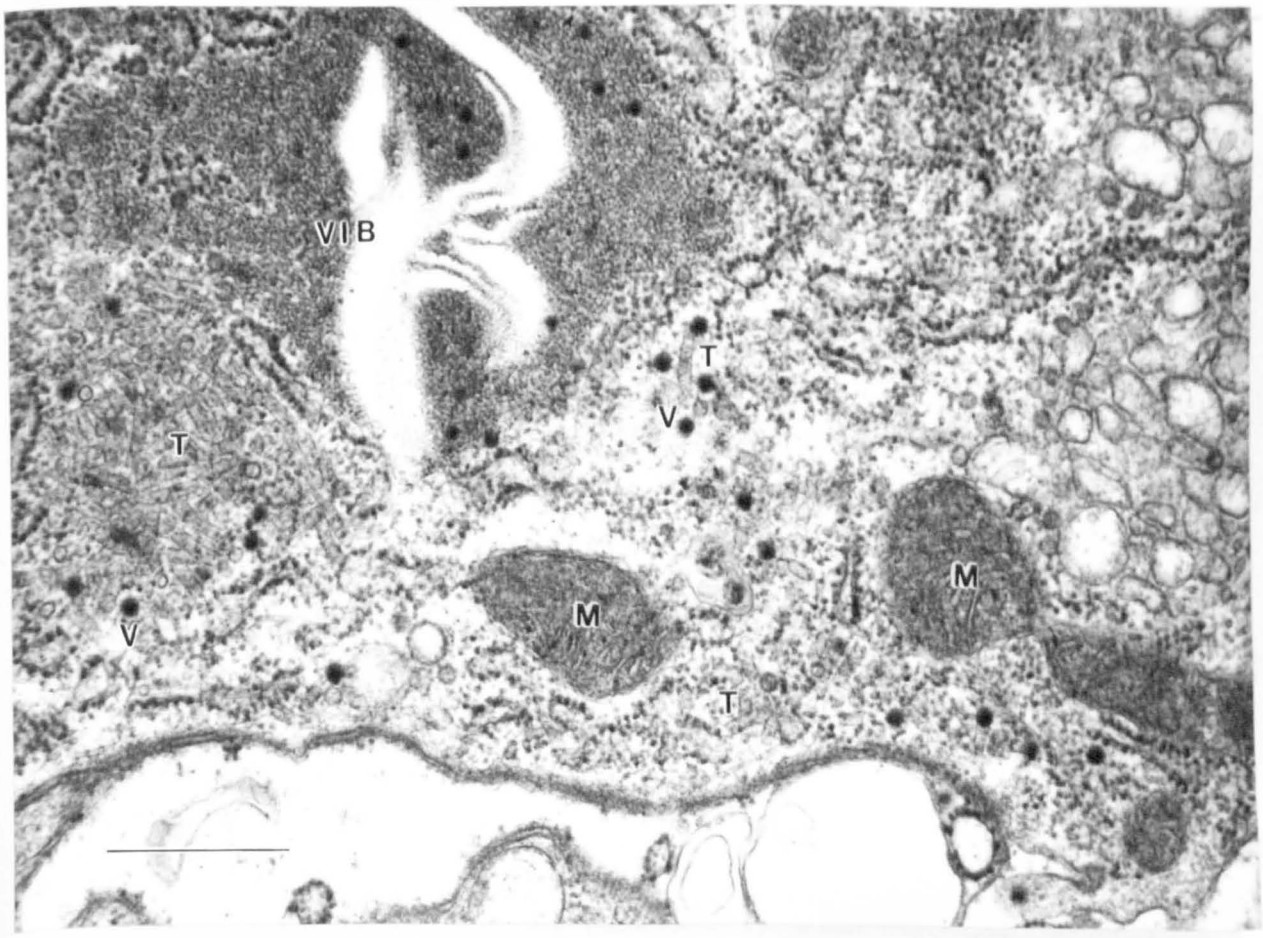

Fig. 4.16 BTV infection in an acinar cell of the salivary gland of *C. variipennis* (10 days pi). **VIB**: virus inclusion body with fracture; **V**: double-shelled BTV particles; **T**: tubules; **M**: mitochondria: bar=500 nm

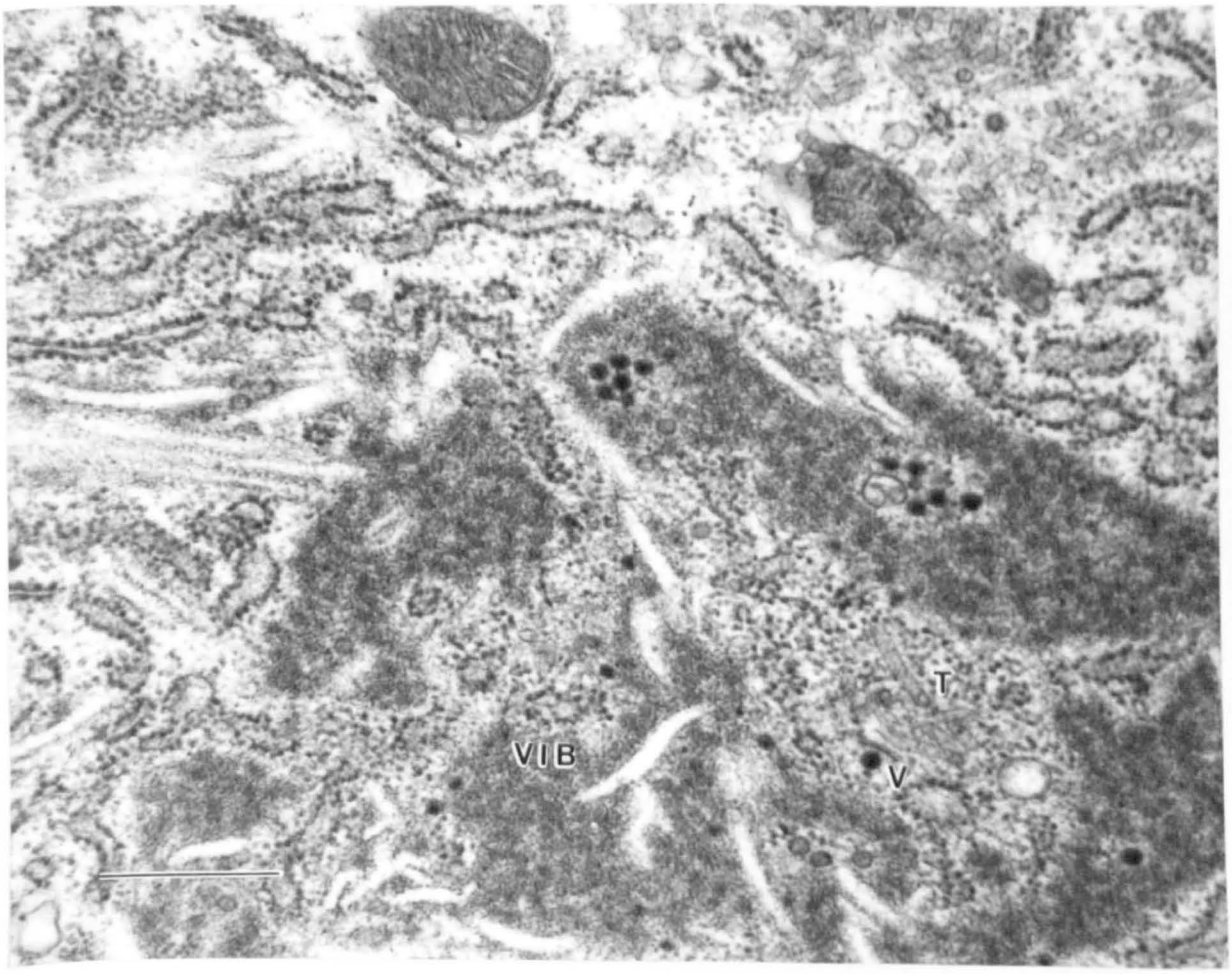

Fig. 4.17 BTV infection in an acinar cell of the salivary gland of *C. variipennis* (10 days pi). **VIB**: virus inclusion body; **V**: virus particle; **T**: tubules; **M**: mitochondria; bar=500 nm

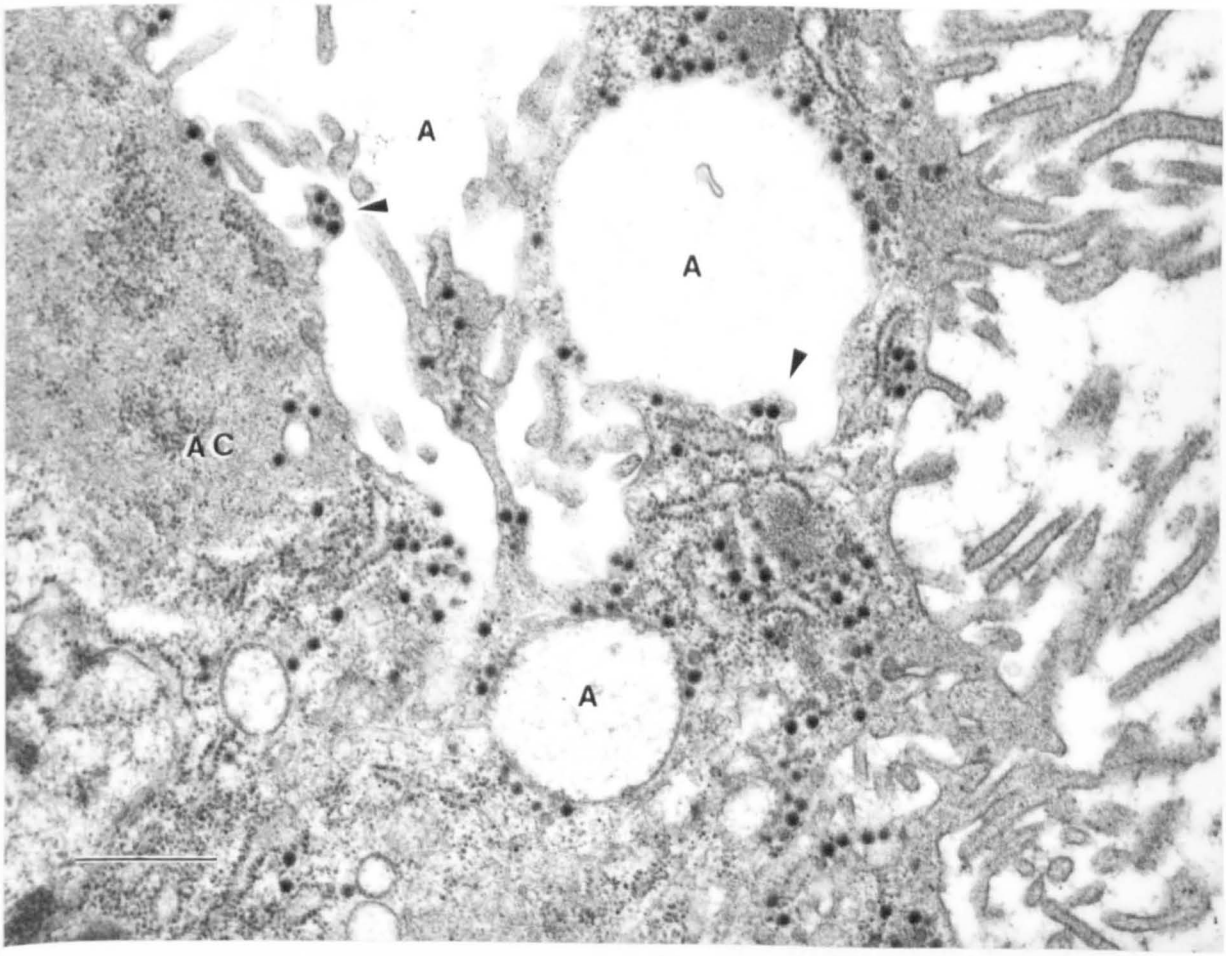

Fig. 4.18 Release of BTV from an acinar cell of a *Culicoides* salivary gland (10 days pi). **AC**: acinar cell; **A**: acini; arrows: virus particles released from acinar cell into acini by membrane 'budding'; bar=500 nm

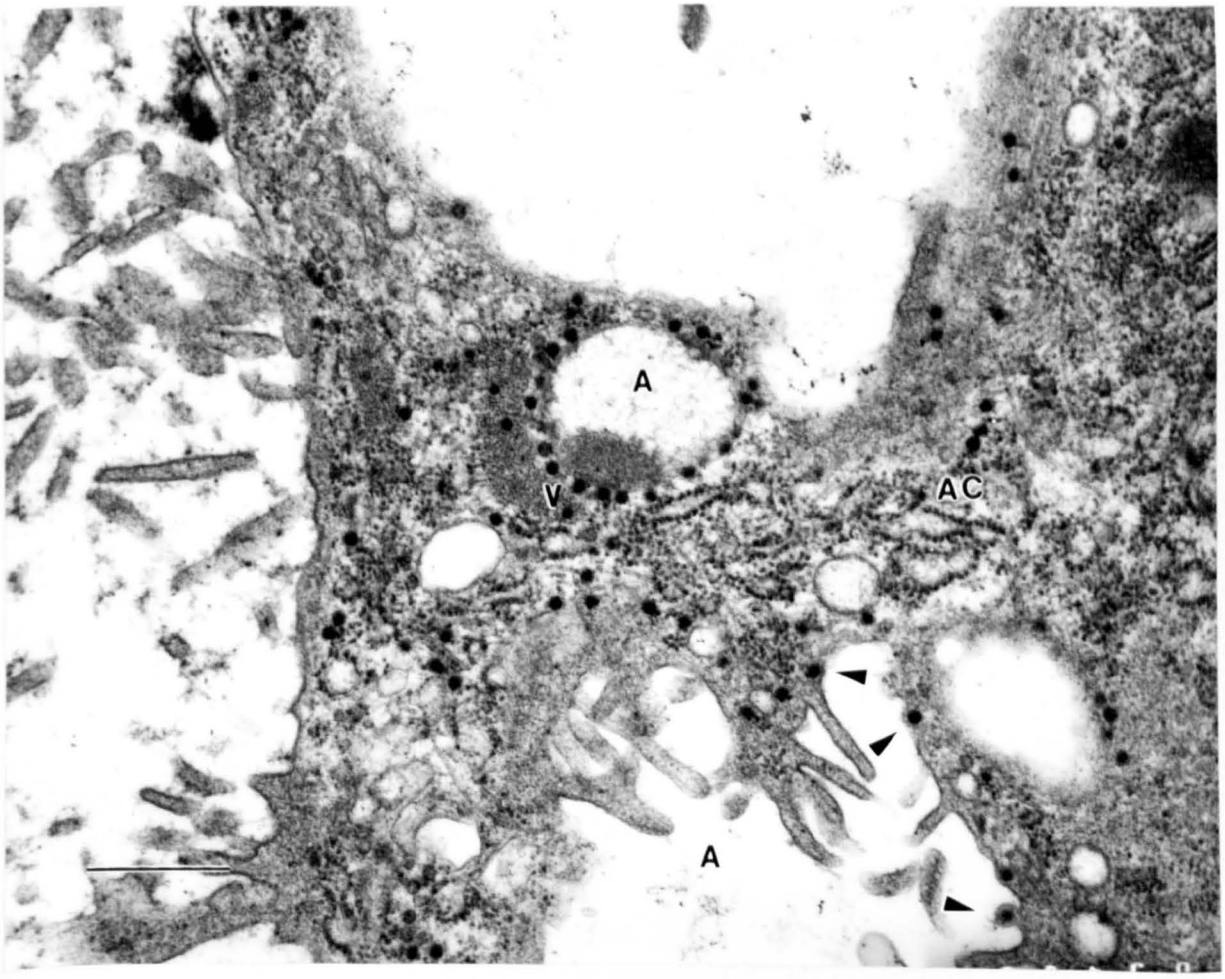

Fig. 4.19 BTV infection of a salivary gland of *C. variipennis* (10 days pi). **AC**: acinar cells; **A**: acini; **V**: virus particles; Arrow: BTV release by membrane 'budding'; bar=500 nm

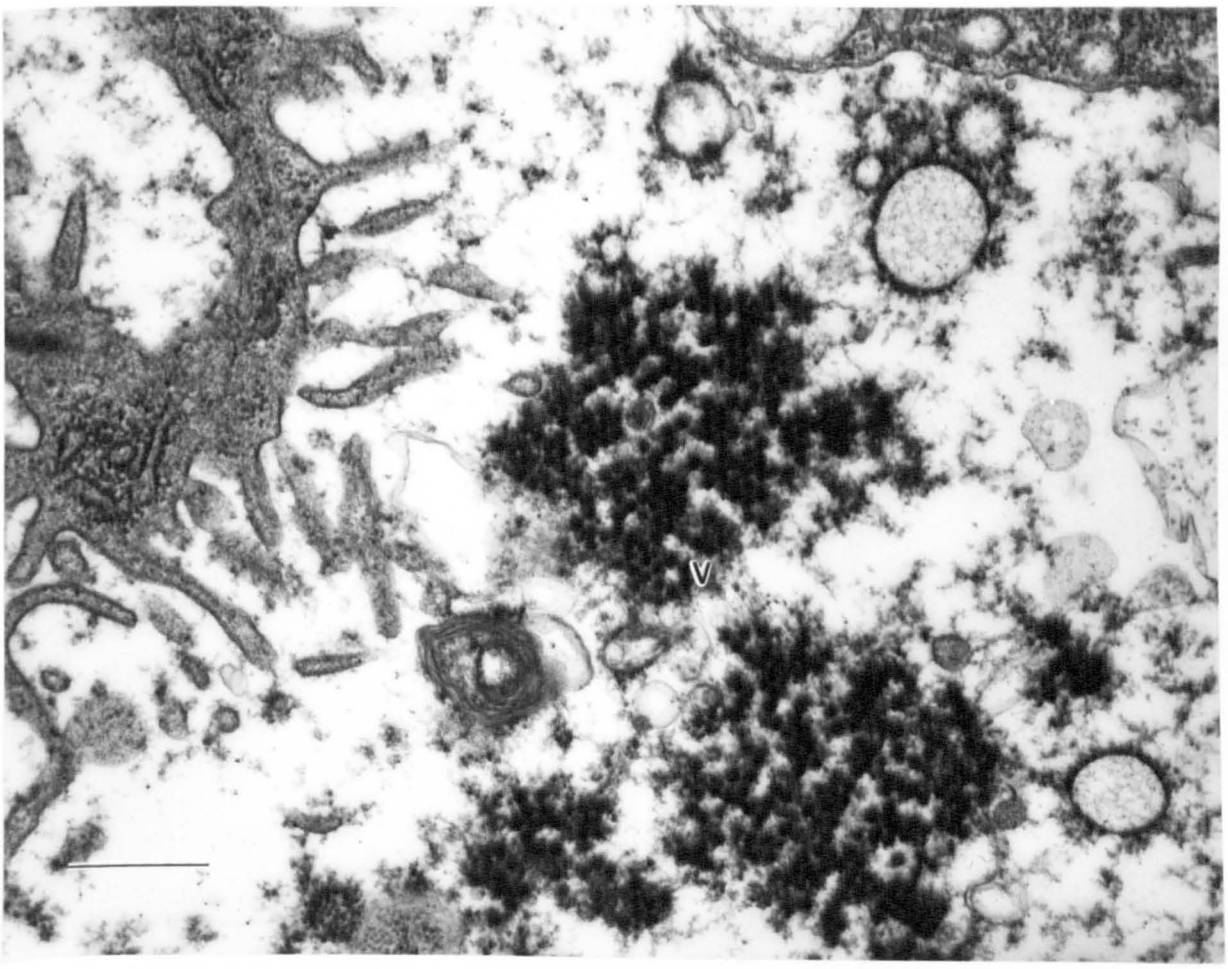

Fig. 4.20 BTV accumulation in the major secretory duct of the salivary gland of *C. variipennis* (10 days pi).
V: virus particles; bar=500 nm

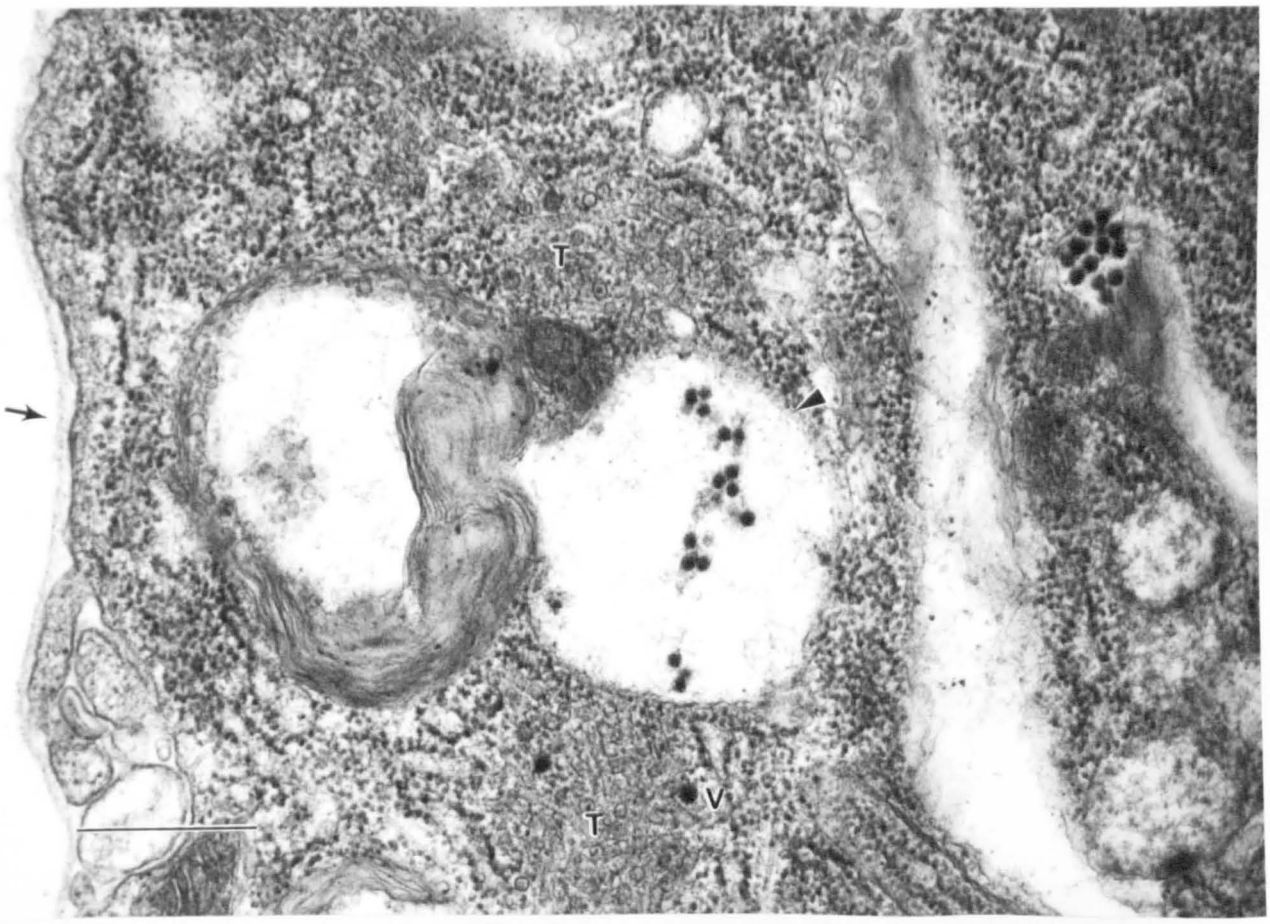

Fig. 4.21 Continuous BTV infection of a *Culicoides* salivary gland (10 days pi) from the haemocoel through the basement membrane. ➔basement surface of salivary gland; ►lysosome containing uncoated BTV particles; **V**: progeny virus particles; **T**: tubules; bar=500 nm

Infection of the salivary gland cells through the basement surface of the glands seemed to be a continuous process. Uptake of virus particles from the basement membrane surface, even late in infection, was clearly observed and the fusion of endosomes with lysosomes containing uncoated virus was also seen (Fig. 4.21). No virus re-entry into the acinar cells from acini was observed.

4.3.4 Detection of BTV infection of the gut of *C. variipennis*

Thirty three gut samples of orally infected *C. variipennis* (7 and 10 days pi) were examined. The ultrastructural morphology of these guts seemed normal in comparison with those of negative control samples from uninfected midges. Fig. 4.22 shows the ultrastructure of the midgut of an uninfected midge. The microvilli, cell membranes and subcellular structures are clear. A characteristic of the midgut structure is that large numbers of vesicles are distributed in the cytoplasm of the gut cells. No obvious evidence was observed to show BTV replication in the gut cells of midges fed with an infectious blood meal. VIBs, tubules and virus particles were not found in any gut cells of infected midges.

4.3.5 BTV infection of nervous tissues of *Culicoides*

Only two thoracic ganglia tissue samples were obtained as an incidental product when the salivary glands were dissected from all the IT inoculated *C. variipennis* at 10 days pi. The nervous tissue was heavily infected (Fig. 4.23) and typical BTV replication was observed in nerve cells and axons. VIB matrices were frequently seen in the cytoplasm of nerve cells and axons (Fig. 4.24, 4.25, 4.26, 4.27). The sizes of VIBs (6 VIBs measured) varied from 1,153x557 nm to 2,576x1,548 nm (length x width) and were bigger than those in infected BHK-21 cells (from 500x340 to 600x480 nm, length x width, see page 72). Nascent cores and virus-like particles were observed in these VIBs (Fig 4.24, 4.25, 4.26) but the numerous double-shelled virus particles were only seen in the cytoplasm close to VIBs (Fig. 4.24, 4.25). No virus release from the infected nerve cells and axons was observed.

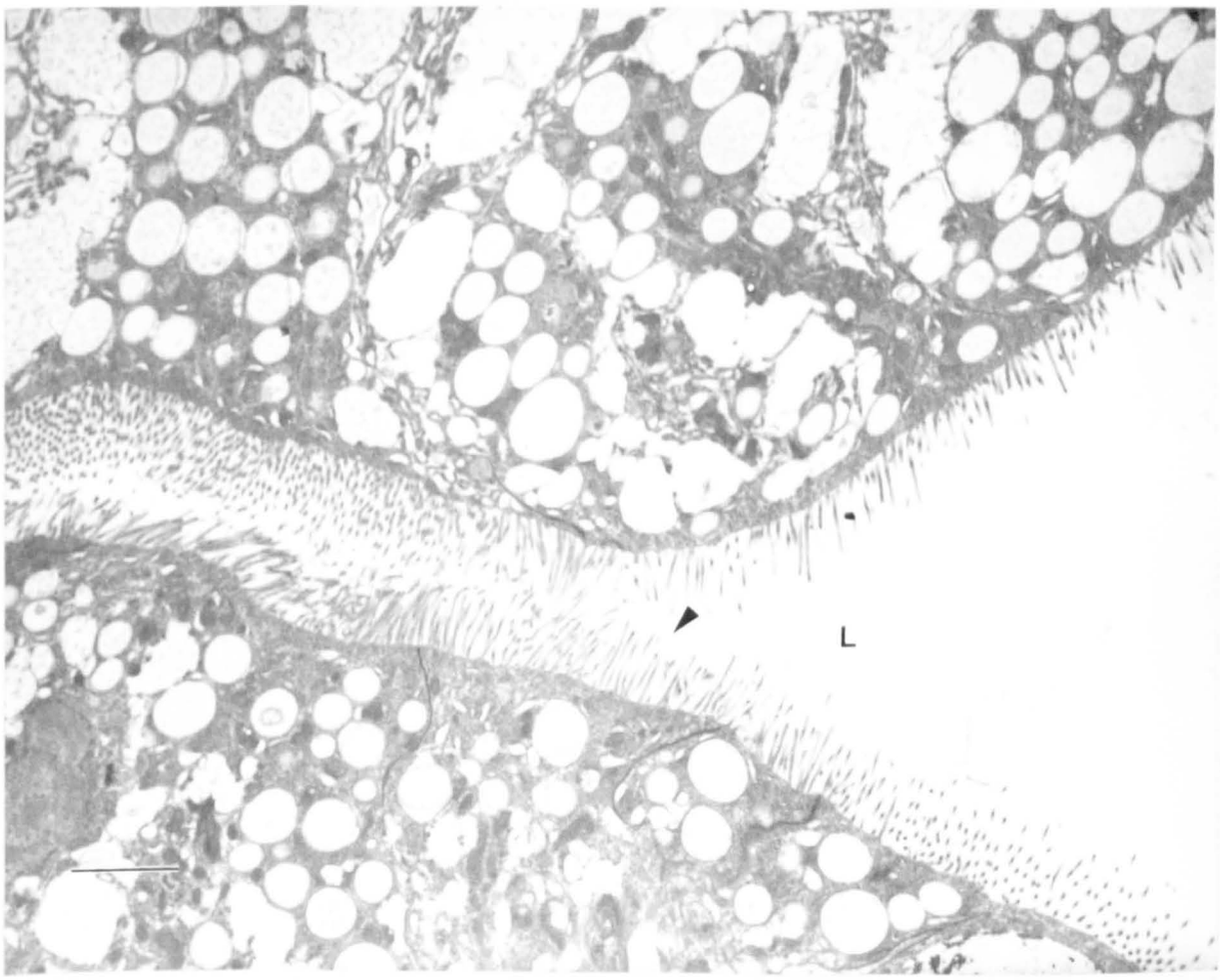

Fig. 4.22 Electron micrograph of normal midgut of *C. variipennis*. **L**: lumen; arrow: microvilli; bar=2 μm

Large amounts of tubules formed in BTV infected nerve cells and axons (Fig. 4.23, 4.24, 4.25, 4.26, 4.27). These tubules were distributed in the cytoplasm of infected cells and axons and were often seen in the vicinity of VIBs (Fig. 4.24, 4.25, 4.26). As in BHK-21 cells and in the acinar cells of the salivary glands, tubules seemed to be closely associated with double-shelled progeny virus particles (Fig. 4.24, 4.25). The width of the tubules ranged from 58 to 65 nm.

No obvious pathological changes were observed in the nervous tissues. The ultrastructure of the mitochodria remained clear and normal.

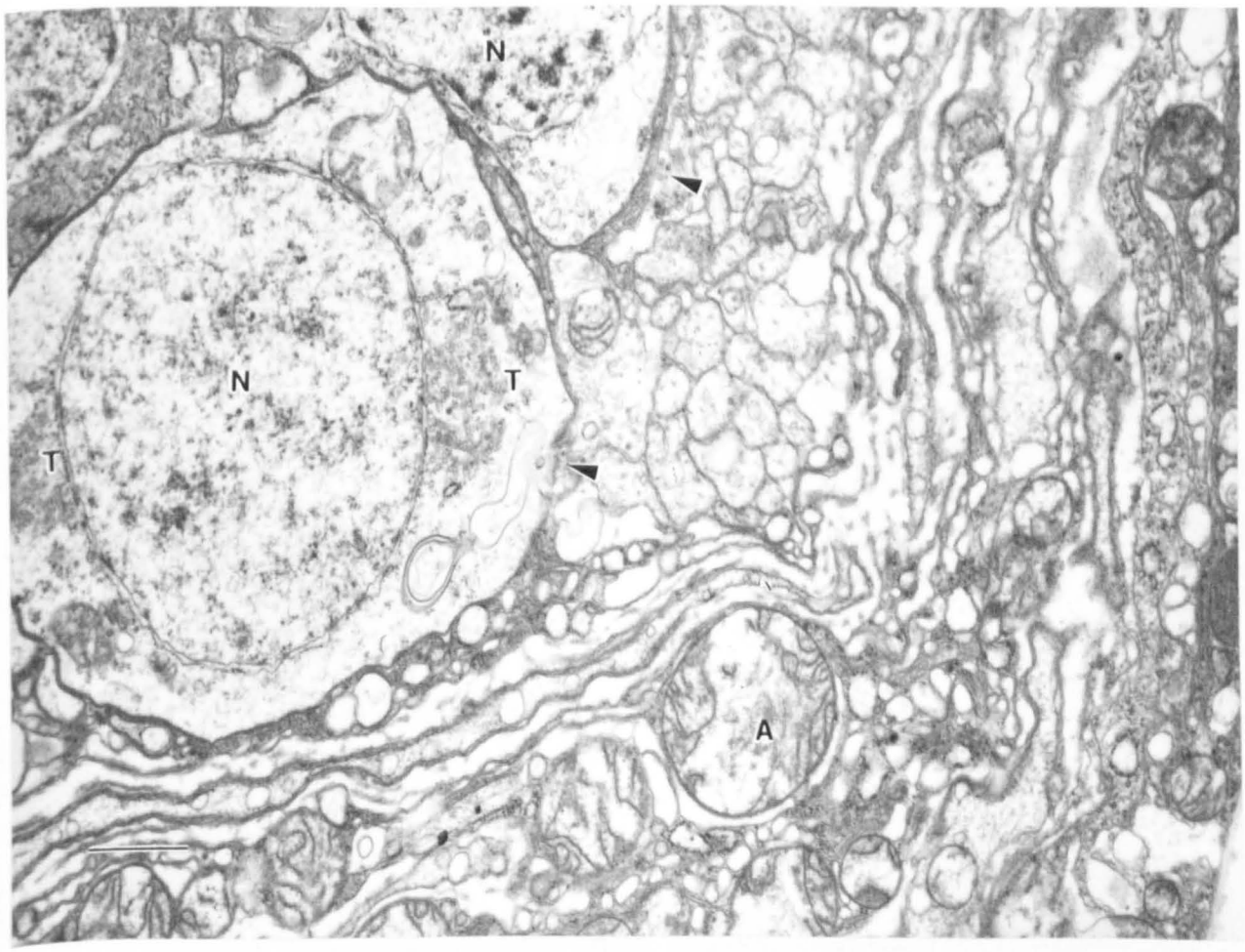

Fig. 4.23 BTV infection of thoracic ganglia of *C. variipennis* (10 days pi). ► nerve cell; **A**: axon; **N**: nucleus; **T**: tubules; bar=500 nm

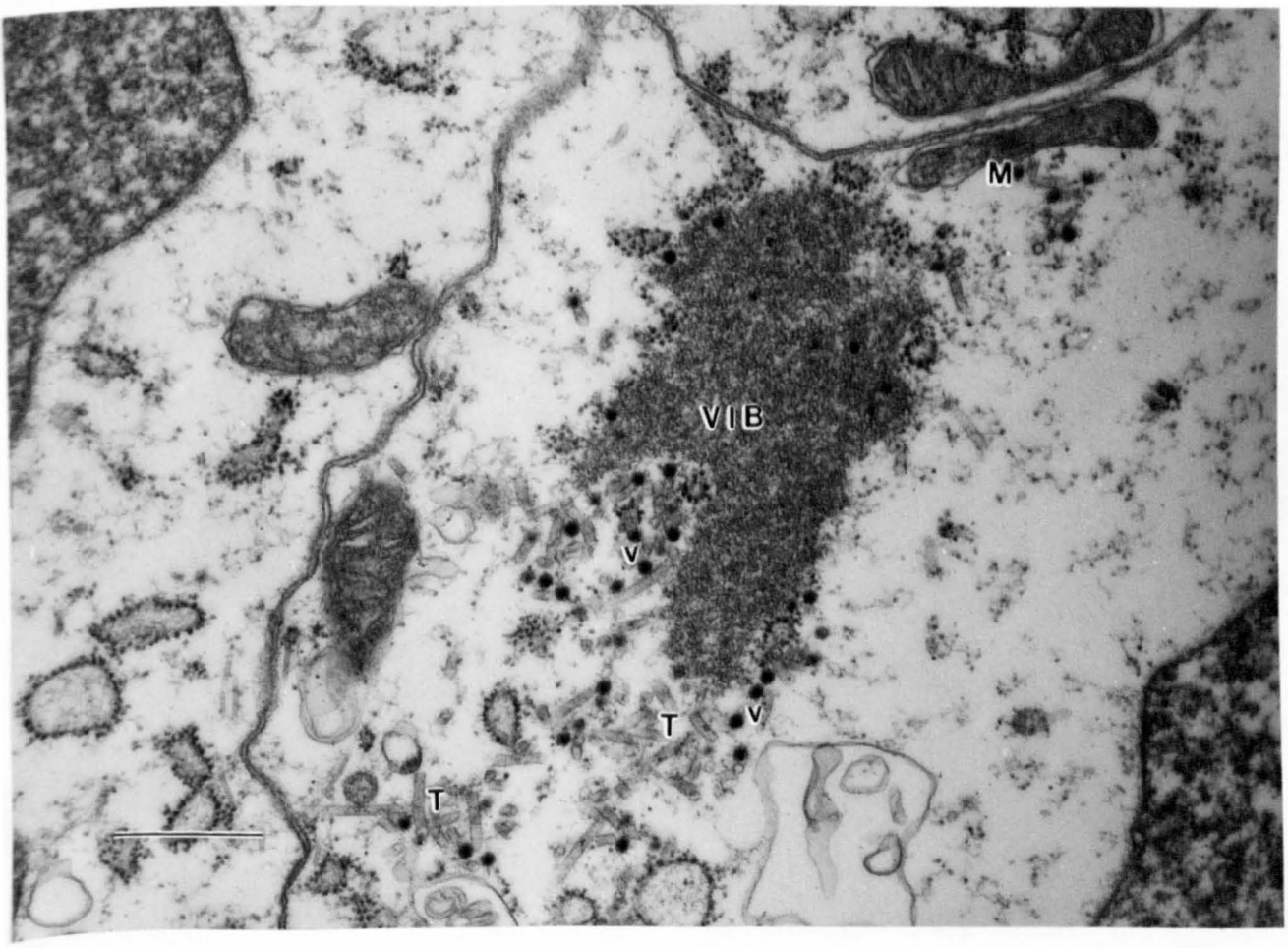

Fig. 4.24 BTV infection of a nerve cell in a thoracic ganglion of *C. variipennis* (10 days pi). **VIB**: viral inclusion body; **V**: virus particles; **T**: tubules; **M**: mitochondria; bar=500 nm

Fig. 4.25 BTV infection of a nerve cell in a thoracic ganglion of *C. variipennis* (10 days pi). **VIB**: viral inclusion body; **T**: tubules; **M**: mitochondria; bar=500 nm

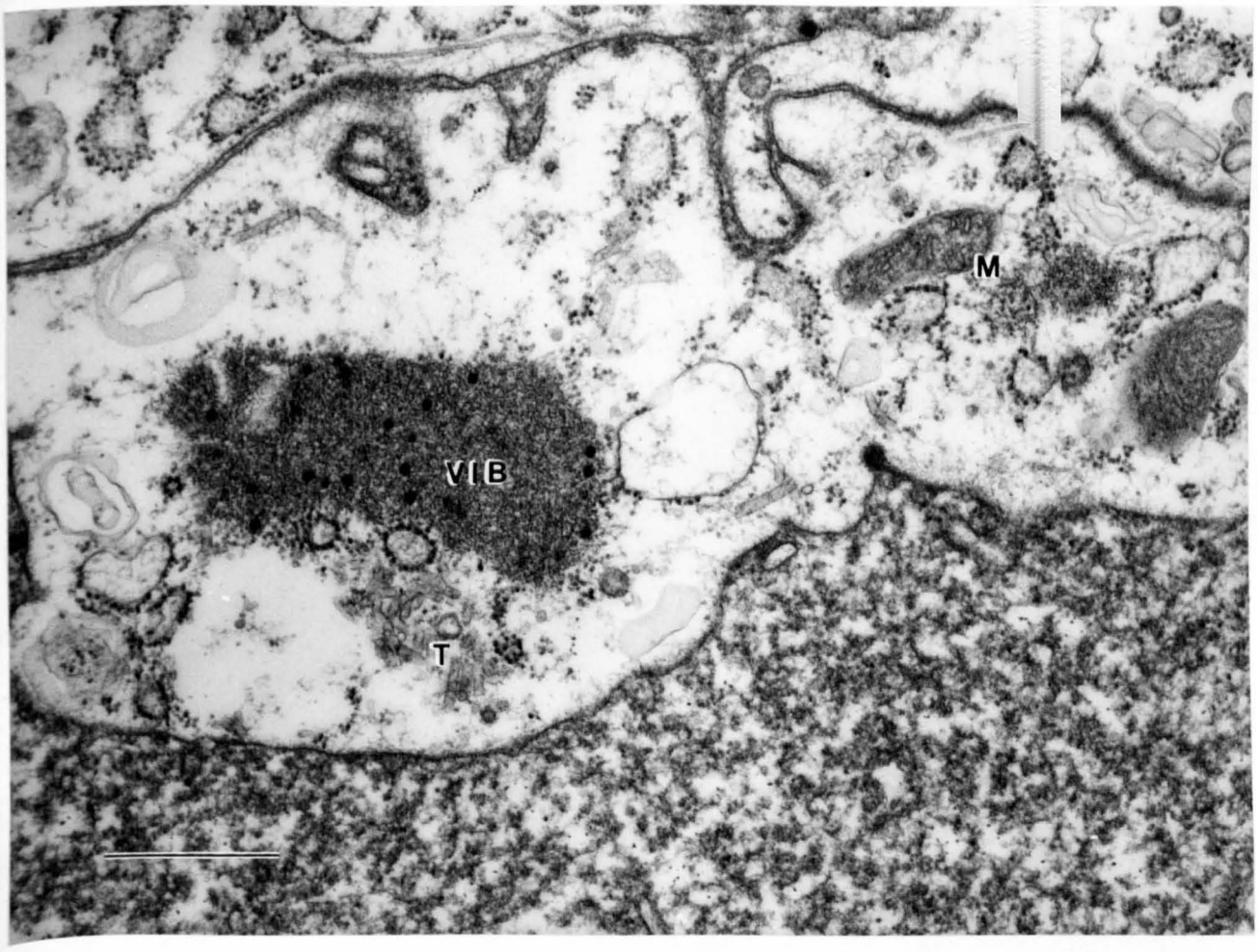

Fig. 4.26 BTV infection of an axon in a thoracic ganglion of *C. variipennis* (10 days pi). **VIB**: viral inclusion body; **T**: tubules; **M**: mitochondria; bar=500 nm

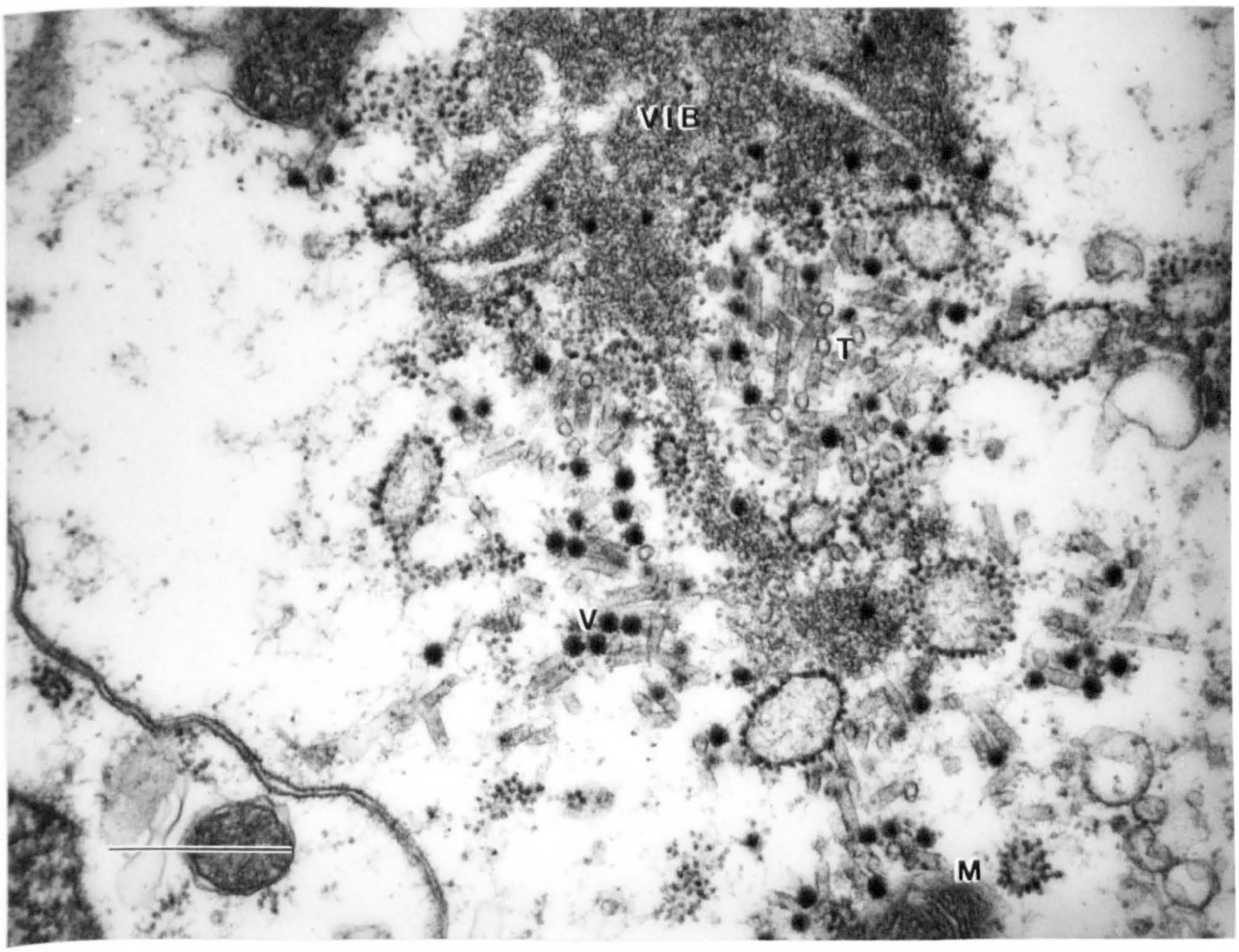

Fig. 4.27 BTV infection of an axon in a thoracic ganglion of *C. variipennis* (10 days pi). **VIB**: viral inclusion body; **T**: tubules; **M**: mitochondria; **V**: virus particles; bar=500 nm

4.3.6 Detection of BTV infection in BHK-21 and KC cells by Immunogold TEM

4.3.6.1 Pre-embedding staining

Only those virus particles or viral antigens, in the process of being released at the cell surface, will be available to be labelled with immunogold conjugates when using the pre-embedding assay. In infected BHK-21 cells, non-enveloped double-shelled virus particles, released onto the cell surfaces, were detected by using both the PAbV and the MAbA3, and the surfaces of these virus particles were consequently labelled with gold particles

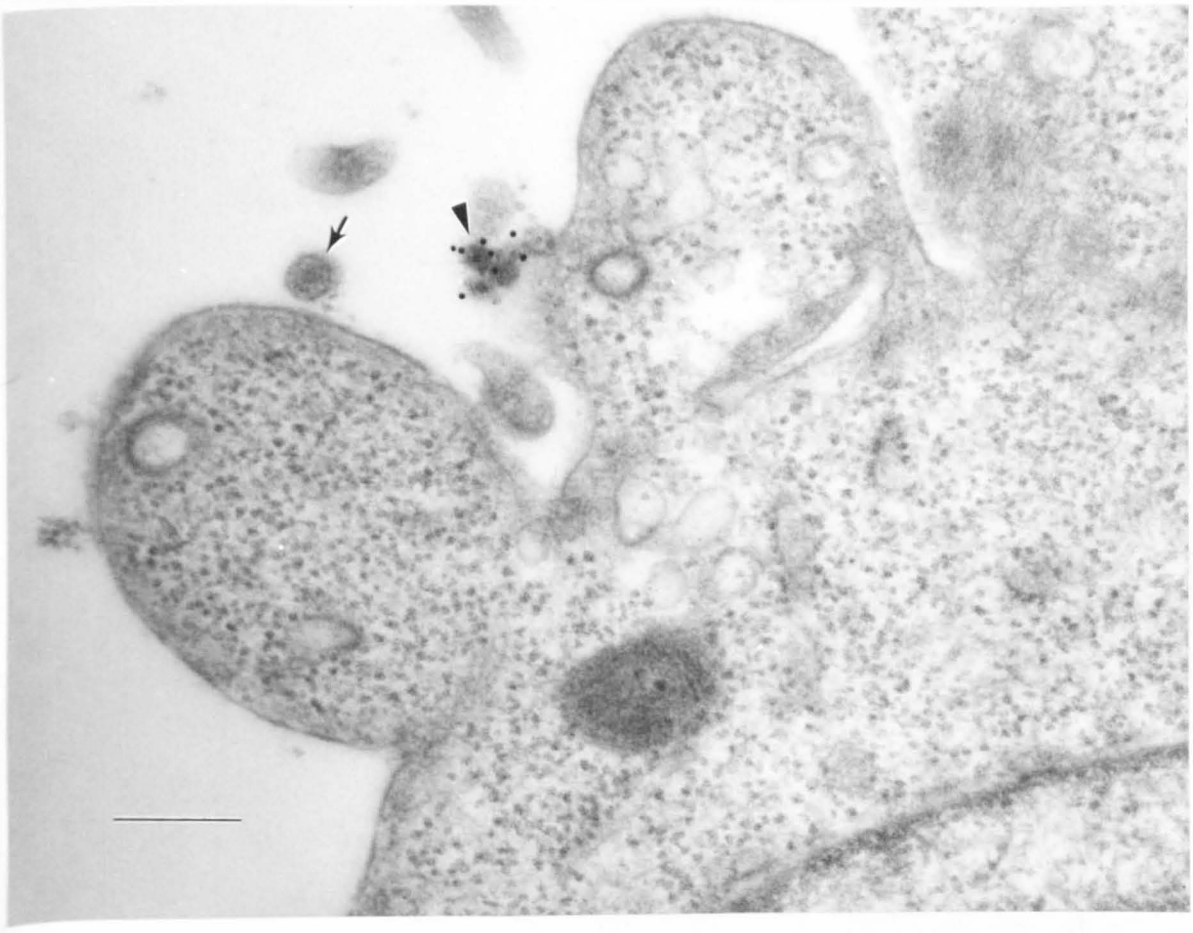

Fig. 4.28 Pre-embedding immunogold staining of BTV infected BHK-21 cell (20 hours pi). ► virus particles labelled with gold conjugate (incubated with PAbV); → An enveloped virus particle is not labelled; bar=200 nm

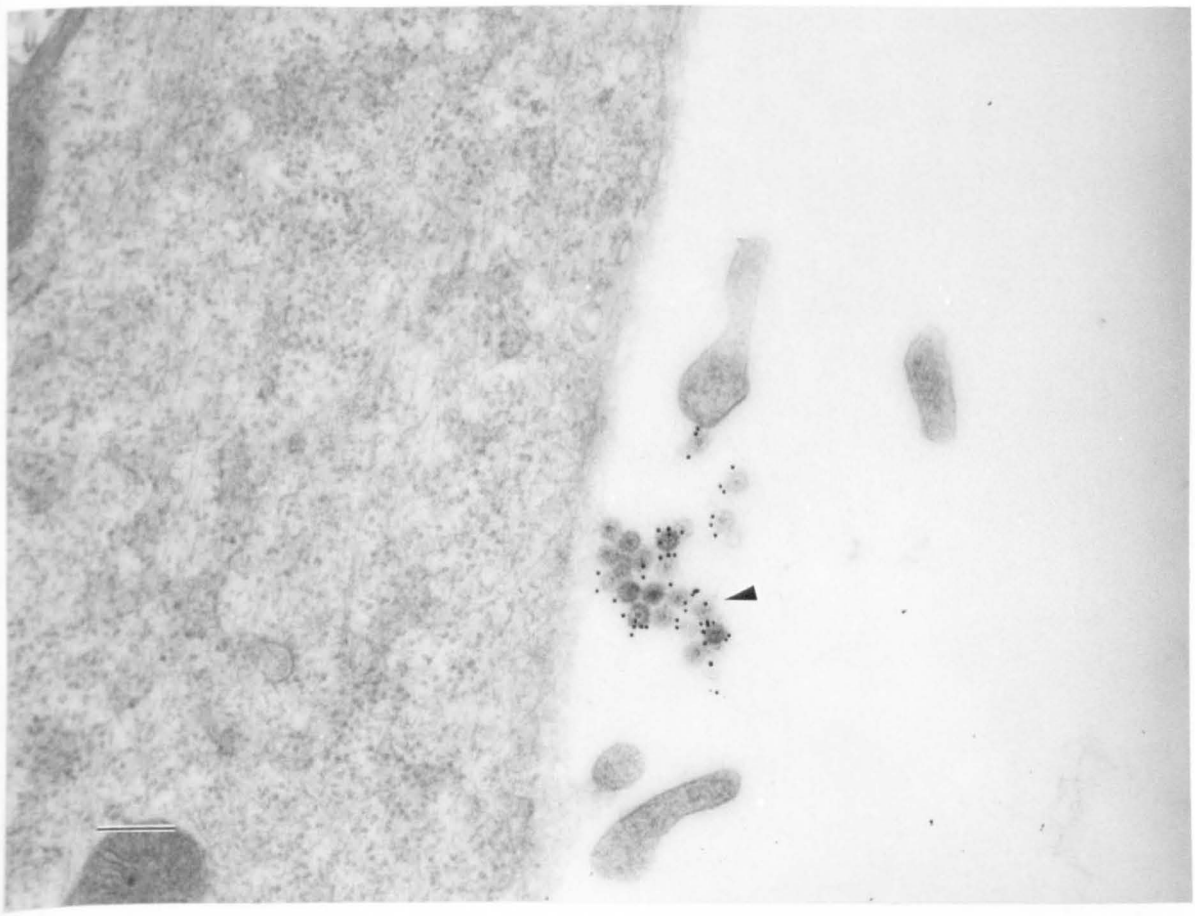

Fig. 4.29 Pre-embedding immunogold staining of BTV infected BHK-21 cell (20 days pi). arrow: gold labelled virus particles (incubated with PAbV); bar=200 nm

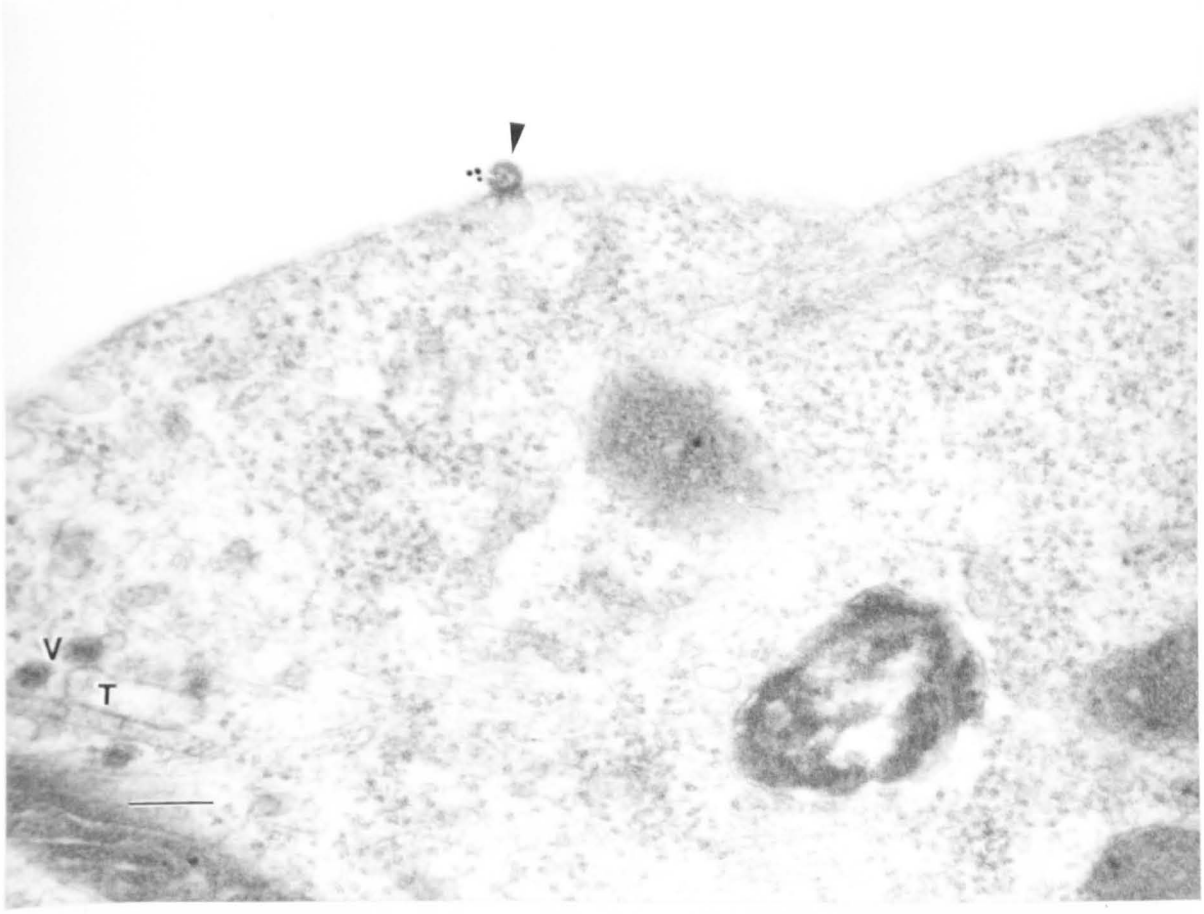

Fig. 4.30 Pre-embedding immunogold staining of BTV infected BHK-21 cell (20 hours pi). arrow: gold labelled virus particle (incubated with MAbA3); V: virus particles; T: tubules; bar=200 nm

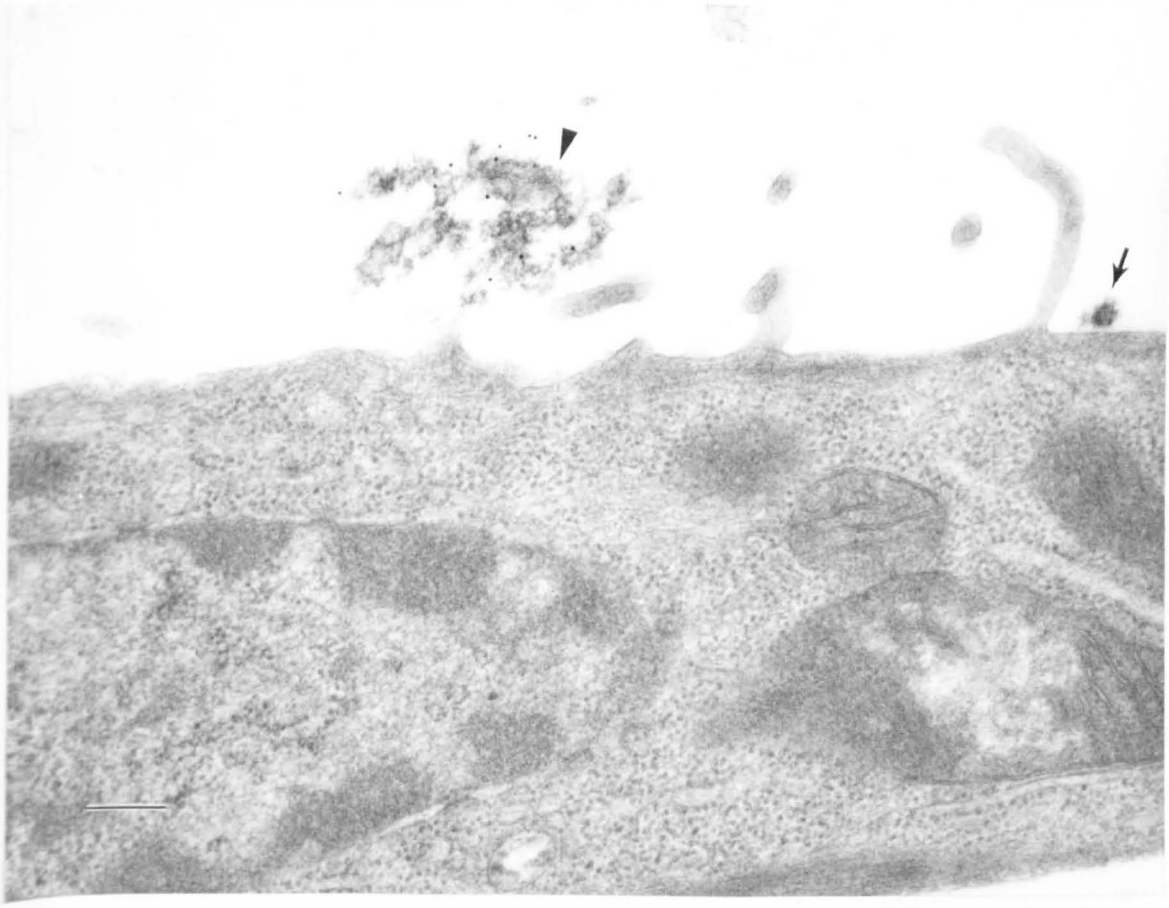

Fig. 4.31 Pre-embedding immunogold staining of BTV infected BHK-21 cell (20 hours pi). ► membrane-like structure labelled with gold conjugate (incubated with PAb10C); → virus particle; bar=200 nm

when either of these antibodies was applied as the first antibody (Fig. 4.8, 4.28, 4.29 and 4.30). Enveloped virus particles, which were released by budding, were not labelled with gold particles (Fig. 4.28). The polyclonal antibody against the viral non-structural protein NS3 polypeptide 10C (PAb10C) did not label any virus particles but did bind to the membrane structures extruding from the cell surface close to where virus particles were budding (Fig. 4.31). No endogenous virus was seen in uninfected BHK-21 cells.

Similar work was carried out with infected KC cells. However, no positive labelling was obtained possibly because so few infected KC cells seem to contain developing virus particles (see Section 4.3.2).

4.3.6.2 Post-embedding staining

Post-embedding immunogold labelling was carried out on the ultrathin sections of both infected BHK-21 and KC cells. However, no BTV-related structures were observed to be labelled with gold particles when using this technique.

4.4 DISCUSSION

Ultrastructural studies of BTV infected cells have shown that there is a series of events from initiation of virus replication to the production of progeny virions (Bowne and Jochim 1967; Lecatsas 1968; Eaton *et al* 1990; Brookes *et al* 1993; Gould and Hyatt 1994). The major events in BTV replication are: adsorption and endocytosis; uncoating; formation of VIBs and tubules; and release of progeny virus particles from the cell surface. After adsorption, endocytosis and uncoating of the infecting virus particles, VIBs form in which progeny virus particles are produced. Previous studies have proved that VIBs contain three populations of virus particles: sub-cores, cores and virus-like particles, in addition to structural and nonstructural viral proteins (VP2, VP3, VP5, VP6, VP7, NS1 and NS2) (Eaton *et al* 1987; Gould *et al* 1988; Eaton *et al* 1988; Eaton and Hyatt 1989; Thomas *et al* 1990; Hyatt *et al* 1991a; Brookes *et al* 1993; Gould and Hyatt 1994).

The results of this study support the premise that VIBs are virus factories of BTV in both

mammalian cells and *Culicoides* salivary gland cells although these structures are bigger in persistently infected KC cells and *Culicoides* salivary gland cells than in BHK-21 cells. VIBs were shown to contain immature virus particles at different stages of development, including nascent core particles and virus-like particles with the more mature particles being towards the periphery, while double-shelled virus particles were only seen outside these structures. This suggests that the production of BTV was in process in VIBs and that progeny virus particles seem to move towards the periphery of the VIBs as they are assembling. Gould *et al* (1988) reported that the outer coat proteins VP2 and VP5 are added at the periphery of VIBs.

The present study confirms the observation of Hyatt *et al* (1989) that progeny virus particles are released through the cell membrane of infected BHK cells either by membrane budding as enveloped particles, or by extruding without acquiring an envelope. As BTV is a non-enveloped virus, the cell membrane envelope surrounding virus particles released by budding is presumably discarded sometime after this event. The virus particles, released by extrusion, were associated with cell surface debris. These two types of virus release are clearly different and may involve different mechanisms. It is also of significance to note that progeny virus particles were released from infected KC cells and salivary gland cells of *Culicoides* only by membrane budding. The non-enveloped extrusion that occurred in BHK cells has not been seen in *Culicoides* cells. The reason for the difference and whether this affects the characteristics of the subsequent stages of BTV infection for each cell type is unknown. It has been reported that the release of virus is mediated by the BTV non-structural protein NS3 (Hyatt *et al* 1991a; Hyatt *et al* 1993; Gould and Hyatt 1994). In this context it was also observed in the present study that NS3 is associated with virus release from mammalian cells. The results of immunogold labelling in my work have shown that NS3 is released along with membrane-like structures, from the surface of infected BHK-21 cells adjacent to where the virus particles are released by budding and by extrusion. Similar work was also done with KC cells but no positive results were obtained because of the characteristically low proportion of cells persistently infected with BTV in infected KC cell culture.

Tubules have long been known to be characteristic structures in cells infected with BTV. Tubules have also been purified (Huismans and Els 1979; Mertens *et al* 1987) from BTV-infected cells and shown to consist predominantly of the nonstructural protein NS1. Furthermore, probing of cytoskeletons of BTV-infected cells using monoclonal antibodies, against specific virus proteins, has confirmed that tubules contain NS1 (Eaton *et al* 1988; Hyatt and Eaton 1988, Eaton *et al* 1990; Gould and Hyatt 1994). The function(s) of the tubules and NS1, which forms the tubular structure, are unknown and virus has not been reported to be associated with these structures (Gould and Hyatt 1994) although NS1 antigen has been reported in BTV particles (Eaton *et al* 1988). In the present study, ultrastructural results showed that large amounts of tubules formed in both mammalian and *Culicoides* cells infected with BTV. These tubules seemed to be associated with VIBs since they were found in the cytoplasm surrounding the VIB matrices. Previous *in situ* hybridisation results have also shown that VIBs contain NS1 mRNA (Gould and Hyatt 1994). These results indicate that NS1 is produced in and is released from VIBs. The results of the present study also suggest that tubules may be related to the double-shelled virus particles since they were frequently co-located with these double-shelled virus particles, but not with subcores, cores or subviral particles. Mature virus particles were frequently seen attaching to tubules and sometimes a number of virus particles lined up on tubules with an identical distance in between. The function (s) of these tubules and the relationship between them and mature virus particles are unclear. However, studies of kinesin, the main protein constituent of microtubule-based molecular motors (Spudich 1994), may suggest a mode of action. This molecular motor works to transport molecules in cells, single molecules moving along a microtubule track for many seconds before dissociating. It is possible that the tubules in BTV-infected cells play a similar role in facilitating the intracellular transport of progeny virus particles.

The present study has shown that the characteristics of BTV replication in a *Culicoides* cell line, KC cells, are different from those in BHK-21 cells and are also different in some aspects from replication in *Culicoides* salivary glands and *Culicoides* nervous tissues. VIBs were only seen in a small proportion (27/200) of KC cells in an infected culture, whereas most cells in infected BHK-21 cell cultures contained these structures, and this was also the case in infected *Culicoides* salivary glands. A low rate of infection of KC cells in

infected cultures (about 50%) was also observed by using immunohistochemistry (see Chapter 2, Fig 2.3). This may be because the KC cell line was originally derived from larval *Culicoides* tissues and still shows a mixed cell population (Fig. 4.14). It is likely that different cell types vary in their susceptibility to BTV.

Large amounts of tubules were always present in the cytoplasm near VIBs. The sizes of the VIBs in persistently infected KC cells, salivary gland cells and nerve cells were much bigger than those in BHK-21 cells and newly infected KC cells (6 hours pi). This suggests that VIBs may be built up during persistent infection. Most of the VIB matrices in the insect systems were irregular and were highly characteristic in appearance with distinctive 'fractures' within their structures. No similar observation has been reported previously. The origin of these 'fractures' is uncertain but it is probably an artifact produced during the processing of the samples for TEM.

Unlike BHK-21 cells, *Culicoides* salivary gland cells and *Culicoides* nerve cells, almost half of the VIBs in KC cells did not contain any obvious viral structures. Nascent core particles and virus-like particles were only observed in 59% of the VIBs in the infected KC cells and these were at a much lower density than those in infected BHK-21 cells and *Culicoides* salivary gland and nerve cells. Progeny virus particles were also seen at low frequency (19/200, Table 4.1) in the cytoplasm of KC cells whereas they were frequently seen in BHK-21 cells (about 70%, Section 4.3.2) and 100% of *Culicoides* salivary gland and nervous tissue samples. Virus particles were rarely seen being released from newly infected KC cells and when they were seen, release was only by membrane budding (Fig. 4.12). However, in BHK-21 cells, virus particles are released either by membrane budding or by extrusion without acquiring an envelope. Virus release was not detected at all from persistently infected KC cells. The nature of these differences is unknown. No similar observations on BTV infection in insect cells have yet been published. However, the results of this study (see Chapter 2) also showed that BTV grew rapidly in KC cells to a level of about $10^{5.0}$ $TCID_{50}$/ml (in media) by 24 hours pi and then increased slowly to reach a peak of $10^{6.5}$ $TCID_{50}$/ml by 48 hours pi. The infection of KC cells was persistent and could last for months at a level around $10^{6.0}$ $TCID_{50}$/ml if the cells were passaged regularly and well maintained. Therefore, there is no doubt that BTV replication is a

continuous and productive process in infected KC cells. A similar phenomenon, concerning an apparent paucity of virus particles, also occurs in mosquito cells infected with Semliki Forest virus and dengue virus (Lehane and Leake 1982). The ability of insect cell lines to produce high titres of virus over extended periods of time, as detected by titration, apparently in the virtual absence of virus particles in infected cells, as detected by electron microscopy seems to be a paradox. It may be related to the low proportion of cells infected at any one time, or to a low level of virus production per infected insect cell. It may also be because replication and release of BTV by insect cells involves mechanisms which are totally different from those in BHK-21 (mammalian) cells. Clearly, the mechanisms controlling or modulating virus production in insect cells are still poorly understood and remain to be elucidated.

The results of the *in vitro* transmission work in Chapter 3 showed that the salivary glands of *C. variipennis* do not present either salivary gland infection or escape barriers to BTV. Based on those results, the salivary glands of IT infected *C. variipennis* were studied using transmission EM. BTV infection of the salivary glands was found to follow a pattern of the virus movement through the basement membrane → acinar cells → the lumen of the ducts (Fig. 4.15). Virus enters the acinar cells from the haemocoel passing through the basement membrane of the salivary glands by endocytosis. BTV then localises and replicates in VIBs in the cytoplasm of serous acinar cells. Mature double-shelled progeny virus particles leave the VIBs to enter the cytoplasm of the cells and are then released into the serous alveoli (acini) that are located on the opposite side of the acinar cells from the basement membrane. The virus particles are then transported through intermediate ducts and accumulate in crystalline arrays in the lumen of the major secretory ducts. No virus is released back to the haemocoel through the basement membrane. In addition, no virus re-enters acinar cells from the acini. This polarised nature of BTV infection in the salivary glands of *C. variipennis* accounts for the accumulation of progeny virus particles in the secretory ducts. This mechanism makes the transmission of BTV through the saliva more likely by ensuring that the maximum number of virus particles is available for transmission during biting.

Thirty three gut samples of *C. variipennis* orally infected with BTV were examined but no evidence has been observed to show BTV replication in the gut cells. Neither have VIBs, tubules or virus particles been found in these gut preparations. The ultrastructural morphology of the guts from orally infected *Culicoides* seemed no difference from those of negative control samples from uninfected midges. This negative result may be due to the relatively low infection rate exhibited by our *Culicoides* colony so that no infected samples were actually examined. However, it may also due to the nature of the gut infection. The results of virus titration (see Chapters 2 and 3) show that BTV replicates only to very low levels in most midges following an infectious blood meal. Additionally, immunohistochemistry shows that positive virus antigen reactions are qualitatively much weaker in the gut tissues than in other infected tissues/organs such as ganglia, haemocoel and salivary glands, even in individuals with fully disseminated infections (see Section 2.4). The combination of low infection rate and low level of observed BTV replication by titration or by IHC inevitably causes considerable difficulties in detecting evidence of virus particles in gut cells. Previous ultrastructural studies of BTV infection of the midgut of *C. variipennis* (Sieburth *et al* 1991) described the presence of virus in blood meals and in the peritrophic membrane in the gut lumen but did not show convincing evidence of virus replication in gut cells. These workers concluded that there was no virus accumulation in the gut cells. In view of these facts it will be necessary for very large numbers of gut samples to be screened before the nature of BTV infection in the gut cells of *Culicoides* can be elucidated and the mechanisms involved can be fully understood.

The nervous tissues of *C. variipennis*, particularly the thoracic and cephalic ganglia, are some of the most susceptible tissues to BTV as has been shown by IHC and by TEM in the present study. These tissues were always heavily infected in those *C. variipennis* that exhibited fully disseminated BTV infections. Furthermore clear evidence of typical BTV replication was seen in nerve cells and axons. Nascent cores and virus-like particles were observed in VIBs in *Culicoides* nerve cells and axons, and large numbers of apparently mature double-shelled virus particles were also widely distributed in the cytoplasm of these cells and axons. Though not previously reported in *Culicoides*, the involvement of nerve tissues in arbovirus infection has been recorded in mosquitoes by several authors including Leake and Johnson (1987) and Romoser *et al* (1992). In the present study

although no obvious pathological changes were observed in infected nerve tissues it is possible that the presence of large amounts of replicating virus in these tissues could alter the behaviour of the individuals involved. In addition, the thoracic and cephalic ganglia are located immediately adjacent to the salivary glands which raises the possibility of these tissues being a direct source of salivary gland infection. Furthermore, large amounts of progeny virus particles released into the haemocoel from infected nerve tissues would also tend to increase the chance of salivary gland infection. Further study on BTV infection of the nervous system of *Culicoides* may help to determine the importance of the role played by these tissues in BTV infection and transmission.

CHAPTER 5
ANALYSIS OF BLUETONGUE VIRUS PROTEINS WHICH BIND TO THE CELL SURFACE

5.1 INTRODUCTION

A key factor in BTV-infection of cells is the initial binding of BTV to the cell surface. In the present study, radiolabelled BTV 1SA lysates were used in adsorption tests to detect viral proteins that bind specifically to the cell surface of BHK-21 and KC cells by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The major purpose of this work was to identify the BTV virus protein(s) which bind to the cell surfaces of mammalian and *Culicoides* host cells and to further investigate the significance of specific virus-insect interactions in determining the susceptibility of *C. variipennis* to BTV.

5.2 MATERIALS AND METHODS

5.2.1 Virus, cells and antibodies

BTV 1SA, BHK-21 cells and KC cells used in this study were as described in Chapter 2.

A polyclonal antibody against BTV 1SA (PAbV) and a monoclonal antibody against the BTV major core protein VP7 (MAbA3) were provided by Dr. J. Anderson and Mrs J. Thevasagayam at the IAH Pirbright. Polyclonal antibodies against VP2 (PAbVP2), VP5 (PAbVP5), NS2 (PAbNS2) and a monoclonal antibody against NS1 (MAbNS1) were provided by Dr. A Wade-Evans also at Pirbright.

5.2.2 Preparation of the radiolabelled BTV infected cell lysates

BTV viral proteins were labelled with ^{35}S-express by using a method modified from those of Lee *et al* (1981), Greenberg *et al* (1983) and Bass *et al* (1990). BHK-21 cells were grown in petri dishes (100 cm) and the cell monolayers were infected with 10 $TCID_{50}$

virus particles per cell of BTV 1SA in serum-free Eagle's medium. After adsorption for an hour at 37°C, the monolayers were washed three times (5 minutes each) and then refed with Eagle's medium containing 2 mM glutamine and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). The cells were then incubated at 37°C for 6-8 hours before being washed three times (5 minutes each) and refed with Eagle's medium minus methionine and cysteine. After 45 minute incubation at 37°C, the cells were again washed once (5 minutes), refed with Eagle's medium minus methionine and cysteine and were then incubated at 37°C. Forty five minutes later, [^{35}S]-express (Du Pont NEN) (20 μCi/ml) was added to the medium and the radiolabelling was carried out at 37°C for 3 hours. Alternatively, the infected cells were radiolabelled for 2-3 h at 20 h pi. The cells were washed three times (1 minute each) with PBS (pH 7.3), and were frozen (-70°C) and thawed (room temperature, RT) three times. Finally, the cells were lysed with Nonidet P-40 lysis buffer [120 mM NaCl, 50 mM Tris (pH 8.0) and 1% Nonidet P-40] or with Triton X-100 lysis buffer [1% Triton X-100, 0.8 M KCl, 10 mM Tris (pH 7.8)]. PMSF (Phenylmethylsulfonyl Fluoride, 100 μg/ml), TLCK (Na-p-Tosyl-l-Lysine Chloromethyl Ketone, 50 μg/ml) and TPCK (N-Tonsyl-l-Phenylalanine Chloromethyl Ketone, 100 μg/ml were added to the lysis buffer to inhibit the activity of cellular proteases. The lysate was incubated for 30 minutes at 4°C and then centrifuged for 5 minutes at 6,000 g. The supernatant was collected in a screw capped Eppendorf tube and stored at -70°C. ^{35}S-labelled lysate from uninfected BHK cells was used as a negative control.

5.2.3 Radiolabelled VP5 and NS1 expressed from cDNA recombinants

^{35}S-methionine labelled BTV structural protein VP5 and nonstructural protein NS1 were both provided by Dr. A. Wade-Evans. They were expressed from cDNA clones using a coupled transcription-translation system in rabbit reticulocyte lysate according to the manufacture's instructions (Promega).

5.2.4 Adsorption assay

Ten millilitres of cold radiolabelled lysate, diluted 1:30-50 in PBS (pH 7.3), was added to a petri dish (100 cm) containing either a BHK-21 monolayer or a KC cell monolayer.

Adsorption was carried out with gentle rocking on a shaker for 2-3 hours at 4°C. The cells were gently suspended by pipette and centrifuged at 4,000 g for 5 minutes. The cell pellets were washed at least three times with cold PBS and were then lysed with 1 ml Laemmli sample buffer for analysis on SDS polyacrylamide gels. The cells were lysed with 1 ml 1% Nonidet P-40 in PBS for 30 minutes at 4°C and then centrifuged at 6,000 g for 5 minutes. The supernatant was used for immunoprecipitation.

5.2.5 Immunoprecipitation

The immunoprecipitation assays were carried out using the method described by Bass *et al* (1990). Radiolabelled BTV infected cell lysate and the cell lysate after adsorption were diluted 1:20 with PBS (1 ml) and were incubated overnight at 4°C with the different antibodies (as detailed above) separately. Thirty µl of Sepharose CL-4 protein A beads (Sigma Chemical Co.) were then added to each reaction. After being incubated at RT for 30-60 minutes, the beads were washed three times with washing buffer (50 mM NaCl, 10 mM Tris, 0.1% Nonidet P-40) and were then washed once with 50 mM Tris buffer (pH 6.8). Laemmli sample buffer was added and the samples were boiled for 10 minutes before the supernatants were loaded onto 10% SDS polyacrylamide gels. Radiolabelled uninfected cell lysates before and after adsorption were used as negative controls. Infected cell lysates were used as positive controls.

5.2.6 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

10% SDS-PAGE gel with a 1% stacking gel was prepared by following the conventional method (Sambrook *et al* 1989). Twenty µl of each infected cell lysate and 80 µl of each lysate of cells after the adsorption test were loaded onto the gel. Electrophoresis was carried out under a constant voltage, 50V, overnight. The gels were fixed in 10% acetic acid-30% methanol overnight, then dried for two hours at 80°C and exposed to X-ray film for 2-7 days at RT. The concentration of protein was not measured since the amounts of protein in the different samples loaded onto the gels were not directly comparable (see footnote on page 123).

5.3 RESULTS

Fig. 5.1, provided through the courtesy of Dr. A Wade-Evans, is used as the reference for the migration of BTV 1SA viral proteins. It shows the SDS-PAGE (10%) profile of BTV 1SA viral proteins (except VP1) expressed *in vitro* from cDNA recombinants.

BHK-21 cells and KC cells were incubated with radiolabelled BTV infected cell lysates at 4°C, washed extensively and then subjected to SDS-PAGE. 1% Nonidet P-40 lysis buffer or 1% Triton X-100 lysis buffer were used. Work carried out at the beginning of this study showed that Triton X-100 lysis buffer was not able to disrupt BTV particles. Therefore a profile of whole virus binding to BHK-21 was consistently observed on SDS PAGE gel when using Triton X-100 lysis buffer (Fig. 5.2). However, BTV virus particles were disrupted when the Nonidet P-40 lysis buffer was applied as shown by the fact that most of viral protein bands disappeared after the adsorption test. Therefore Nonidet P-40 lysis buffer was used for further work throughout this study. Fig.5.2 compares the activity of Nonidet P-40 lysis buffer and Triton X-100 buffer for disrupting BTV, by adsorption experiments on BHK-21 cells. A similar profile of whole virus binding, using viral proteins prepared with Triton X-100 lysis buffer, was also observed for KC cells (results not shown). Viral proteins were identified by their migration on SDS-PAGE gels relative to marker proteins of known molecular weight.

The results showed (Fig.5.2 5.3) that five viral proteins adsorbed to cell surfaces. A 60-kD viral protein, migrating to a position close to both VP5 and NS1, bound to both BHK-21 and KC cells. However it is difficult to identify precisely which protein this band was without further experiments since VP5 (predicted molecular weight 59.4kD)(A.Wade-Evans, personal communication) and NS1 (predicted molecular weight 64.3kD) migrated very closely to each other in the gel. In addition, a second viral protein of 48kD, co-migrating with NS2 (predicted molecular weight 40.7kD), adsorbed to BHK-21 cells but not to KC cells. A viral protein of 39.6kD, which bound to KC cells but not to BHK-21 cells, co-migrated with VP7 (predicted molecular weight 38.6 kD). A viral protein, co-migrating with the minor inner capsid protein VP6, was seen to bind to BHK-21 cells but not to KC cells (Fig. 5.2, 5.3). Another viral protein, co-migrating with the major inner

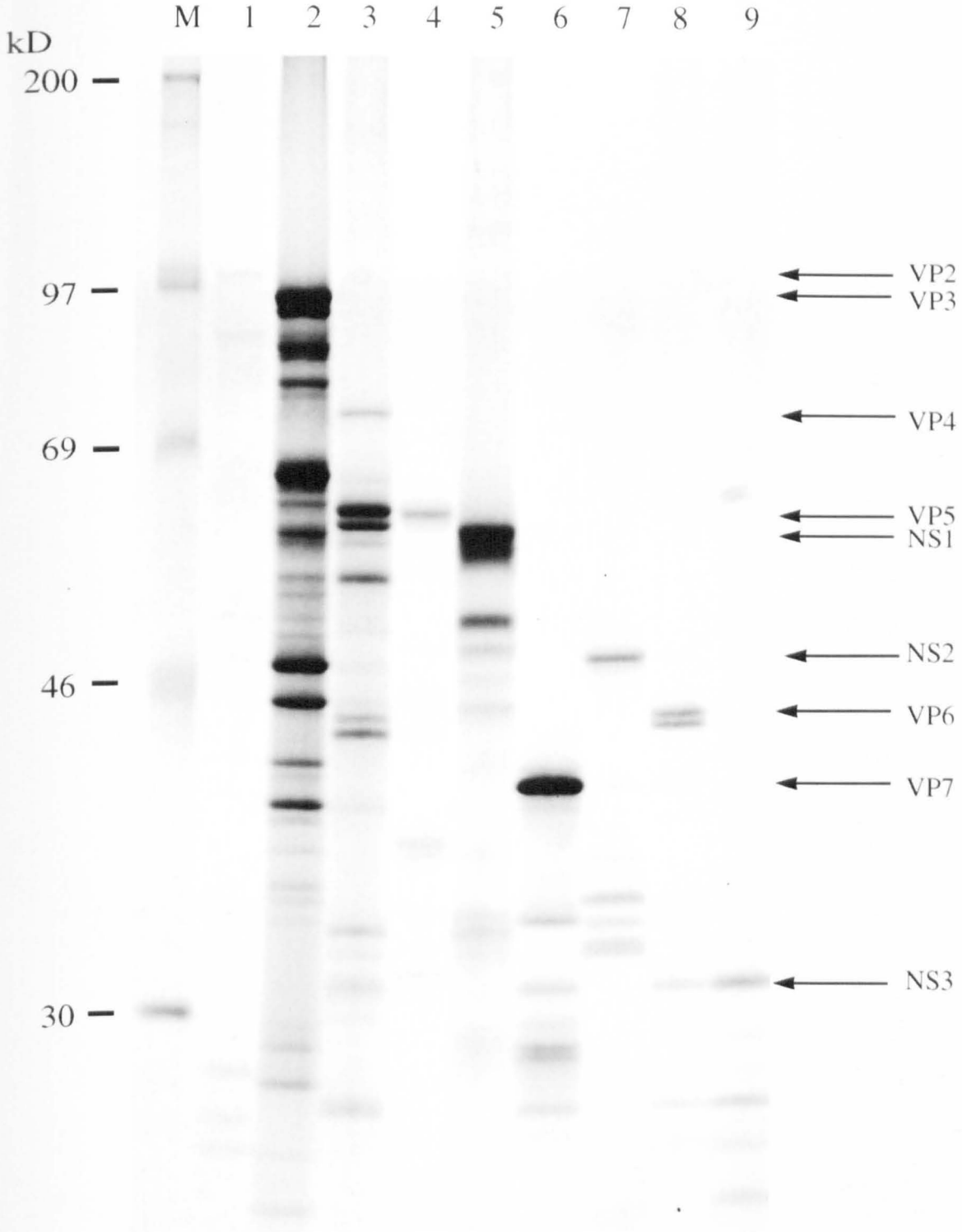

Fig. 5.1 SDS-PAGE profile of BTV proteins expressed *in vitro* from cDNA recombinants. M: molecular marker; Lane 1-9: BTV viral proteins encoded by genome segment 2-10 (courtesy of Dr. A. Wade-Evans).

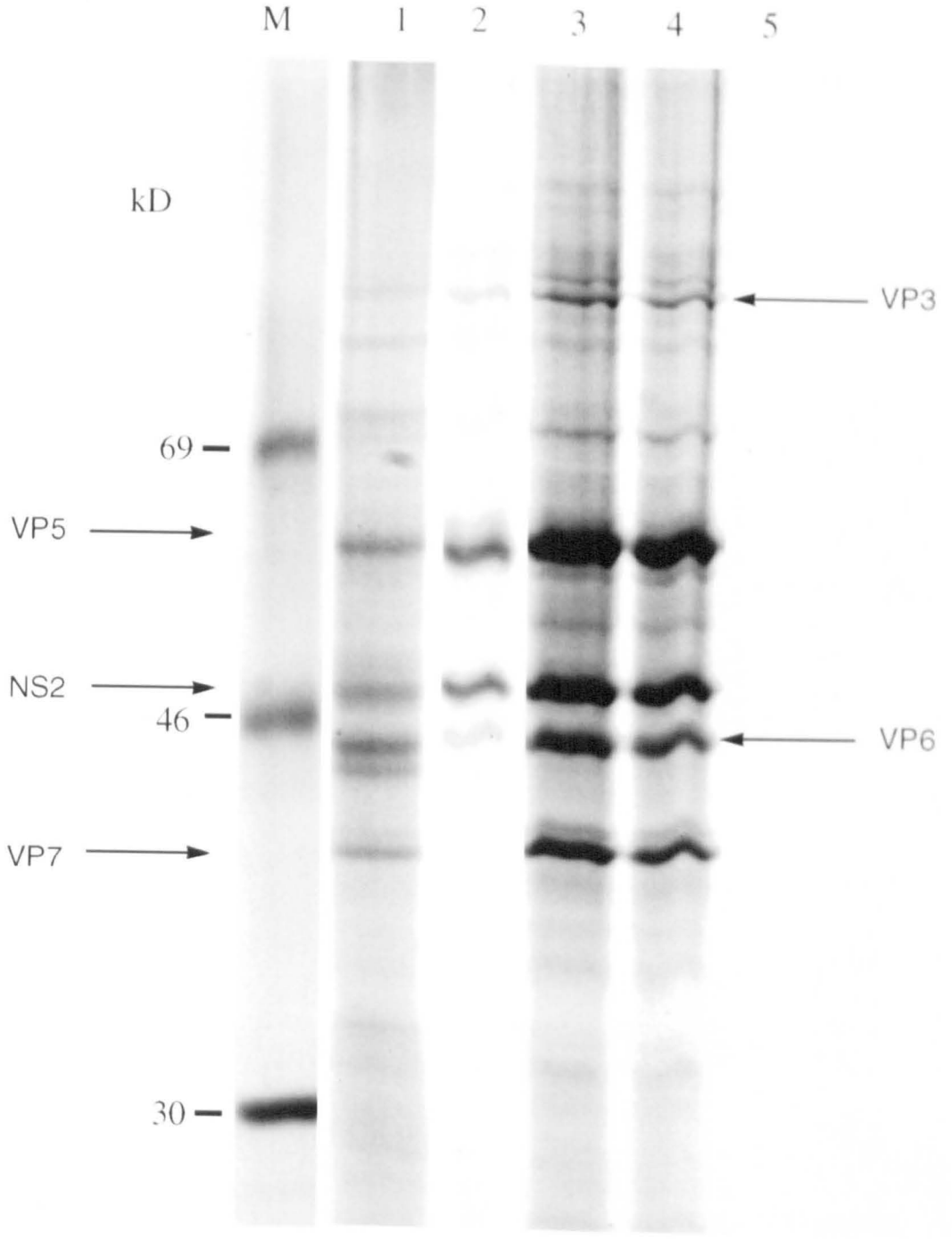

Fig. 5.2 Comparison of the activity of Nonidet P-40 lysis buffer and Triton X-100 lysis buffer for disrupting BTV by SDS-PAGE. Lane 1: BTV infected cell lysate lysed with Nonidet P-40 buffer (20μl); lane 2: lysate of BHK-21 cells (80 μl) after incubation with the BTV infected cell lysate (1:30) in lane 1; lane 3: BTV infected cell lysate lysed with Triton X-100 buffer (20 μl); lane 4: lysate of BHK-21 cells (80 μl) after incubation with the BTV infected cell lysate (1:30) in lane 3; lane 5: BHK-21 cells after incubation with uninfected BHK-21 cell lysate.

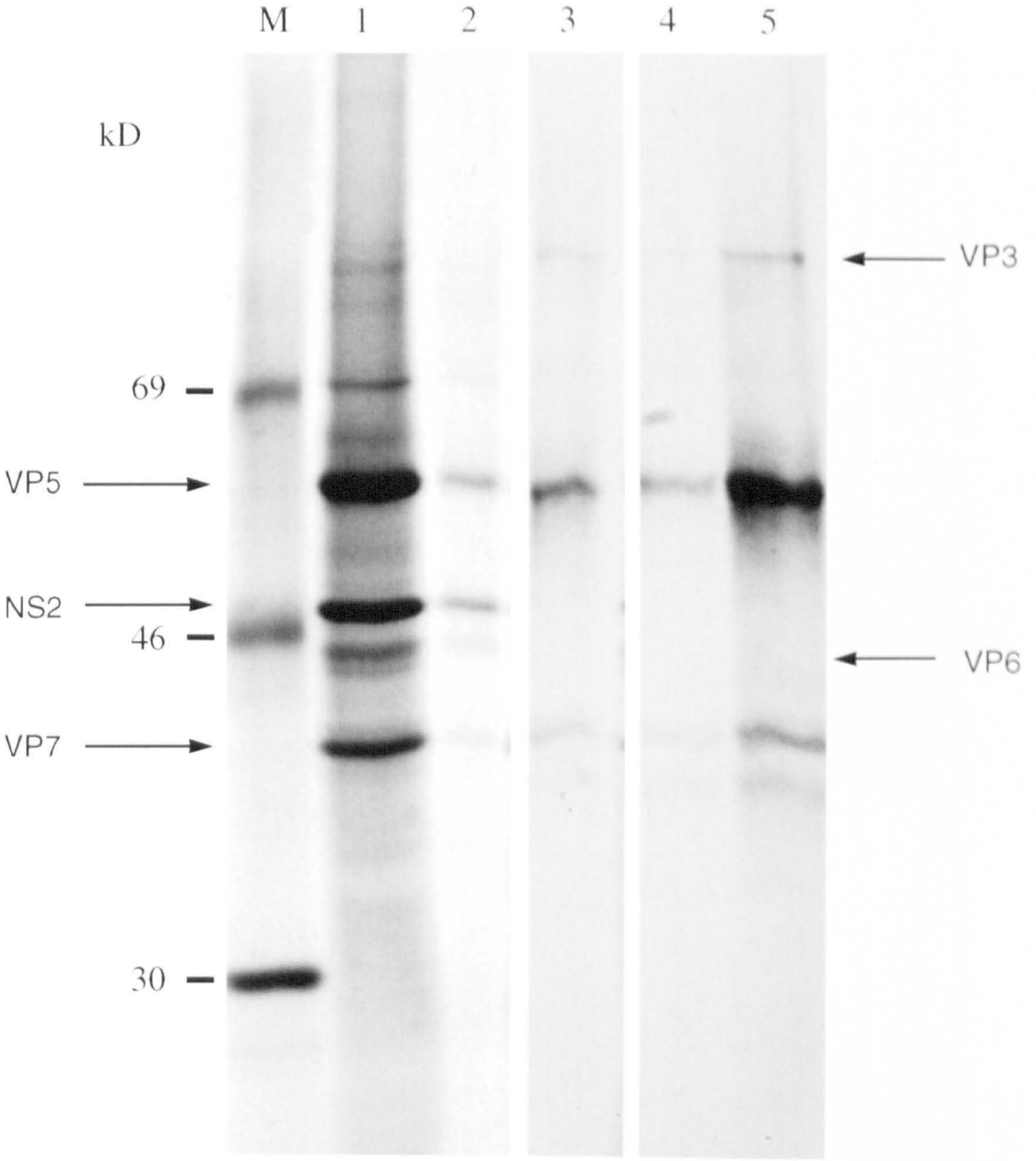

Fig. 5.3 Binding of BTV proteins to cell surfaces (SDS-PAGE). M: molecular marker; lane 1: BTV proteins in infected cell lysate in Nonidet P-40 buffer (20μl); lane 2: lysate of BHK-21 cells (80μl) incubated with th BTV infected cell lysate (diluted 1:30); lane 3: lysate of KC cells (80μl) incubated with the BTV infected cell lysate (diluted 1:50); lane 4: lysate of KC cells (80μl) incubated with the BTV infected cell lysate (diluted 1:60); lane 4: lysate of KC cells (80μl) incubated with the BTV infected cell lysate (diluted 1:30).

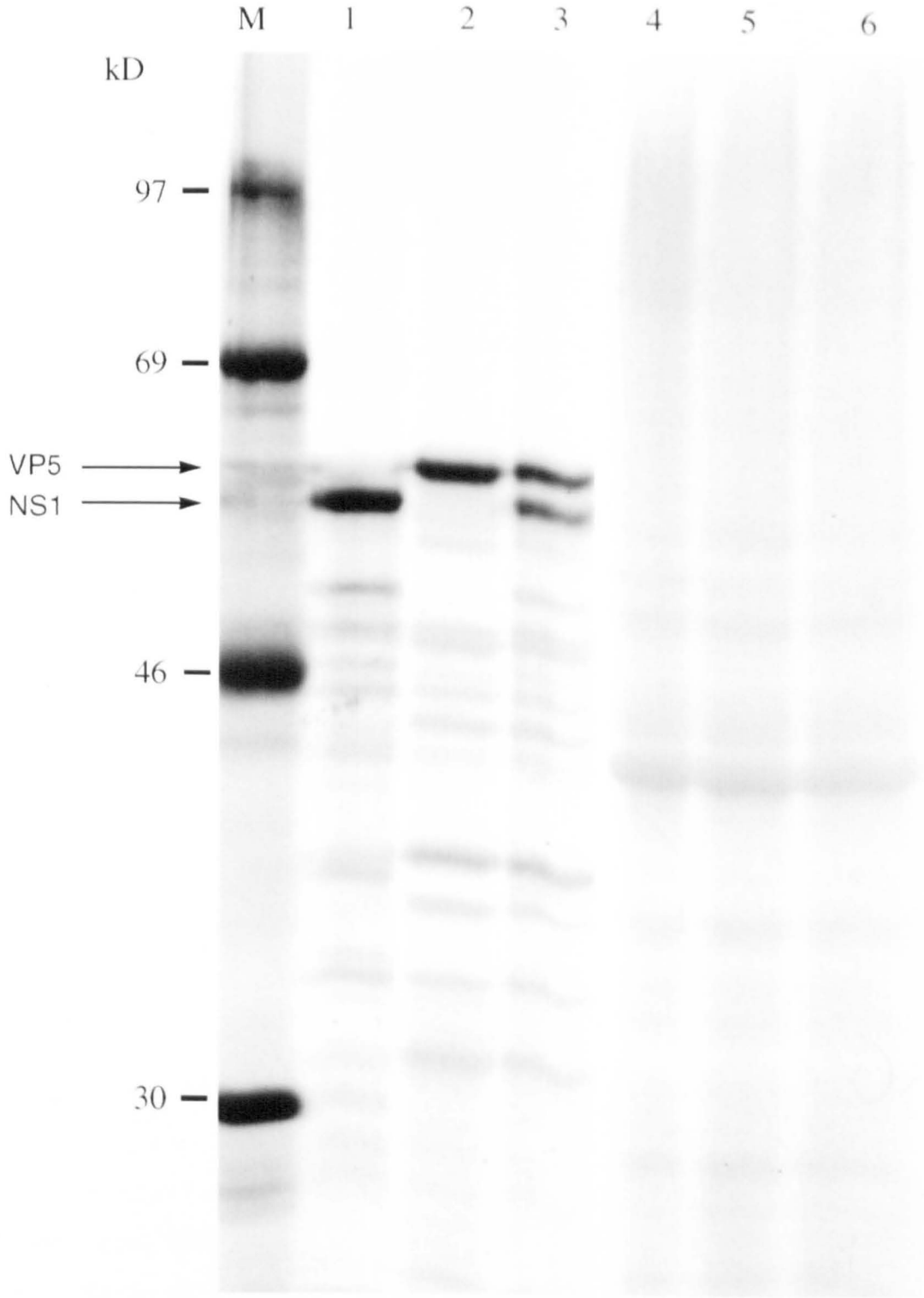

Fig. 5.4 Binding tests of VP5 and NS1 expressed *in vitro* from cDNA recombinants (SDS-PAGE). M: molecular marker; lane 1: expressed NS1; lane 2: expressed VP5; lane 3: expressed VP5 + NS1; lane 4: cells incubated with NS1 in lane one; lane 5: cells incubated with VP5 in lane 2; lane 6: cells incubated with VP5 + NS1 in lane 3.

capsid protein VP3, was also observed in the binding products to both BHK-21 and KC cells (Fig. 5.2, 5.3). No labelled protein from uninfected negative control cell lysates adhered to either BHK-21 or KC cells. No attachment was detected of a labelled protein which migrated with VP2 (predicted molecular weight 112 kD) to either BHK-21 cells or KC cells.

To further investigate the nature of the 60-kD protein which bound to both BHK-21 cells and KC cells, binding tests involving VP5 and NS1 (expressed *in vitro* from cDNA clones in the coupled express system in rabbit reticulocyte lysates), were done in this study (Fig. 5.4). However, the results of these binding experiments using expressed VP5, NS1 and VP5+NS1 were negative. Three *in vitro* expressed lysates with VP5, NS1 or VP5+NS1 showed the same protein profiles after being incubated with cells while no band corresponding to VP5 or NS1 was observed to bind to the cells. It is therefore likely that the protein bands which were detected adhering to the cells in this experiment originated from the rabbit reticulocyte and hence this work should be repeated.

5.4 DISCUSSION

It is believed that BTV initiates infection in both mammalian cells and insect cells by binding to specific receptor(s) on the cell surface (Eaton *et al* 1990; Gould and Hyatt 1994). This is known to be a specific interaction between the cell receptor(s) and BTV viral protein(s). However, no result has been published so far to describe the nature of either the cellular receptors or the viral binding protein(s). However, analysis of BTV-erythrocyte interaction has indicated that the virus binds to specific sialic acid-containing, serine-linked oligosaccharides in the glycophorins of human and a number of animal erythrocytes (Eaton and Grameri 1989). The outer coat protein VP2 is the principal serotype-specific antigen of BTV and induces neutralizing antibodies (Huismans and Erasmus 1981, Kahlon *et al* 1983). VP2 is commonly believed to mediate virus binding to cells (Eaton *et al* 1990) since BTV-10 particles lacking VP2 but containing VP5 were unable to bind to BHK-21 cells in suspension (Huismans *et al* 1983) and the ability of BTV to agglutinate erythrocytes correlates to the presence of VP2 (Cowley and Gorman 1987).

Mertens *et al* (1987, 1993, 1995) have produced infectious subvirus particles (ISVP) and cores of BTV by cleaving VP2 with trypsin and chymotrypsin and by uncoating either ISVPs or intact virus particles respectively. They have shown that changes on the surface of BTV particles can influence the infectivity of the virus for host cells (mammalian and insect cells) and for a vector species of *Culicoides* (*C. variipennis*). ISVPs appear to be much more infectious for mammalian cells, insect cells and vector *Culicoides* than do intact virus particles and core particles. Cores have very limited infectivity for mammalian cells but have a similar infectivity to intact virus particles for vector *Culicoides*. The results of this study (see Chapter 2) also show that ISVPs initiate BTV replication earlier than do intact virus particles in both mammalian cells and *Culicoides* cells. However, cores, at a dose equivalent to 10^3 $TCID_{50}$/ml, cannot induce infection in mammalian cells but are as infective as intact virus particles for *Culicoides* cells. This information suggests that the initial stage of BTV core-insect cell interaction and entry involves different cell receptors, different viral binding proteins or different mechanisms to those involving ISVPs or intact virus particles. It is also suggested that there is a viral protein-receptor interaction or mechanism in *Culicoides* cells, which does not exist in mammalian cells.

It has been previously reported that the host cell translation in mammalian cells is "shut off" during the late stages of BTV infection (Mertens *et al* 1984). Using BTV 1SA at a multiplicity of infection dose of 1 plaque forming unit (PFU) per BHK-21 cell, shut off is effectively complete by 10 hours pi (Mertens *et al* 1984). The proteins labelled after shut off, were also found in *in vitro* translation products from total denatured viral genome RNA and are thought to represent virus encoded proteins (Mertens *et al* 1984). In the present study, the major protein bands which were labelled in infected BHK-21 cells at a late time point (9-11 h pi) and at a higher multiplicity of infection dose 10 $TCID_{50}$/cell (equivalent to approximately 6 PFU/cell) are therefore all believed to be viral proteins (PPC Mertens personal communication). Labelling of BTV proteins at 20 h pi at a multiplicity of infection equivalent to 6 PFU/10^4cells was also carried out in this study and showed the same profile of labelled proteins compared with the early labelling.

The results of the present study are very preliminary but they do suggest some tentative conclusions concerning the binding of BTV proteins to cells. Three viral proteins were

bound to BHK-21 or KC cells. One of them, a 60-kD viral protein was bound to both mammalian cells and *Culicoides* cells. The 60-kD viral protein may correspond to either the outer coat protein VP5 or the nonstructural protein NS1 but it is difficult to be specific because the bands of VP5 and NS1 migrate very closely to each other during SDS-PAGE Further studies would be needed to confirm the identity of this protein band, but this results could indicate some role of VP5, which is an outer capsid protein, in binding of the virus particles to both mammalian or insect cells. NS1 is not thought to be a likely participant in cell attachment by BTV particles since it is exclusively non-structural.To confirm any role on cell attachment and the identity of the protein concerned further studies are required, including immunoprecipitation and western blotting of viral binding proteins and also competition assays using single viral proteins and the different BTV particle types.

A 48-kD viral protein, corresponding to the nonstructural protein NS2, and a 40-kD viral protein co-migrating with NS2A, both bind to mammalian cells but not to *Culicoides* cells. Small amounts of NS2 have been reported to be associated with the outer capsid layer of the purified BTV particle (Mertens *et al* 1987). In addition, Mertens *et al* (1987) showed that BTV ISVPs do not possess haemaglutinating (HA) activity, suggesting that the cleavage of VP2, or loss of NS2 which both occur during conversion of the intact virus particles to ISVP reduces the HA activity of the virus. These authors also found that the highest level of HA occurred with incompletely purified virus while fully purified material had a much lower HA titre. The virus protein which correlated best, in terms of relative concentration, with HA titre was NS2 (PPC Mertens personal communication). It is therefore possible that NS2 may be involved in haemaglutination and virus binding to mammalian cells. A previous study on rotavirus infection showed that a nonstructural protein (NS35) may be involved in attachment to target cells (Bass *et al* 1990). My results suggest that the binding of BTV to the cell receptor(s) of mammalian cells is a mechanism which may involve both the 60-kD protein and NS2. NS2 is a phosphorylated protein and its migration on SDS-PAGE gels varies (Roy 1992). However its position in the order of BTV protein migration on SDS-PAGE gels is constant.

A 39.6-kD viral protein, which adheres to *Culicoides* cells but not to mammalian cells,

co-migrated with the major core protein VP7. This result may explain why core particles of BTV can infect *Culicoides* cells and vector species *Culicoides* but are relatively much less infectious for mammalian cells. It also suggests that *Culicoides* cells present receptors on their surface that recognises VP7, which may not exist on the surface of mammalian cells. This factor may be especially important in the natural, oral infection of vector *Culicoides* because the secretion of digestive enzymes into the midgut in midges, such as occurs in mosquitoes, tsetse flies, tabanids and sandflies (Champlain and Fisk 1956; Akov 1972; Gooding 1972; Spiro-Kern and Chen 1972; Briegel and Lea 1975; Thomas and Gooding 1976; Houseman 1980; McFarlane 1985; Clements 1992) may modify intact virus particles to produce ISVPs or cores. During this process VP7 is likely to become more exposed on the surface of the virus particles as they are digested or modified in the lumen of the gut. Therefore, ISVP (produced naturally in the gut) would be expected to be more infectious than intact virus particles and even if the virus were completely uncoated during digestion, cores, by virtue of the exposed VP7 would still be infectious for the gut cells.

In the present study no viral binding protein has been shown that co-migrates with intact VP2. This may mean that VP2 was not presenting the correct confirmation in the cell lysate and did not adsorb to cell receptors at the initial stage of BTV infection or even that there is no VP2-specific receptor on the surfaces of either mammalian or *Culicoides* cells. However it cannot yet be confirmed that the binding proteins observed in this study are not degradation products of VP2 as suggested by Huismans *et al* (1983), Cowley and Gorman (1987) and Eaton *et al* (1990), or that VP2 is not degraded during any cell binding and uptake mechanism as might be expected if it is taken up by endosomes/lysosomes. In this context, it is possible that the 60-kD viral protein which binds to both mammalian and *Culicoides* cells, could be a degraded VP2 which does not co-migrate with the intact protein. A previous study (Mertens *et al* 1987) has already shown that two cleavage products from VP2, VP2a and VP2b, remain associated with the ISVP. One of them, VP2b migrates to a position very close to VP5 in SDS-PAGE gels. In the light of this information, future work is clearly necessary to identify the origin of the 60-kD viral binding protein and establish its relationship, if any, with VP2 and other viral proteins.

A viral protein, co-migrating with VP3, was also consistently observed to bind to both BHK-21 and KC cells and a viral protein, co-migrating with VP6, was seen to adhere to BHK-21 cells but not to KC cells. VP3 and VP6 are inner core proteins, are not exposed on the surface of the BTV particle and are therefore thought unlikely to be involved in the binding of the virus to cell surfaces. The binding of these proteins may be "real" in the context of these experiments but is probably of limited significance in the natural situation.

In an attempt to confirm the origin of the 60-kD viral binding protein, preliminary adsorption tests have already been done in the present study using VP5, NS1 and VP5+NS1 expressed from cDNA recombinants in rabbit reticulocyte lysates. However neither VP5 nor NS1 were observed to bind to mammalian cells. It is possible that these proteins when expressed *in vitro* may have a conformation which is different from that of natural proteins. It may also be that the binding of the 60-kD viral protein to a cell receptor requires the presence of other viral protein(s) such as NS2 or VP7, i.e. the binding domain of the virus may involve more than one protein. It is also possible that the 60-kD protein is neither VP5 nor NS1 but an entirely different protein, such as a degraded VP2, as has already been suggested. These questions all need to be resolved.

Immunoprecipitation tests have also been carried out in this study to attempt to identify the viral binding proteins but have not yet been successful although the method has been shown to be efficient in a previous study on rotavirus binding proteins (Bass *et al* 1990). The lack of success in the present study is probably related to the ratio of viral binding protein-cell lysates, to the antibodies. This is difficult to adjust but an optimum ratio of antigen to antibody is very important in order to maximise the amount of viral protein being trapped by Protein A. Too much or too little antibody can produce false negative results. In addition, the characteristics or concentration of the detergent in the cell lysates may also affect the antigen-antibody reaction.

In summary, these preliminary results suggest that three BTV viral proteins were shown to be involved in binding to host cell surfaces. A 60-kD viral protein which adheres to both mammalian cells and *Culicoides* cells. A 44-kD viral protein, co-migrating with NS2,

adsorbed to mammalian cells but not to *Culicoides* cells, and a 39.6-kD protein, co-migrating with VP7, which adheres to *Culicoides* cells but not to mammalian cells. However more work needs to be done to confirm and further characterise these BTV proteins and their significance in cell attachment.

* Twenty μl of BTV infected cell lysate and 80 μl of each binding product were loaded on the gels for SDS-PAGE. The amounts of protein in the samples were not measured because: 1) the amounts of viral proteins are not comparable in different samples, since most of the proteins in the samples were cellular; 2) there was a lot of cell debris in the infected cell lysates, which would make the measurement of soluble proteins inaccurate; 3) the binding products were lysed directly with a relatively small volume of SDS-PAGE sample buffer, in order to maintain a high concentration and to obtain as strong a signal as possible. The purpose of this study was to detect the presence of viral binding proteins but not to determine the amount of each protein bound. The amounts of viral proteins binding to the cell surfaces is unknown but is thought to be very small; 4) Since the losses of proteins, due to dilution and washing during the adsorption tests, could not be effectively analysed in these assays, the results obtained must be regarded as qualitative. No attempt at quantisation of binding protein was made.

CHAPTER 6
GENERAL DISCUSSION

During the course of this work particular emphasis was placed on the use of several established techniques and the development of new techniques to study virus infected midges. This has produced a large body of complementary qualitative and quantitative data. Very large numbers of insects were used in many parts of the study to ensure consistency.

Study of BTV replication in cells *in vitro* and in whole midges utilised a combination of techniques involving defined viral and subviral particles, virus titration, immunochemical localisation using monoclonal antibodies, ultrastructural and immunoelectron microscopy, and analysis of viral binding proteins. The results have confirmed and expanded our understanding of events taking place during BTV replication *in vitro* in both mammalian and midge cells but have also been extended to investigating BTV replication in various tissues and organs from intact midges, which has not previously been reported.

Ultrastructurally, VIBs have been confirmed as virus factories of BTV in both mammalian (BHK-21) cells, *Culicoides* (KC) cells, the cells of *Culicoides* salivary glands and nervous tissues (see Chapter 4). The VIBs in infected KC cells, salivary gland cells and nervous tissues were much bigger that those in BHK-21 cells possibly as a result of VIBs being built up during the persistent infection which is a characteristic in insect cells. Most of the VIB matrices in KC cells, salivary gland cells and nervous tissues were irregular and had a characteristic structure containing distinct fractures. The VIBs were usually seen to contain immature virus particles at different stages of development with nascent cores and virus-like particles. The more mature particles were located towards the periphery of the VIBs but double shelled virus particles were only seen outside these structures. However a characteristic of BTV infected KC cells was that 40.7% of VIBs did not contain any recognisable nascent cores and virus-like particles. This suggests that BTV replication in at least some KC cells may be non-productive. A possible reason for this is that the KC cell line originated from

macerated first instar larval *Culicoides* tissues. Therefore the KC cell line consists of mixed cell types, some of which may be unable to support BTV replication.

Tubules are characteristic structures in cells infected with BTV and consist predominantly of the nonstructural protein NS1 (Hyatt and Eaton 1988; Eaton *et al* 1990; Gould and Hyatt 1994) but the function(s) of tubules and NS1 are unknown and virus particles have not previously been reported to be associated with these structures. However, in this study it was striking that large amounts of tubules were observed in both mammalian and *Culicoides* cells, including the cells of the salivary glands of *C. variipennis*, infected with BTV. These tubules were clearly associated with VIBs usually being found in the cytoplasm surrounding the VIB matrix. Mature virus particles were frequently observed attaching to tubules sometimes with a number of virus particles lined up on tubules with an identical distance in between each particle. This strongly suggests a functional association and studies of kinesin, the main protein constituent of microtubule-based molecular motors (Spudich 1994), may suggest a mode of action. Molecular motors work to processively transport molecules in cells, with single molecules moving along a microtubule track for many seconds before dissociating. It is therefore possible that the tubules in BTV-infected cells play a similar role in facilitating the intracellular transport of progeny virus particles.

The data in the present study confirm a single earlier observation (Hyatt *et al* 1988) that progeny virus particles are released through the cell membrane of infected BHK-21 cells either by membrane budding, as "enveloped" particles, or by extrusion without acquiring an envelope. These two types of virus release are clearly different and may involve different mechanisms. As BTV is a non-enveloped virus, the cell membrane "envelope" surrounding virus particles released by budding is presumably discarded shortly after being released. Virus particles, released by extrusion, were always associated with cell surface debris which may represent this discarded material. It is also significant to note that progeny virus particles were released from infected salivary gland cells of *C. variipennis* and a *C. variipennis* cell line (KC cells) at an early stage (6 hours postinfection) and only by membrane budding. The non-

enveloped extrusion that occurs in BHK-21 cells has not been seen in either *Culicoides* salivary glands or KC cells. No virus release has been observed from persistently infected KC cells probably because the typical signs of BTV replication were only observed in a minority of these cells. However persistent infection can last for months with a relatively high level of virus production (about $10^{6.0}TCID_{50}$/ml) in the absence of any cytopathology which suggests that the virus-cell interaction is well-balanced.

This is the first report of immmunohistochemical localisation of viral antigen in infected midges. Prior to the present work there have been few published studies on dipterans and these have dealt with mosquitoes infected with arboviruses. Previous work has focused on wax embedding techniques at relatively high temperatures followed by standard sectioning followed by immunochemical staining. Whilst this has been a pioneering technique problems have been experienced with a loss of detectable antigenicity probably due to heating during the wax-embedding process. The method was used originally because considerable difficulty was experienced in cutting cryostat sections of infected mosquitoes. Indeed this was also experienced initially with midges in my study. The principal problems were that at standard cryostat temperatures (-20^{0}C) both mosquitoes and midges were dislodged from the embedding medium by the cryostat knife with only occasional clean sections being cut. In this study it was found that lowering the cryostat temperature to -24^{0}C resulted in excellent cutting characteristics enabling serial sections to be cut and also ensuring that minimal antigenic losses were encountered due to temperature effects. This allowed the use of a monoclonal antibody in the histochemical assay and yet still provided adequate signal to be achieved. The technique should now be explored in other virus-vector combinations.

Using this localisation method extensive studies backed up by comprehensive conventional feeding, injection and titration studies quantified in detail virus kinetics in midges. It was remarkable that the data revealed a pattern of viral replication and dissemination very similar to that recorded for many unrelated arboviruses in mosquitoes.

In oral infection experiments a high proportion of individuals from both susceptible and refractory colonies could not be infected although the whole gut epithelium, including both the anterior midgut and the foregut-midgut junction, and the posterior midgut were exposed to high levels of the virus. This reflects the presence of a midgut infection barrier (MIB). It is significant that preliminary binding experiments in vitro have indicated for the first time the possible involvement of several binding proteins. Although much more detailed studies will be necessary to confirm these preliminary data interesting differences were noted between the proteins binding to mammalian and midge cells *in vitro*.

In midges successfully infected via the oral route, it was confirmed that the anterior midgut epithelium and the cells at the foregut-midgut junction of *C. variipennis* were infected as frequently and as early as the posterior midgut with the main barrier to dissemination and transmission of BTV resulting from a mesenteron escape barrier (MEB). Despite feeding midges blood with levels of virus that were considerably higher than might be encountered naturally only 35.4% of the susceptible and 31.3% of the refractory colonies of *C. variipennis* showed evidence of initial infection of the midgut characterised by imunochemical staining as; 1) A virus positive reaction in gut cells ranging from very low to higher levels and spreading throughout the cytoplasm. 2) A virus positive reaction restricted to endosome-like structures in the cytoplasm of infected gut cells.

In infected susceptible individuals virus dissemination from the gut showed the following patterns of replication: 1) virus was detected only in a few sporadic fat body cells beyond the gut; 2) virus disseminates from the gut cells into the haemocoel and replicates in secondary organs/tissues but at low levels; 3) virus disseminates from the gut cells into the haemocoel and replicates in secondary target organs/tissues at high level. Patterns 1 and 2 suggest that factors in the haemocoel may be able to stop the dissemination of the virus which enters the haemocoel from the gut cells by trapping small amounts of BTV in fat body cells. The same phenomenon of 'virus trapping' by fat body cells did not occur in IT inoculated midges probably because IT inoculation results in relatively large amounts of virus being introduced directly into the haemocoel. Therefore in circumstances when only small amounts of virus

enter the haemocoel, the fat body cells may operate as a 'self-clearing mechanism' acting as part of a *Culicoides* 'immune system'. This could prevent infection of the salivary glands and thereby preclude transmission. If the virus enters the haemocoel at a level above the threshold at which the 'immune system' is able to inactivate it, the barrier is 'swamped', infection of the salivary glands proceeds normally and transmission can occur. In this respect, it is known that insects have an efficient cellular and humoral 'immune system' which defends the host against bacteria and parasites (Lackie 1988; Hultmark 1993; Tanada and Kaya 1993). Haemocytes, usually circulating within the haemolymph, have phagocytosis and encapsulation functions which comprise aspects of the cellular immunity of insects. Insects can also produce certain 'low molecular weight' peptides or proteins which may play a role against invading pathogens similar to that of antibodies in mammalian systems. However, there is only very limited information on antiviral activity in insects infected with arboviruses (Chadwick and Dunphy 1986). Nevertheless Luo and Brown (1993) have reported that Sindbis virus can induce the production of an antiviral peptide during infection of mosquito cells. Such a study has yet to be carried out on any species of *Culicoides*.

In totally susceptible individuals (replication pattern 3) a fully disseminated infection was observed with virus replicating to high levels in secondary target organs/tissues. The gut cells (including the anterior, and posterior midgut epithelium, and the cells of the foregut-midgut junction), the fat body, cephalic and thoracic ganglia, salivary glands and ommatidia of the compound eyes are commonly infected organs/tissues. However, the hindgut cells, muscles, Malpighian tubules and oocytes/nurse cells of the ovaries are not susceptible to BTV infection.

Using the salivation induction and transmission techniques it was possible to obtain the first detailed report of virus transmission in midge saliva. No evidence was obtained for the presence of salivary gland infection or escape barriers (SGIB, SGEB) as 100% of BTV intrathoracically (IT) inoculated *C. variipennis*, whether from a susceptible or a refractory colony, were able to develop a persistent and fully disseminated infection and to transmit the virus through their saliva. No individuals from the refractory colony of *C. variipennis* were

able to transmit BTV subsequent to oral infection, and only 12.12% of persistently infected susceptible colony midges transmitted the virus although the infection rates of these two colonies were similar after ingestion an infectious blood meal. However this situation is related to the presence of a MEB and/or a dissemination barrier in the haemocoel but not to SGIB or SGEB

So far, little has been published to describe the characteristics of BTV infection in the salivary glands of vector *Culicoides* (Foster and Jones 1981). In the present study, by using electron microscopy, BTV infection of the salivary glands of *C. variipennis* was described in detail for the first time. Development was typically polarised following a pattern of virus movement from the haemocoel through the basement lamina → acinar cells → acini → intermediate ducts → the lumen of the secretory ducts → host animals. Virus enters the acinar cells from the haemocoel passing through the basement membrane of the salivary glands by endocytosis. BTV then localises and replicates in viral inclusion bodies (VIB) in the cytoplasm of serous acinar cells. Mature progeny virus particles are then released into serous alveoli (acini) that are located on the opposite side of the acinar cells to the basement membrane. The virus particles are then transported through intermediate ducts and accumulate in crystalline arrays in the lumen of the major secretory ducts. No virus appears to be released back into the haemocoel through the basement membrane although the infection and reinfection of the salivary glands through the basement surface of the glands is a continuous process. Neither has virus been seen to re-enter acinar cells from the acini. This polarised nature of BTV infection in the salivary glands of *C. variipennis* accounts for the massive accumulation of progeny virus particles in the secretory ducts and means that transmission of BTV through the saliva during biting is assured.

Both laboratory and field studies dealing with *Culicoides*-BTV have indicated that vertical transmission does not appear to occur. In *C. variipennis* BTV has not been detected in the progeny of parental midges infected with the virus (Jones and Foster 1971b; Nunamaker *et al* 1990; PS Mellor personal communication). In the present study, oocytes/nurse cells have

never been found to become infected with BTV in either IT inoculated or orally infected *C. variipennis*, even in the individuals with fully disseminated infections. This strongly supports the assertion that vertical transmission of BTV does not occur in *C. variipennis* because of the presence of a transovarial transmission barrier which prevent access of the virus to the egg cells. No insight was obtained into the nature of this barrier however.

Nervous tissues of *C. variipennis* were proven in this study to be heavily infected in individuals with a fully disseminated BTV infection, using both IHC and transmission electron microscopy. Ultrastructural results showed typical and active virus replication in nerve cells and axons. The influence of nervous tissue infection on virus dissemination and on insect behaviour needs to be elucidated in the future.

In conclusion, the transmission barriers presented by *C. variipennis* to BTV are: mesenteron infection barrier, mesenteron escape barrier, fat body mediated dissemination barrier in the haemocoel and transovarial transmission barrier. The salivary glands of *C. variipennis* present neither a salivary gland infection barrier nor a salivary gland escape barrier. Further work should now be directed at elucidating the mechanisms upon which these barriers are based. This work should focus on the MIB and MEB because my work has shown that these are the principal barriers in this BTV vector species of midge.

APPENDICES

Appendix 1 Replication of BTV in BHK-21 cells infected with IVP, ISVP and CP of BTV1SA

Time pi (hour)	Mean log_{10} $TCID_{50}$/ml [IVP]	SEM	Mean log_{10} $TCID_{50}$/ml [ISVP]	SEM
0	3.75	0.00	3.00	0.00
1	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00
3	0.00	0.00	0.00	0.00
4	1.50 (1.25-1.75)	0.14	0.00	0.00
5	2.50	0.00	0.00	0.00
6	2.25	0.00	0.00	0.00
7	2.33 (2.25-2.50)	0.08	0.00	0.00
8	3.00 (2.75-3.25)	0.14	1.50	0.00
9	2.75	0.00	2.00 (1.75-2.25)	0.14
10	3.25	0.00	2.00	0.00
12	3.25	0.00	2.00	0.00
14	3.50	0.00	3.00	0.00
16	4.00 (3.75-4.25)	0.14	2.83 (2.75-3.00)	0.08
18	5.50	0.00	3.00	0.00
20	7.00	0.00	4.00	0.00
22	6.50	0.00	4.00	0.00
24	7.00	0.00	5.00	0.00
26	8.00 (7.75-8.25)	0.14	6.50	0.00
28	7.00	0.00	7.08 (7.00-7.25)	0.08
30	8.50	0.00	6.00	0.00
48	7.50 (7.25-7.75)	0.14	7.00	0.00

IVP: intact virus particles
ISVP: infectious subviral particles
CP: core particles
pi: postinfection
SEM: standard error of mean

BTV titration results in BHK-21 cells infected with IVP, ISVP and CP of BTV 1SA at different times postinfection from three experiments (see Chapter 2). The data in brackets show the range of titres from the three titration experiments, otherwise the data of the three replicates were the same. CP were not infectious for BHK-21 cells and therefore no data are shown in this table.

Appendix 2 Replication of BTV in KC cells infected with IVP, ISVP and CP

Time pi (hour)	Mean $\log_{10}$ $TCID_{50}$/ml (IVP)	SEM	Mean $\log_{10}$ $TCID_{50}$/ml (ISVP)	SEM	Mean $\log_{10}$ $TCID_{50}$/ml (CP)	SEM
0	3.75	0.00	3.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	0.00	0.00	0.00	0.00
5	0.00	0.00	0.00	0.00	0.00	0.00
6	2.00	0.00	0.00	0.00	0.00	0.00
7	2.25	0.00	0.00	0.00	0.00	0.00
8	3.25*	0.14*	1.75*	0.14*	2.25	0.00
9	2.50	0.00	2.25	0.00	2.25	0.00
10	4.75	0.00	3.00	0.00	2.25*	0.14*
12	4.83**	0.08**	3.00*	0.14*	3.00	0.00
14	4.75	0.00	3.00	0.00	2.75	0.00
16	5.00	0.00	3.50	0.00	3.00	0.00
18	4.50	0.00	3.75	0.00	2.75	0.00
20	4.75	0.00	3.50	0.00	3.58**	0.08**
22	4.25	0.00	3.83**	0.08**	4.00*	0.14*
24	4.25	0.00	4.00	0.00	4.00	0.00
26	5.00*	0.14*	5.00	0.00	4.50	0.00
28	4.83**	0.08**	5.00	0.00	5.00*	0.14*
48	6.50*	0.14*	6.75	0.00	6.00	0.00

IVP: intact virus particles
ISVP: infectious subviral particles
CP: core particles
pi: postinfection
SEM: standard error of mean

BTV titration results of KC cells infected with IVP, ISVP and CP of BTV 1SA at different times post infection from three experiments (see Chapter 2). The data marked with '*' are means and SEM of the titres which varied in the three experiments (in a range of $10^{0.5}TCID_{50}$/ml), the data marked with '**' are means and SEM of the titres which also varied in the three experiments (in a range of $10^{0.25}TCID_{50}$/ml), otherwise the data of the three replicates were the same.

Appendix 3 Replication of BTV 1SA in IT inoculated *Culicoides variipennis*

Time pi days	hours	No. midges (BTV+)	Mean $\log_{10}$ $TCID_{50}$/midge	SEM
< 1				
	1	10	3.50 (3.00-3.75)	0.08
	2	10	3.51 (3.00-3.75)	0.08
	4	10	3.48 (3.25-3.75)	0.09
	8	10	3.75 (3.50-4.00)	0.09
	12	10	3.73 (3.25-4.00)	0.11
	16	10	3.50 (3.25-4.00)	0.11
	20	10	3.79 (3.20-4.50)	0.13
	24	10	4.00 (3.50-4.50)	0.17
1		20	4.25 (3.75-5.00)	0.13
2		18	4.65 (4.00-5.50)	0.14
3		24	4.50 (3.75-5.50)	0.12
4		21	4.77 (3.50-5.25)	0.13
5		21	5.04 (4.25-5.50)	0.12
6		19	4.75 (3.75-5.25)	0.15
7		20	5.08 (3.25-5.50)	0.17
8		18	4.50 (3.50-5.25)	0.19
9		19	5.21 (4.00-5.50)	0.18
10		22	4.96 (4,25-5.50)	0.17
14				
Total		282		

pi: postinfection
IT: intrathoracic
SEM: standard error of mean

BTV titration results of 282 IT inoculated *C. variipennis* at different times postinfection (see Chapter 2). Each midge was inoculated with approximately $10^{3.0}TCID_{50}$ of BTV suspension. The mean titres and SEM of the titres per midge are shown in this table. The ranges of the titres of BTV per midge are shown in brackets.

Appendix 4 Titration of BTV 1SA from orally infected *Culicoides variipennis*

Time pi days	hours	No. midges (BTV+)	Mean $\log_{10}$ $TCID_{50}$/midge	SEM
< 1	1	20	3.14 (2.50-3.50)	0.08
	2	20	2.96 (2.50-3.50)	0.08
	4	62	3.06 (1.50-3.50)	0.09
	8	67	2.81 (1.50-3.50)	0.09
	12	63	1.96 (1.50-2.75)	0.11
	16	66	2.82 (1.50-3.25)	0.11
	20	68	2.98 (1.00-3.00)	0.13
	24	59	1.35 (0.75-2.75)	0.17
1	28	69	1.84 (-0.25-4.50)	0.17
	32	30	2.75 (-0.50-3.75)	0.17
2		29	2.78 (-0.25-3.75)	0.22
3		26	3.49 (-0.25-4.75)	0.21
4		27	3.35 (-0.25-5.00)	0.24
5		23	3.44 (-0.25-4.50)	0.23
6		19	3.50 (-0.25-5.00)	0.26
7		54	3.31 (-0.50-4.50)	0.22
8		31	3.05 (-0.25-5.25)	0.26
9		36	3.35 (-0.25-5.00)	0.25
10		29	3.30 (-0.25-4.75)	0.27
11		35	3.05 (-0.25-5.25)	0.25
12		20	3.01 (-0.25-5.00)	0.23
14		46	2.97 (-0.25-5.50)	0.28
Total		899		

pi: postinfection
SEM: standard error of mean

BTV titration results of *C. variipennis* after being fed on a suspension of BTV in sheep blood, containing $10^{6-7}TCID_{50}$/ml of the virus, at different times postinfection (see Chapter 2). A total of 1675 midges were tested. The mean titres and SEM of 899 BTV positive midges are shown in this table. The ranges of the titres of BTV per midge are shown in brackets.

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