STUDIES ON THE INFECTION OF SANDFLIES AND BITING MIDGES WITH ARBOVIRUSES

by

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Abstract

Studies on the infection of sandflies and biting midges with arboviruses

The research for this thesis was carried out in the Entomology department of the Animal Virus Research Institute, Pirbright.

Membrane feeding and intrathoracic inoculation techniques were developed and used successfully for the first time to infect sandflies with viruses. These infection techniques were used to investigate the susceptibility of a laboratory colony of the sandfly Lutzomyia longipalpis to infection with three viruses of the Phlebotomus fever group, Sicilian and Naples sandfly fever, and Pacui. The suitability of the colony for use as a laboratory model for studies on the development of such viruses in an insect host was then assessed. Sicilian and Naples sandfly fever viruses are Old World viruses transmitted in nature by the Old World sandfly Phlebotomus papatasi. Pacui virus is a New World virus isolated from the New World sandfly L.flaviscutellata. A limited number of P.papatasi were obtained and both infection techniques were applied equally successfully to this species. The development of the New and Old World viruses in New and Old World sandflies was then compared.

The susceptibility of two Culicoides species to the Phlebotomus fever group viruses was tested as part of a general programme of research into the specificity of arboviruses with respect to the insect host. Several Culicoides species are important as vectors of the orbivirus Bluetongue. The vector potential of sandflies for this virus was assessed and the development of Bluetongue virus in Culicoides and sandflies was compared. The distribution of virus in infected insects on successive days after infection was determined by infectivity titrations of dissected heads, thoraces and abdomens.

A fluorescent antibody test was developed and used to detect viral antigens in tissue smears from infected Culicoides and sandflies. The possibility of using such a test to detect viral antigens in paraffin wax sections of infected insects was investigated.

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Declaration

Some of the work presented in this thesis has already been published or accepted for publication under the following titles.

1. The susceptibility of Lutzomyia longipalpis (Lutz and Neiva), Diptera, Psychodidae, to artificial infection with three viruses of the Phlebotomus fever group.
Jennings, M., and Boorman, J., (1980) Annals of the Society of Tropical Medicine and Parasitology, in press.

2. Use of the indirect fluorescent antibody technique for the detection of Bluetongue virus antigen in tissue smears from Culicoides variipennis (Diptera, Ceratopogonidae).
Jennings, M., and Boorman, J., (1980) Veterinary Microbiology 5, 13-18

3. The susceptibility of the sandfly Lutzomyia longipalpis (Lutz and Neiva), Diptera, Psychodidae, to laboratory infection with Bluetongue virus.
Jennings, M., and Boorman, J., (1980) Archives of Virology 64, 127-131

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Part 1

General Introduction

1.1. Definition and classification of arboviruses

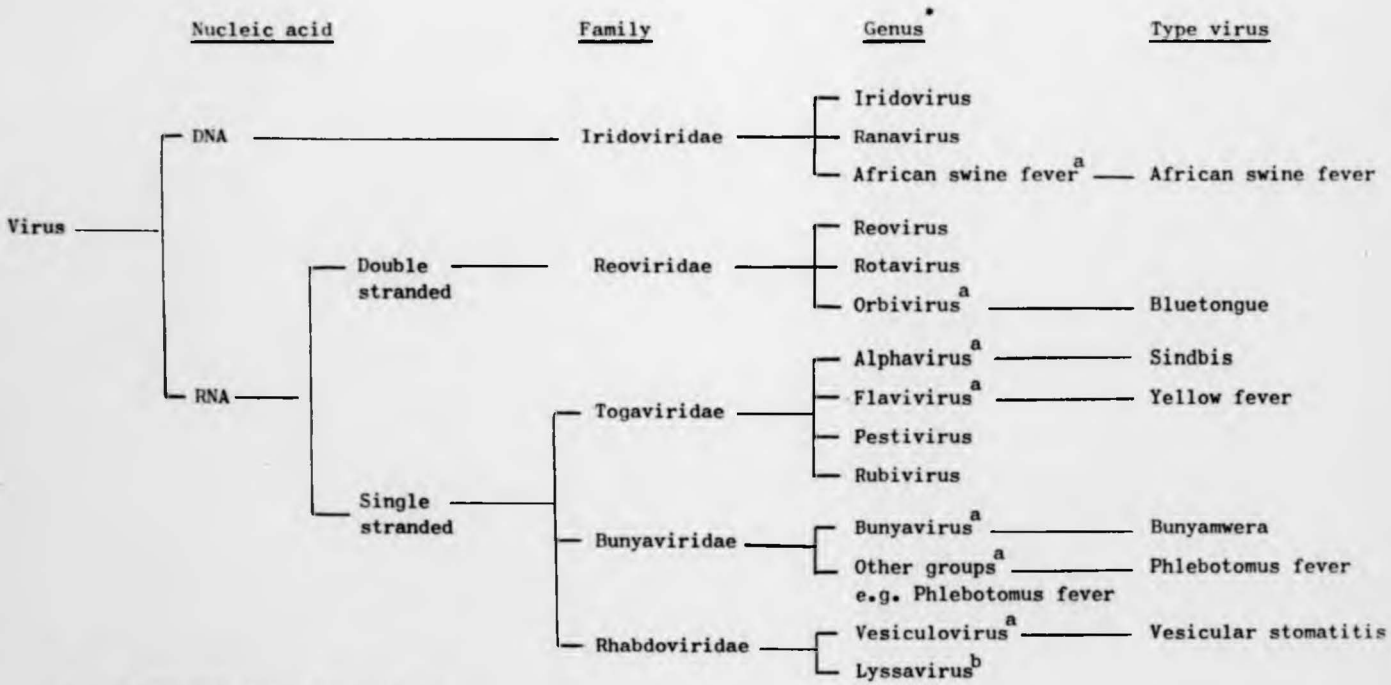
The arbovirus group is defined by biological and epidemiological criteria. It comprises those animal viruses which are transmitted between vertebrate hosts by blood-sucking arthropods. Virus multiplication occurs in both the vertebrate and the invertebrate host.

Classification of arboviruses on the basis of their morphological and physicochemical properties (Figure 1) shows that five of the families described by the International Committee for the Taxonomy of Viruses (Wildy, 1971; Fenner, 1976; Matthews, 1979) are represented in the group.

There are 423 arboviruses listed in the Arthropod-borne Information Exchange, Number 38, 1980. Many of the viruses listed are known only as isolates either from arthropods or from vertebrates. Nevertheless they are included in the arbovirus group because they are related to confirmed members by their morphological, physicochemical, and antigenic properties. For example, Rio Grande (RG) has been isolated only from rodents and not as yet from any arthropod. The virus has been placed in the Phlebotomus fever group because of its antigenic relationship with other members of this group (Calisher et al., 1977).

Arboviruses infect a wide range of vertebrate hosts including many different types of mammals and birds. They often cause little or no clinical disease in the vertebrate host and these animals may then act merely as reservoirs for the virus. Many arboviruses however are of considerable medical and veterinary importance because of the disease they cause in man (e.g. dengue, sandfly fever, and yellow fever), and domestic livestock (e.g. bluetongue disease of sheep, African horse sickness, Rift Valley fever of sheep and cattle, and Nairobi sheep disease). Their distribution is widespread and therefore they constitute a major health and economic problem on an international scale.

Figure 1. Classification of families and genera of viruses containing arboviruses (Based on Matthews, 1979)



a Genus containing arboviruses

b Genus containing possible arboviruses

• Only genera containing vertebrate and invertebrate viruses included

1.2. Arthropod vectors

The two major groups of arbovirus vectors are ticks, and insects belonging to the order Diptera. A few virus isolations have been made from mites, for example Junin (JUN) virus, the cause of Argentinian haemorrhagic fever, has been isolated from Echinolaelaps mites (in Theiler and Downes, 1973). Insect vectors are mainly considered in this thesis.

The three most important families within the Diptera which are involved in virus transmission are : mosquitoes (Culicidae), biting midges of the genus Culicoides (Ceratopogonidae), and Phlebotomus and Lutzomyia sandflies (Psychodidae). Of these three families the mosquitoes have been found to transmit the largest number of known arboviruses and have been studied in greatest detail. It is possible that Simulids, Tabanids, or other blood sucking insects can act as vectors but few isolations have been made from such insects. A California group virus has been isolated from Tabanids; and African horse sickness virus has been isolated from Tabanids and Stomoxys in Turkey (in Andrewes et al., 1978) but it seems likely that the role of these insects is as mechanical, rather than true biological vectors.

With the exception of the alphaviruses which are all mosquito-borne, there is no precise correlation between the virus group and the insect vector species. Table 1.1.illustrates the systematic range of arboviruses transmitted by each of the three insect families mentioned above.

All the alphaviruses and many of the flaviviruses are mosquito-borne. The majority of viruses in the Bunyamwera supergroup and the virus groups classified as possible Bunyaviridae have been isolated from mosquitoes. Three Phlebotomus fever group viruses, Arumowot (ART), Itaporanga (ITP), and Rift Valley fever (RVF) have been isolated from these insects; and two other members of this group,

Table 1.1. Systematic range of arboviruses transmitted by mosquitoes, Culicoides, and sandflies

Insect group	Virus		Examples
	Family	Genus	
Mosquitoes	Togaviridae	Alphavirus	Sindbis, Semliki Forest, Chikungunya, Eastern equine encephalomyelitis
		Flavivirus	West Nile, Yellow fever, Dengue, St.Louis encephalitis
	Reoviridae	Orbivirus	Corriparta, Lebombo, Palyam, Eubenangee, Orunga
	Rhabdoviridae	Vesiculovirus	Vesicular stomatitis Indiana
	Bunyaviridae	Bunyavirus	Bunyamwera, Capim, Koongal, Tensaw
		Bunyavirus-like	Rift Valley fever, Arumowot, Anopheles A, Turlock
Culicoides	Reoviridae	Orbivirus	Bluetongue, Ibaraki, African horse sickness
	Rhabdoviridae		Bovine ephemeral fever
	Bunyaviridae	Bunyavirus	Akabane, Maindrain
		Bunyavirus-like	Nairobi sheep disease
Sandflies	Reoviridae	Orbivirus	Changuinola, Irituia
	Rhabdoviridae	Vesiculovirus	Vesicular stomatitis Indiana
	Bunyaviridae	Bunyavirus-like	Aguacate, Pacui, Phlebotomus fever

Chagres (CHG) and Icoaraci (ICO), have been recovered from both mosquitoes and sandflies (cited in Berge, 1975). Mosquito-borne Reoviridae include Corriparta (COR) (Whitehead et al., 1968) and Palyam (PAL) (Dandawate et al., 1969) both of which are orbiviruses. Only a few rhabdoviruses have so far been isolated from mosquitoes. These include Vesicular stomatitis Indiana (VSI) (Sudia et al., 1967), and Cocal (COC) (Jonkers et al., 1965).

Culicoides are most important as vectors of orbiviruses such as Bluetongue (BT), African horse sickness (AHS) and Epizootic haemorrhagic disease of deer (EHD) (see Table 1.2.). Several bunyaviruses have also been isolated from these insects. Examples are Akabane (AKA) and Samford (SAM) (Doherty et al., 1972), both members of the Simbu group. Maindrain (MD), a Bunyamwera supergroup virus, has been isolated from C.variipennis Coquillet (Hardy, 1970) and this species has been shown to transmit virus in the laboratory (Mellor et al., 1974). Rift Valley fever virus (RVF), which has recently been included in the Phlebotomus fever group, has also been isolated from Culicoides (Lee in Fagbami et al., 1973). The third family of viruses to include agents isolated from Culicoides is the family Rhabdoviridae. Two viruses, Bovine ephemeral fever (BEF) and Kotonkan, have been recorded from Culicoides (Davies and Walker, 1974; Kemp et al., 1973) although the latter virus has not been proved to be biologically transmitted by these insects.

Sandflies are associated with three groups of viruses from three separate families: the Phlebotomus fever group (Bunyaviridae), the Orbivirus group (Reoviridae) and the Vesiculovirus group (Rhabdoviridae). They are most important as vectors of the Phlebotomus fever group viruses (see Table 1.3.) which include Naples and Sicilian sandfly fever (SFN and SFS), Pacui (PAC), Karimabad (KAR) and Aguacate (AGU). Only two orbiviruses, Changuinola (CGL) (Tesh et al., 1974) and Irituia (IR), have so far been isolated from sandflies (in Andrewes et al., 1978).

Table 1.2. The Orbivirus group of viruses
(Compiled from Berge, 1975 and Matthews, 1979)

Virus or subgroup of viruses		Arthropod vector	Vertebrate host
Bluetongue subgroup[T]	20[+]	Culicoides	Sheep, cattle, goats, wild ungulates.
Eubenangee subgroup	3	Mosquitoes	Cattle, kangaroo, wallaby.
Corriparta subgroup	2	Mosquitoes	Human, cattle, horse, kangaroo, birds.
Changuinola subgroup	8	Sandflies	Human, arboreal mammals, rodents.
Colorado tick fever subgroup	2	Ticks	Human, rodents
Kemerevo subgroup	18	Ticks	Human, rodents, birds, ungulates.
Palyam subgroup	5	Mosquitoes	Cattle, sheep,human.
Epizootic haemorrhagic disease of deer subgroup	4	Culicoides	Deer
Abadina subgroup	4	Mosquitoes	-*
Warrego subgroup	2	Culicoides	Cattle, kangaroo, wallaby.
Wallal subgroup	2	Culicoides	Kangaroo, wallaby.
African horse sickness virus	9	Culicoides	Horse
Equine encephalosis virus	-	-	Horse
X (from bovine serum)	-	-	Bovids
Undescribed Aus MK 6357	-	Mosquitoes	-

Cont'd.....

Table 1.2. Continued.....

Virus or subgroup of viruses		Arthropod vector	Vertebrate host
Undescribed SUA T5-0616[a]	-		
Undescribed USA 69V2161[a]	-		
Ungrouped : Ibaraki	-	-	Cattle
Japanaut	-	Mosquitoes	Bats
Lebombo	-	Mosquitoes	-
Orunga	-	Mosquitoes	Human, cattle, sheep
T5-0616	-	-	Skunk
Umatilla	-	Mosquitoes	Birds

T Type virus

\+ Number of viruses in subgroup or number of virus types

-* Not known

a Neither arthropod nor vertebrate host listed

Table 1.3. The Phlebotomus fever group of viruses
(Compiled from Berge, 1975 and Matthews, 1979)

Virus	Arthropod vector	Vertebrate Host
Aguacate	Lutzomyia	Human
Anhanga	Lutzomyia ?	Sloth
Arumowot	Mosquitoes	Rodents
Bujaru	Lutzomyia ?	Rodents, human
Cacao	Lutzomyia	Human
Caimito	Lutzomyia	-*
Candiru	Lutzomyia	Human, penguin
Chagres	Lutzomyia, mosquitoes	Human
Chilibre	Lutzomyia	-
Frijoles	Lutzomyia	-
Gordil	-	Rodents
Icoaraci	Lutzomyia, mosquitoes	Rodents
Itaituba	-	Marsupials
Itaporanga	Mosquitoes	Rodents
Karimabad	Phlebotomus	Human
Nique	Lutzomyia	-
Pacui	Lutzomyia	Rodents
Punta Toro	Lutzomyia	Human
Rio Grande	Lutzomyia ?	Rodents
Saint Floris	-	Gerbil
Salehabad	Phlebotomus	Human
Sandfly fever Naples	Phlebotomus	Human
Sandfly fever Sicilian	Phlebotomus	Human
Urucuri	-	Rodents
Undescribed ISS Phl 3[a]		
Undescribed Sud An 754-61	-	Rodents

? Possible vector

-* Unknown vector or vertebrate host

a Neither vertebrate nor invertebrate host listed

Three Phlebotomus fever group viruses are listed in the Arbovirus information exchange No.38, (1980) which are not included above. These are Alenquer, isolated from man, Buenaventura, from Lutzomyia, and a new classification of Rift Valley fever.

The sandfly-borne vesiculoviruses include VSI (Shelakov and Peralta , 1967; Tesh et al., 1974), Chandipura (CHP) (Vijai et al., 1970), and Isfahan (ISF) (Tesh et al., 1977).

The isolation of a virus from an insect captured in the wild does not necessarily mean that the insect species involved is a vector of that virus, or that the virus itself is a true arbovirus. The insect may merely be an incidental carrier not normally involved in a biological transmission cycle. Laboratory experiments are required to prove that an insect, (or other arthropod), can act as a true vector of virus. These must include the demonstration of virus multiplication in the insect following ingestion of virus, and also the subsequent transmission of virus to a susceptible vertebrate host, or substitute system such as provided in artificial feeding techniques. Similar experiments are also necessary to satisfy the accepted biological criteria for classification of a viral isolate as a true arbovirus. The virus in question must be shown to multiply in both vertebrate and invertebrate tissues, and to be transmissible by an invertebrate host.

1.3. Host range and vector susceptibility

Arboviruses vary individually in their specificity for the invertebrate host both under natural conditions and in laboratory experiments. This subject has been reviewed by Varma (1972). An arbovirus may naturally infect a number of different species and genera of insects. This has been well documented in the case of the mosquito-borne arboviruses, and the information contained in the Arbovirus catalogue (Berge, 1975) illustrates the broad host range of many of these viruses. Sindbis (SIN), Yellow fever (YF), West Nile (WN), and Western equine encephalomyelitis (WEE) for example, have all been isolated from several mosquito species, the latter virus having been recovered from five separate genera. Some of the

California encephalitis group viruses have a similar wide host range. La Crosse (LAC), Keystone (KEY), and Trivittatus (TVT) virus have been isolated from six different genera of mosquitoes including at least twelve species (Sudia et al., 1971). Isolations of an arbovirus from different families of insects have also been reported. For example VSI has been recovered from Phlebotomus sandflies (Shelakov and Peralta, 1967) and Aedes mosquitoes (Sudia et al., 1967).

However, insects from closely related species can differ considerably in their ability to support multiplication of virus, and to transmit virus, both under laboratory conditions and in the wild. For example, several species of Culicoides act as the vectors of BT virus. C.variipennis is the vector in North America (Foster et al., 1963), and in Africa the vector is C.imicola Kieffer (Dutoit, 1944). However the virus will not multiply in C.nubeculosus Meigen after infection by the natural oral route (Dr. P. Mellor personal communication, 1978).

Individual insects from a single population, (either wild or colonised), may also vary in their susceptibility to infection with a particular arbovirus. Only 30% of a colony of C.variipennis became infected with BT after ingestion of this virus (Foster et al., 1968, Jones and Foster, 1971) and only 7% of a colony of the same species were found to be susceptible to oral infection with Eubenangee (EUB) virus (Mellor and Jennings, 1980). It has been shown that this variation in susceptibility may have a genetic basis and susceptible and resistant colonies of insects can be established by selection (Jones and Foster, 1974).

It is important to be aware that much of the information on the host range of different arboviruses has been derived from virus isolation studies in the field, and proof of the vector status of

different insect species for the arbovirus in question is not always available. The natural host range of an arbovirus may merely reflect the geographical distribution of that virus and of the potential vector. Laboratory experiments may reveal a much less marked specificity of a virus for the insect host than results obtained during virus isolation studies on wild-caught insects would indicate.

Arboviruses are not necessarily static in their geographical distribution, and modern methods of transport for both man and domestic animals increase the chances of the spread of disease to areas previously free from infection. There is furthermore the possibility of the spread of infections through dispersal of infected insects on the wind (Sellers, 1975). It is therefore important to be aware of the vector potential of the haematophagous arthropods in areas likely to become involved during the spread of an arbovirus disease.

Little is known about the factors which limit the susceptibility of an insect to virus infection. In a susceptible insect the development of virus as determined by the titration of infected individuals on successive days after infection progresses as follows. After the initial ingestion of virus there is usually a latent period of from one to two days during which the concentration of virus in the insect decreases. Virus multiplication then becomes evident, and an increase in virus concentration occurs over a period of several days. The concentration reaches a maximum, and may remain at the same level for a number of days or weeks depending on the life span of the insect. During this period virus will be present in the salivary glands, a pre-requisite for transmission. The length of time required for the virus concentration to reach a peak and for the salivary glands to become infected, (the extrinsic incubation period), depends upon factors such as the environmental temperature, the virus, and the insect species concerned. It may be as little as

three days. For example, Lutzomyia trapidoi Fairchild and Hertig is able to transmit VSI within three days of ingesting the virus (Tesh et al., 1971). A gradual decrease in the overall virus concentration may be observed as the insect ages.

The insusceptibility of an insect to virus infection may be due to the presence of a "gut barrier" since methods of infection that bypass the gut, such as intrathoracic inoculation, often result in a virus multiplying in an insect which does not become infected by the more natural oral route. Bluetongue, for example, will multiply to a high concentration in C.nubeculosus after inoculation of the virus directly into the haemocoele (Dr. P. Mellor, personal communication).

The nature of the gut barrier is uncertain. It may be quantitative or qualitative. In the former case the virus concentration in the blood meal must equal or exceed a certain "threshold" level in order to establish infection in an insect. For example, when C.variipennis females were fed on AHS virus at concentrations below 5.7 $\log_{10}TCID_{50}$/ml the initial infection rate on day 0 was 0%. At concentrations above 5.7 $\log_{10}TCID_{50}$/ml the percentage of infected insects increased in a linear relationship with the virus concentration. However the infection rate never exceeded 40% even when the blood meal contained 7.9 $\log_{10}TCID_{50}$ of virus per ml (Mellor et al., 1975).

Hypotheses put forward to explain the qualitative nature of the gut barrier include : the impermeability of a peritrophic membrane ; the absence of specific receptor sites on the gut cells ; variations in permeability of gut cell membranes ; inactivation of viruses by digestive fluids ; and the presence of a surface type defence mechanism (Chamberlain and Sudia, 1961). These will be discussed

in more detail in section 2.4. There may of course be a combination of factors limiting susceptibility. The "gut barrier" would be difficult to demonstrate by normal light microscopical means unless it is a physical barrier.

1.4. Effect of arbovirus infection on the insect host

Arboviruses do not appear to be pathogenic to their insect hosts. However this topic has not been extensively investigated. None of the viruses used in this laboratory to infect Culicoides species (e.g. BT, AHS, MD, or EUB) affected the longevity of these insects. According to LaMotte (1960) high concentrations of Japanese encephalitis (JE) virus persist throughout the life of infected Culex pipiens Linn. without any apparent effect on the insect. Similar results were obtained with Cx.tarsalis Coquillet infected with WEE virus (Thomas, 1963). However, the criteria used in such experiments have generally been crude, with no detailed measure of the biological performance of infected and uninfected insects (e.g. the ability to fly or bite). Tesh and Chaniotis (1975) investigated the effect of VSI virus infection on the fertility of female sandflies. The virus did not appear to have an adverse effect on the sandflies, and in fact the number of FI adults produced by infected females was slightly higher than the number produced by control insects. Printz (1969) found that serial passage of VSI virus 20 to 35 times in Drosophila melanogaster, an abnormal host, modified the virus such that a reduction in the longevity and fertility of infected D.melanogaster females was observed. Recent studies on Ae.triseriatus mosquitoes infected with LAC virus have shown that the feeding behaviour of infected flies may be modified by the virus infection. Infected mosquitoes probed more frequently and did not engorge so fully as uninfected mosquitoes. This had two effects; the likelihood of virus transmission was increased, and infected females produced less

offspring than uninfected females because of the smaller size of the bloodmeal (Grimstad et al., 1980).

Studies of arbovirus infections in insect tissue culture such as reviewed by Singh (1972), have shown that virus replication does not usually result in severe damage to the cells, although a marked cytopathic effect (CPE) may be observed in similarly infected vertebrate cells. For example neither BT nor AHS virus caused any CPE in an Ae.albopictus Skuse cell line (Jennings and Boorman, 1979; Jennings, unpublished results), although both these viruses are cytopathic in BHK 21 (baby hamster kidney) cells. Continuous passage of arbovirus infected insect cell lines may give rise to a persistent inapparent infection of the cells. This does not appear to have any effect on the cells and may not prevent their infection with other viruses (Peleg, 1969; Singh, 1972; Stollar et al., 1975; Libikova and Buckley, 1971).

1.5. The study of arboviruses in the insect host

Much of the information concerning arboviruses of medical and veterinary importance results from the study of the virus in the vertebrate host. Details of virus development in the invertebrate host are more limited and generally derive from studies of viruses in mosquitoes. It is essential to have as complete an understanding as possible of the relationships between arboviruses and confirmed or potential vectors before control programmes directed against the vector can be instigated.

There are many different approaches to the study of arbovirus infections in insects. Valuable information can be obtained by collecting insects from wild populations and testing these for the presence of virus. Field and laboratory studies of the biology of potential vectors are also of great importance. Information is required on such aspects as: adult and larval habitats including

breeding places, general and feeding behaviour, host preferences, longevity, and population dynamics.

Taxonomic studies enable vector species to be identified and establish relationships between confirmed and potential vectors.

The vector potential of suspected species requires confirmation in the laboratory. The factors which directly influence the efficiency of a species as a vector for a particular virus should also be investigated. These include the infectivity threshold (i.e.the level of viraemia in the vertebrate host necessary to infect the vector), the rate of virus multiplication in the insect, infection and transmission rates, and the length of the extrinsic incubation period. The effect of varying environmental conditions, such as temperature, on vector efficiency should also be considered.

The possible occurrence of transovarial transmission should be investigated. The latter has important ecological implications since it provides an additional mechanism for the persistence of a virus in nature if the vector is seasonal in appearance. Transovarial transmission of arboviruses has been shown to occur in a number of arthropod vectors including ticks and insects (see Burgdorfer and Varma, 1967 for a review). Watts and Eldridge (1975) and Tesh and Chaniotis (1975) have reviewed the evidence for the occurrence of transovarial transmission in mosquitoes and sandflies respectively. There is as yet no evidence for such a cycle occuring in any other group of insects. Examples of viruses transovarially transmitted by mosquitoes include several of the California encephalitis group e.g. KEY in Ae.atlanticus Dyar and Knab (Le Duc et al., 1975), LAC in Ae.triseriatus (Watts et al., 1973) and Ae.albopictus (Tesh and Gubler, 1975) and TVT in Ae.trivittatus Coquillet (Christensen et al., 1978). Transovarial transmission of YF by experimentally

infected Ae.aegypti has recently been demonstrated by Aitken et al., (1979). Vesicular stomatitis Indiana is transmitted transovarially by the sandflies L.trapidoi and L.ylephilator (Tesh et al., 1972). The epidemiology of some of the Phlebotomus fever group viruses including Sandfly fever may also involve a transovarial cycle. There have been several reports of isolations of SFS from male sandflies indicating that transovarial transmission of the virus does occur (e.g. Barnett and Suyemoto, 1961; Schmidt et al., 1971), but this has never been conclusively demonstrated. This is discussed in more detail in section 2.1.

In vivo studies of the effect of virus infections on the insect host are also of importance. As mentioned previously the effect of virus infections on such aspects as the longevity, fertility, flight, and feeding behaviour, should be studied since these can directly affect the vector potential of an insect. Knowledge of the distribution of virus in the insect's tissues and organs during the early stages and throughout the period of infection is desirable since this may help to explain the problems of susceptibility. Demonstration of virus in salivary glands or ovaries can provide presumptive evidence of transmission to a vertebrate host or of transovarial transmission of virus, obviating the need for lengthy laboratory transmission studies.

1.6. Problems of arbovirus study in insects

Very few arbovirus-vector relationships have been studied in the detail suggested above. Two notable exceptions are the detailed investigations of LaMotte (1960) and Thomas (1963) respectively into JE and WEE virus infections in mosquitoes.

Laboratory studies on insect-virus relationships are limited by many problems associated with the nature of the vector. These include the availability, size, and ease of handling and infection

of the insects under investigation.

1.6.1. Availability and colonisation of insects

For any long-term study on an insect-virus relationship it is necessary to have access to large numbers of the insect species at frequent and regular intervals. The distribution of many species is both seasonally and geographically restricted, and therefore they are most conveniently studied in laboratory colonies. However not all insect species are easy to colonise.

It has been shown that significant variation in susceptibility to oral infection with arboviruses occurs among geographically isolated strains of a single insect species (Gubler and Rosen, 1976; Hardy et al., 1976; Aitken et al., 1977; Gubler et al., 1979). There is also evidence that individual populations of insects are polymorphic in their susceptibility to an arbovirus infection and that this polymorphism may be genetically controlled (Jones and Foster, 1974; Hardy et al., 1978). Ideally therefore, a colony should be started from the largest numbers of individuals available in order to provide as wide a gene pool as possible in the subsequent population. The initial problem is usually to obtain a sufficient number of living specimens with which to proceed, particularly if the species in question is not endemic in the area where the research is to be carried out. Imported insects should always be screened to test for the presence of pathogenic agents, and a secure insectary should be available to protect against the possibility of a non-endemic species escaping to establish colonies in the wild.

Once adult insects have been obtained there may be difficulty in promoting blood feeding, mating, or egg laying, under laboratory

conditions. For example, some insect species mate only while swarming (Downes, 1969). Even successfully established colonies may have restrictions imposed on their use should diapause or aestivation occur during the life cycle thus limiting the continued supply of adults for research.

Many species of mosquitoes have been colonised and are easily maintained in the laboratory (Gerberg, 1970). Examples include Ae.triseriatus (Richardson and Romoser, 1972), Ae.albopictus (Rosen and Gubler, 1974) and Cx.fatigans Weidemann (Tesh , 1975). However few colonies of sandflies or Culicoides are available that are sufficiently productive to supply the large numbers of surplus insects required for virological research.

Sandflies exhibit a narrow range of tolerance to environmental conditions and are thus difficult to maintain in the laboratory. The first rearing of a sandfly species was reported in 1907 by Grassi, who reared some larvae of Phlebotomus mascitti Grassi (in Ward, 1977). Since that time numerous species have been reared in the laboratory. Methods of colonisation have slowly developed and improved, however the number of productive colonies available are still limited. Old World sandflies which have been maintained as laboratory colonies for more than three generations include P.papatasi Scopoli (Unsworth and Gordon, 1946; Schmidt, 1964; Modi personal communication, 1979; Killick-Kendrick personal communication, 1979); P.orientalis Parrot (Schmidt, 1964); P.argentipes Annandale and Brunetti (Killick-Kendrick et al., 1977); and P.longipes Parrot and Martin (Gemetchu, 1976). Of approximately 300 Neotropical sandfly species only 16 have been maintained beyond one generation in closed laboratory colonies. Of these only Lutzomyia longipalpis Lutz & Neiva, L.gomezi Nitzulescu, L.sanguinaria Fairchild & Hertig, and L.flaviscutellata Mangabeira, have been produced in sufficiently large numbers for experimental

work (in Ward, 1977).

Colonisation of Culicoides species has developed more recently. The first reported laboratory colony was of C.nubeculosus (Megahed, 1956). Jones (1957) colonised C.variipennis, and since then several species have been successfully maintained as laboratory colonies. These include C.guttipennis Coquillet (Hair and Turner, 1966); C.furens Poey (Linley, 1968); C.arakawae Arakawa and C.schultzei Enderlein (Sun, 1969); and C.riethi Kieffer, C.nubeculosus and C.variipennis (Boorman, 1974). The last two species have been established in the laboratory at Pirbright for many years and normally produce insects far in excess of the numbers required for routine maintenance of each colony. They have been used increasingly in recent years for the study of arboviruses.

Because of the problemsinherent in the colonisation and continued laboratory maintenance of many insect species, a satisfactory colony of the natural vector of a particular virus may not be available for study. It may therefore be necessary to construct a "model" system with an alternative species. The susceptibility of the available colonies to the virus under investigation must first be determined. It is essential to do this even if a colony of the natural vector can be obtained, since as previously stated different laboratory populations can vary in their susceptibility to infection with a particular virus. Furthermore, individual virus stocks, (ie. different passage levels of virus from different animal or cell culture systems), may vary in their infectivity for insects.

1.6.2. Experimental infection of insects with arboviruses

The second problem which arises when studying arboviruses in insects concerns the initial infection of insects in the laboratory.

Insects become infected in nature by ingesting virus with blood from a viraemic vertebrate host. Provision of a suitable host as a source of infection may not be possible if the arbovirus in question is pathogenic in man or the larger domestic animals and does not cause a detectable viraemia to develop in the standard small laboratory animals. Even when the natural vertebrate host is available the viraemia obtained under laboratory conditions may be of a short duration and of an insufficient level to infect the insect. For example, in this laboratory it has proved extremely difficult to infect C.variipennis, the natural vector of BT, with this virus by feeding insects on infected sheep. Only 4 out of a total of 400 females were found to contain virus after feeding on several sheep with blood virus concentrations of the order of 4.5 $\log_{10}TCID_{50}/ml$, as determined by titration in BHK21 cells (Dr. P.Mellor personal communication, 1979). The use of artificial methods of infection such as membrane feeding or intrathoracic inoculation may overcome a problem of this nature. The success of the former method depends partly on whether the presence of the blood alone is sufficient to initiate a feeding response, or whether other undetermined stimuli may be necessary before insects will ingest blood. Any number of factors may be involved. The natural stimulus to feed may be the carbon dioxide exhaled by the host animal. There may be a specific light intensity and temperature required. Insects may not feed in the relatively confined space of laboratory cages. They may need to walk on the "membrane" or animal skin rather than find access through the mesh of a cage top. The success of the inoculation technique depends both on the skill of the operator and on the robustness of the insect. Both techniques involve a certain amount of manipulation of the insects. Many insects are fragile and may not survive such procedures at all, or at least without extremely careful handling. Thus these basic

procedures require modification and adaption to the species used.

1.6.3. Size of insect vectors

Mosquitoes, Culicoides, and sandflies are all very small insects and are therefore difficult to handle. This applies to studies both of the living insect, and of the pathogenesis of viral infections in the vector. It is partly because of the difficulties imposed by the size of insects that few studies have been made on the sequential stages of virus infections in the tissues and organs of infected insects. Such work involves determination of the virus content of individual organs on successive days after the initial infection and in vertebrates would usually be carried out by dissecting and titrating each separate tissue. Estimation of the virus content of parts of infected insects in this manner is complicated both by the limitations that vector size impose on dissection techniques and by the appearance of virus in the haemolymph soon after infection. The haemolymph, and thus the virus it contains, contaminates the surface of most tissues. The size of insects renders difficult both the initial dissection and the washing procedures necessary to remove surface contaminating virus.

Most of the information on the distribution of virus in the vector has been obtained from the study of mosquitoes, since the majority of these insects are considerably larger than Culicoides or sandflies and are therefore easier to dissect. The respective lengths of Ae.aegypti, L.longipalpis and C.variipennis for example are 5.6mm, 2.0-2.5mm, and 1.2-1.5mm.

Electron microscope studies of arboviruses in insects are possible, although this assumes the possession of such an instrument, and involves lengthy processing of specimens. Several arbovirus infections of mosquitoes have been investigated by electron microscopy.

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Examples include studies on the distribution of WEE and Dengue (DEN) in mosquitoes (Larsen and Ashley, 1971;Sriurairatna and Bhamarapravati, 1977), and on salivary glands infected with Chikungunya (CHIK), CE and JE viruses (Janzen et al., 1970; McLean et al., 1975; Takahashi and Suzuki, 1979). The only report of such work with Culicoides or sandflies infected with arboviruses is by Bowne and Jones (1966) who completed an electron microscope study of BT virus in the salivary glands of C.variipennis. Routine histological techniques of sectioning and staining for light microscopical work cannot be used to demonstrate virus. However, recently developed immunological methods such as the fluorescent antibody or immunoperoxidase techniques, can be used to demonstrate a variety of antigenic substances, including viral antigens, in animal cells and tissues (see part 3.1 and 3.6.). Such methods have as yet had only limited use in the study of arbovirus infections of insects. It is possible that they may provide an ideal means of studying the pathogenesis of arbovirus infections in Culicoides and sandflies.

1.7. Organisation and aims of the thesis

Research in the Entomology department at the A.V.R.I. Pirbright is concerned with the study of selected arboviruses and the relationships of these viruses with their insect vectors and other possible insect hosts. Emphasis is placed on the study of two groups of insects, Culicoides and sandflies. Culicoides are studied because several vectors of orbiviruses of economic importance such as BT and AHS are included in the genus. Research on sandflies is carried out because several species are important as vectors of viruses of man and domestic animals yet very little is known about the behaviour of arboviruses in

these insects.

The research presented in this thesis was undertaken firstly to investigate some of the problems involved in laboratory studies on arbovirus infections in sandflies in order that the development of some of the Phlebotomus fever group viruses in these insects could be studied. The supply of sandflies was initially limited and irregular, and therefore a second line of research was followed, examining the possibility of using a fluorescent antibody technique for the detection of arboviruses in insects at a histological level. In this part of the project emphasis was placed on the study of BT virus in the vector C.variipennis since this virus is of particular importance in the research programme at A.V.R.I. and the insects were readily available. The two sections were linked by interest in the specificity of arboviruses with respect to the insect host, and in the susceptibility of the two insect groups to viruses from two different groups.

The aims of this thesis are summarised as follows:

1. To determine whether sandflies can be infected with viruses using artificial methods such as membrane feeding and intrathoracic inoculation.
2. To investigate the susceptibility of the sandfly Lutzomyia longipalpis to infection with three viruses of the Phlebotomus fever group: SFS, SFN, and PAC, to determine whether this species could be used as a laboratory model for studies of these viruses.
3. To investigate the susceptibility of sandflies to infection with an orbivirus BT; and of Culicoides species to the above three Phlebotomus fever group viruses, as part of a general programme of research into the specificity of the arboviruses with respect to their insect host, and into

the vector potential of these insects with respect to these viruses.

4. To determine whether the fluorescent antibody technique could be applied to the study of viruses in Culicoides and sandflies with emphasis on the detection of BT viral antigen in the vector C.variipennis.

The work is presented in two parts. The first includes all studies on the development of viruses in the insect species (i.e. numbers 1 to 3 above), the second contains the immunofluorescence studies (i.e. number 4 above).

Part 2

Studies on the artificial infection of sandflies and *Culicoides* with three Phlebotomus fever group viruses, Sicilian and Naples sandfly fever, and Pacui, and with an Orbivirus, Bluetongue

2.1. Introduction

There are at least 27 antigenically related viruses in the Phlebotomus fever group most of which have been isolated from sandflies (Table 1.3.). The group has a widespread geographical distribution in both the Old and New World. Vertebrate hosts include man (e.g. Candiru, Chagres, Sicilian and Naples sandfly fever, Punta Toro), rodents (e.g. Arumowot, Bujaru, Gordil, Icoaraci, Itaporanga and Pacui), and sloth (Anhanga) (Andrewes et al., 1978). At least five of the viruses which infect man give rise to an acute febrile disease of short duration. The classical form of this disease was first described in the Mediterranean area where its association with Phlebotomus sandflies gave rise to the names sandfly, Phlebotomus, or Pappataci fever. Sandfly fever occurs in those parts of Europe, Asia, and Africa lying between 20 and 45 degrees latitude (Sabin et al., 1944). The disease was of considerable military importance during both world wars since it was prevalent in many areas where troops were stationed. Although not fatal it incapacitated large numbers of men for 7 to 14 days at times when their services were most needed. The viral aetiology of sandfly fever was demonstrated by Doerr et al. in 1909. The virus responsible for the disease was designated Sandfly fever virus. In 1951 Sabin differentiated two immunologically distinct "strains" of Sandfly fever virus - the Sicilian (SFS) and Naples (SFN) strains. These are in fact different viruses, which are antigenically distinct both from each other, and from the other viruses in the Phlebotomus fever group. The Naples virus is more closely related to the other members of the group than is the Sicilian virus.

The vector of Sandfly fever virus, at least of SFS, is the sandfly Phlebotomus papatasi. Doerr et al. (1909) first showed that

these insects could transmit the disease. This was confirmed by later workers such as Whittingham (1924) and Sabin et al. (1944). Evidence for the vectorial capacity of P.papatasi for SFN virus is more limited than for SFS virus. The Naples virus has been isolated from P.papatasi in India (Goverdhan et al., 1976) and in Egypt (Schmidt et al., 1971). In Egypt SFN virus was only isolated from freshly engorged sandflies. This does not confirm the vector status of the insect since the isolated virus may have been ingested with a recent blood meal. The Sicilian virus was isolated from non-engorged sandflies, and from sandflies which had digested their blood meal, indicating that these insects were acting as true biological vectors. Sicilian sandfly fever virus has been isolated from wild-caught P.papatasi in other areas including West Pakistan (George, 1970), Iran (Tesh et al., 1977), and India (Goverdhan et al., 1976).

A transovarial transmission cycle is probably involved in the natural history of sandfly fever. This was first suggested by Doerr et al. in 1909. Evidence for such a cycle occuring was provided by Whittingham (1924) who produced clinical symptoms of the disease in human volunteers bitten by the laboratory reared offspring of infected flies. He believed that the virus was not transovarially transmitted but that the larvae became infected after ingesting the remains of infected adult flies. Two groups of Russian workers, Moshkovsky et al. in 1937 and Petrischeva and Alymov in 1939, provided additional evidence for the occurrence of transovarial transmission. The latter workers obtained transovarial transmission of Sandfly fever virus through two successive generations of flies (in Burgdorfer and Varma, 1967). Sabin et al. (1944) were unable to confirm these results. The early experiments were carried out before it was realised that

more than one virus could cause the disease, and it is not known which virus any of these workers were using. Isolations of SFS virus from wild-caught male sandflies (Barnett and Suyemoto, 1961; Schmidt et al., 1971) strengthens the supposition that transovarial transmission of the virus does occur, but conclusive evidence to this effect has not yet been produced. Several other Phlebotomus fever group viruses have been isolated from male sandflies suggesting that a transovarial cycle may be common to members of this virus group (Tesh et al., 1974; Aitken et al., 1975, Tesh et al., 1977).

There is no detailed information concerning the development of any of the Phlebotomus fever group viruses in sandflies. Doerr et al. (1909) and Whittingham (1924) suggested that the extrinsic incubation period of sandfly fever virus in P.papatasi was at least 7 days, but this has not been proved satisfactorily. Other factors, such as the rate and level of virus multiplication in the insect, the amount of virus ingested or transmitted, the threshold levels for infectivity and associated infection rates, the transmission rate, and details of a possible transovarial transmission cycle, have not been investigated.

Most of the information concerning these and other sandfly-borne viruses has been derived from virus isolation studies on wild-caught insects. Laboratory studies on sandflies as vectors of viruses have been limited, partly by the lack of suitable viraemic vertebrate hosts with the consequent difficulty of infection of the vector. The Sandfly fever viruses, SFS and SFN for example, normally infect man and do not cause a detectable viraemia in the standard small laboratory animals. Previous observations on the development of these viruses in their sandfly vectors have been made using human volunteers as the source of infection (e.g. Whittingham, 1924; Petrischeva and Alymov, 1939;

Sabin et al., 1944). However, even in man the viraemia produced may be low and of short (48 hour) duration. In volunteers infected with SFS for example, the concentration of virus in the blood did not exceed $10^{3.4}$/ml as assayed in tissue culture and in human subjects (Bartelloni and Tesh, 1976). This is below the theoretical threshold level of $10^{4.0}$/ml suggested in Tesh and Chaniotis (1975) to be required to infect the sandfly vector.

The development of a convenient artificial method for infecting sandfly species with viruses would be of great advantage for the study of vector-virus relationships in this group of insects. Artificial infection techniques such as membrane feeding or direct inoculation of virus into the vector, have not been used to study virus infections in sandflies, despite their application to other groups of biting flies such as Culicoides and mosquitoes.

A further limiting factor in the study of sandflies as vectors of viruses, particularly in laboratories in this country, has been the scarcity of productive colonies for experimental work. At the start of this project the only sandflies available for study were from a colony of L.longipalpis.

The following work was undertaken to determine whether membrane feeding and inoculation methods could be used to infect sandflies such as L.longipalpis with viruses without affecting the subsequent survival and behaviour of these rather fragile insects. The susceptibility of this sandfly colony to infection with the two viruses of Sandfly fever, SFS and SFN, was then tested to determine whether the colony would prove to be an efficient laboratory vector for these viruses. If so, it would provide a useful "model" system for the study of the behaviour of these viruses in an insect host.

Both SFS and SFN viruses are confined to the Old World, L.longipalpis however is a New World sandfly species. The susceptibility of this species to Pacui (PAC), a Phlebotomus fever group virus from the New World (Tesh et al., 1975), was therefore also assessed. Pacui was originally isolated from small forest rodents in Brazil (Woodall, 1967 in Aitken et al., 1975), and Trinidad (Jonkers et al., 1968). It has been isolated from both male and female L.flaviscutellata (Aitken et al., 1975) which suggests that a transovarial cycle may be involved in the epidemiology of this virus. Pacui has not yet been isolated from L.longipalpis, but the possibility of the species forming a laboratory "model" for study of the virus, was considered.

A limited number of colonised P.papatasi became available after completion of the major part of this work. Since the original colony was already reported as being susceptible to infection with Sandfly fever (Modi personal communication, 1979), the susceptibility of these insects to infection with PAC virus was tested so that the fate of the Old and New World viruses in Old and New World sandfly species could be compared. Sufficient insects later became available to perform a limited number of experiments with the viruses of SFS and SFN. These were intended mainly as a positive control, to check the infectivity of the two virus stocks for insects by observation of their behaviour in a susceptible vector. To overcome any possible problem of a low oral infection rate, or reluctance of sandflies to feed, the inoculation technique was used to infect P.papatasi with the SFS and SFN viruses in these experiments.

The behaviour of the three Phlebotomus fever group viruses, SFS, SFN and PAC, in two Culicoides species was also investigated. With the exception of RVF virus, which has recently been included in

the Phlebotomus fever group, none of this group of viruses have yet been isolated from Culicoides species, although these insects do occur in areas where viruses from the group are found. The distribution of C.nubeculosus in Southern Europe for example overlaps with the distribution of sandfly fever in that area. A closely related species, C.puncticollis Becker, occurs in the Middle East where sandfly fever is endemic. Both species will bite man and could therefore conceivably act as vectors of the disease. Many Culicoides species will take several blood meals during their life span and are therefore potentially efficient virus vectors.

The study of Culicoides is of particular importance at A.V.R.I. Pirbright since several confirmed vectors of BT virus are included in the genus. Bluetongue virus causes an acute disease in susceptible sheep. The virus will infect other ruminants such as cattle and goats, although these animals are less likely to exhibit clinical signs of the disease. It has a widespread geographical distribution occurring throughout Africa and the Middle East, in India, Pakistan, the Eastern Mediterranean basin including Cyprus, Northern Australia, Central, South and North America. Bluetongue virus antibodies have also been detected in cattle in Arabia. The virus is of major economic importance in parts of these countries where susceptible ruminants are reared. So far a total of 20 different virus types have been distinguished by serological tests.

Bluetongue virus has been isolated from a number of Culicoides species, however the vector potential of many of these remains to be determined. For example, in Australia the virus has been isolated from a mixed pool of Culicoides containing C.actoni Smith, C.brevitarsis Kieffer, C.bundyensis Lee and Reye, C.marksi Lee and Reye, C.perigrinus

Kieffer and C.schultzei Enderlein (St.George et al., 1978). The vector potential of each of these species has not yet been established. Confirmed vectors of BT virus include C.variipennis in North America (Foster et al., 1963), and C.imicola in Africa (Dutoit, 1944). C.obsoletus Meigen and C.imicola may act as vectors in Cyprus (Mellor and Pitzolis, 1979). In some areas a vector has yet to be established.

Bluetongue virus has not been isolated in the wild from any insect other than Culicoides and few laboratory infections of genera other than Culicoides have been reported. Phlebotomus and Lutzomyia sandflies occur in areas where BT is endemic, Phlebotomus in the Old World and Lutzomyia in the New World. Sandflies feed on a wide variety of vertebrates including rodents, dogs, and man. Some species, for example, P.papatasi, P.argentipes and P.perfiliewi Parrot, regularly feed on domestic ruminants (Javadian et al., 1977, Dr. R. Killick-Kendrick personal communication, 1979). Bluetongue is mainly associated with ruminants but the virus has been isolated from rodents e.g. Rhabdomys pumilio and Otomys irroratus (Dutoit and Goosen, 1949 in Neitz, 1960). It is possible that sandflies could act as direct vectors of the disease, or at least play some part in the maintenance of virus in the wild, however no investigation of the susceptibility of these insects to BT virus has been made. The susceptibility of L.longipalpis and P.papatasi to infection with BT virus was therefore assessed to determine the vector potential of these two species with respect to this virus.

Details of the multiplication rate of BT virus in C.variipennis after infection by membrane feeding and intrathoracic inoculation have been published (Foster and Jones 1979; Jochim and Jones, 1966), and certain aspects have been studied at Pirbright (Mellor, unpublished results). It was however considered necessary to repeat these

experiments, and to obtain details of the development of BT virus in C.nubeculosus, so that a more direct comparison of the behaviour of this virus in the two groups of insects, i.e. Culicoides and sandflies, could be made in this project.

Included at the end of this section is a preliminary investigation into the distribution of BT and PAC viruses in the insects in which they multiply. The fate of BT virus in L.longipalpis and C.variipennis after intrathoracic inoculation; of BT virus in C.variipennis and C.nubeculosus after membrane feeding;and of PAC virus after intrathoracic inoculation into L.longipalpis; were compared.

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2.2. Materials and methods

2.2.1. Insects

Sandflies

Adult sandflies from a colony of L.longipalpis were obtained from Dr. R. Killick-Kendrick at Imperial College Field Station, Silwell Park, Ascot. This colony was started in 1972 from 53 females collected in the Lapinha caves, Lagoa Santa, Minas Gerais, Brazil (Killick-Kendrick et al., 1977). A subsidiary colony was established at Pirbright and was maintained through six generations in the manner described in appendix 1. The colony was never sufficiently productive for experimental use, and since the continued maintenance was excessively time-consuming adult flies were obtained from Imperial College whenever possible. They were collected in batches of from 50 to 200 insects contained in $18cm^3$ gauze covered wire cages. They were removed from these cages by gentle suction, using an aspirator with a 5mm diameter glass end tube, and were transferred to smaller cages made from waxed card pill boxes

(diameter 65mm) capped with nylon stocking. Each cage contained a plastic pot (diameter 25mm) filled with moist cotton wool and inserted into a hole in its base (Figure 2.1). This provided a damp resting site for the sandflies. A cotton wool pad soaked in 30% (w/v) sucrose solution was placed on top of the nylon stocking, and approximately 99% humidity was maintained by keeping the cages in plastic boxes containing a small pad of damp cotton wool. Flies were held at 25°C ± 1°C and in the dark except during experimental manipulation.

P.papatasi adults were also supplied by Dr.Killick-Kendrick at Imperial College. His colony was established in 1979 with insects imported by Dr. C. Modi from a colony at the National Institute of Virology, Pune, India. These sandflies were maintained in the same way as L.longipalpis.

The average lifespan of adult sandflies in the laboratory was 12 days. Some individuals survived for up to 22 days.

Culicoides

C.variipennis and C.nubeculosus adults were obtained from colonies in the Entomology department at A.V.R.I. Pirbright. These colonies have been described fully by Boorman (1974). The colony of C.variipennis were established here in 1967 using eggs supplied by Dr. R. Jones from his colony in Denver, USA. C.nubeculosus has been reared in this department since 1971. The colony was started from larvae obtained from Dr. P. Mellor who had initiated a colony two years previously using adults collected near Winches Farm Field Station, Hertfordshire.

The Culicoides adults were kept in the same type of waxed card pill box cages as sandflies. Approximately 100 males and females were contained in each cage. Ten per cent rather than 30% sucrose was

Figure 2.1. Insect holding cages and Hilton pot

Figure 2.2. Membrane feeding unit with chick skin membrane

Figure 2.1. Insect holding cages and Hilton pot

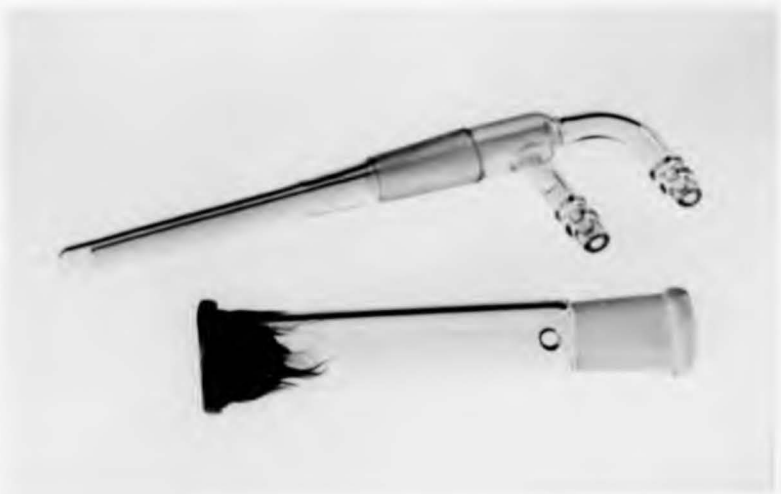

Figure 2.2. Membrane feeding unit with chick skin membrane

provided as a source of carbohydrate. The average survival time at 25°C was 12 days. Some individuals survived for up to 22 days.

2.2.2. Viruses

Phlebotomus fever group viruses

Both SFS and SFN viruses were obtained as freeze dried mouse brain preparations from Dr. J.S. Porterfield, then at the National Institute for Medical Research, Mill Hill.

A similar preparation of PAC virus was supplied by Dr. R. Shope at Yale University, U.S.A.

All three viruses were reconstituted in a 1% solution of bovine albumin in phosphate buffered saline, pH 7.2, (1% BA), and were passaged intracerebrally (i.c.)in suckling mice prior to their use in these experiments. The total number of mouse brain passages for each virus were as follows : SFS 48, SFN 62, and PAC 4. All three viruses were stored at -70°C.

Bluetongue

Bluetongue virus type 4, strain A/SOT 1 was used throughout these experiments. This strain was isolated from sheep in Cyprus in 1969 (Parker et al., 1975). The initial isolation was made by intravenous inoculation into embryonated chicken eggs. The virus was then passaged in BHK 21 (baby hamster kidney) cells (Macpherson and Stoker, 1962), and was stored at -70°C as a suspension in Eagles maintenance medium (EMM) containing Eagles basal medium (Flow) 89%, tryptose phosphate broth (Flow) 10%, foetal bovine serum (Seraservices) 1%, with 52,000 i.u. neomycin (Upjohn) and 25,000 i.u. mycostatin (Squibb) per litre. BHK 21 cell passage levels 9 to 13 were used in these experiments.

2.2.3. Infection of Insects

Insects were infected with viruses using the routine membrane feeding and intrathoracic inoculation techniques described below. Although both methods are regularly used to infect C.variipennis and C.nubeculosus with viruses in this laboratory, neither method had previously been applied to sandflies. The modifications to the basic procedures which were developed to ensure the maximum utilisation and survival of available sandflies will be described in the results section.

2.2.3.1. Membrane feeding

The membrane feeding apparatus and units employed were of the type modified by Boorman from those used by Mellor (1971). They are illustrated in Figures 2.2. and 2.3.

Each membrane feeding unit consisted of an outer glass cylinder (diameter 2cm, length 10cm) and a detachable inner glass "finger". A membrane was stretched over the lower end of the cylinder and was secured with a rubber band. The membranes used in these experiments were prepared from skins of one day old chicks. The skins were plucked before use and all subcutaneous fat was removed. They were then attached to the cylinder with the epidermis on the outside. Care was taken that the skin did not dry out during preparation. Skins could be stored at -20^{o}C for several months but began to deteriorate after about a year and became unattractive to insects. With the membrane in place, feeding mixtures consisting of fresh heparin-treated mouse blood (final concentration of heparin 25 units per ml of blood) and virus, usually in equal volumes of between 0.2 and 0.4 ml, were introduced into the cylinder. The inner finger was inserted and the unit was connected by rubber tubing to a thermostatic water pump. Warm water at a temperature

of 40 to 44°C was circulated through the finger providing a temperature of approximately 37°C at the membrane. The assembled units were mounted vertically in a sealable cabinet. They were designed so that they could be connected in series to enable several cages of insects to be fed at once. The cabinet was fitted both with a 50 watt light and a detachable black cloth covering to enable feeding experiments to be conducted either in light or dark conditions. Assembled units were placed on top of the pillbox cages (Figure 2.3) as soon as the blood-virus mixtures they contained had reached the required temperature. The insects fed through the stocking on top of the cage, and the membrane.

Culicoides and sandflies were membrane fed between 3 and 12 days post emergence. The virus concentrations of feeding mixtures were between 6.0 and 9.5 $\log_{10}MLD_{50}/ml$. The concentrations used in individual experiments are described in the results section.

After feeding, insects were anaesthetised with CO_2 and were transferred for sorting to a brass turntable lined with filter paper (Figure 2.4.) under a dissecting microscope (Hewlett and Lloyd,1960). A constant stream of CO_2 was passed over the insect to maintain anaesthesia. Ten to 20 engorged females were removed and killed by freezing at -70°C. They were stored at this temperature in small glass vials, and were processed later to confirm that virus as well as blood had been ingested. The remaining blood fed females were transferred into clean pillbox cages.

Sandflies would oviposit within 4 days of feeding. It was therefore important at this stage to prevent any contact of the females with damp surfaces in order to discourage egg laying and prolong survival. This is essential in experiments requiring a

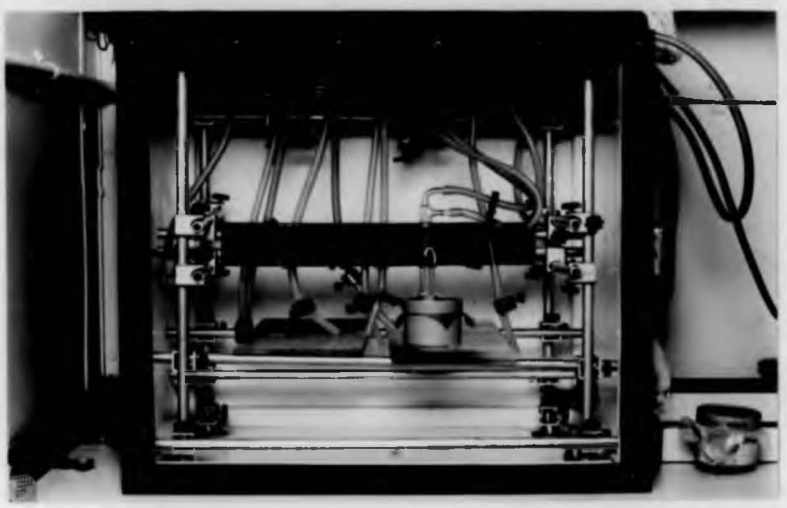

Figure 2.3. Membrane feeding apparatus and cabinet

Figure 2.4. Turntable for sorting insects, fine forceps and fine brush

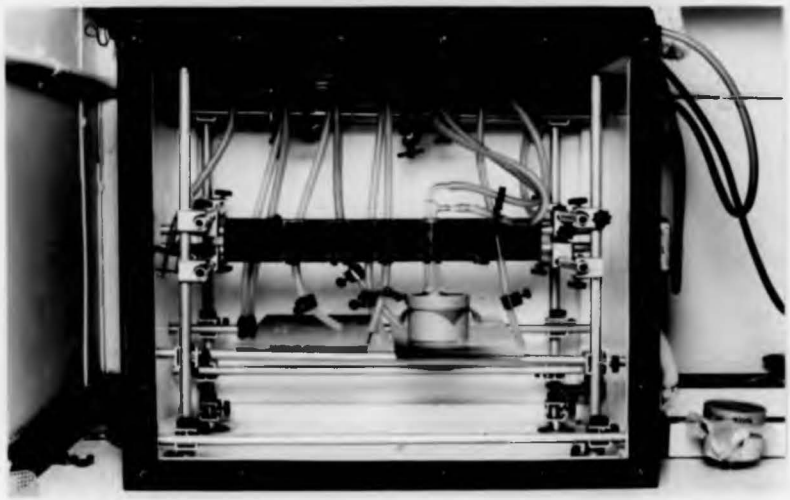

Figure 2.3. Membrane feeding apparatus and cabinet

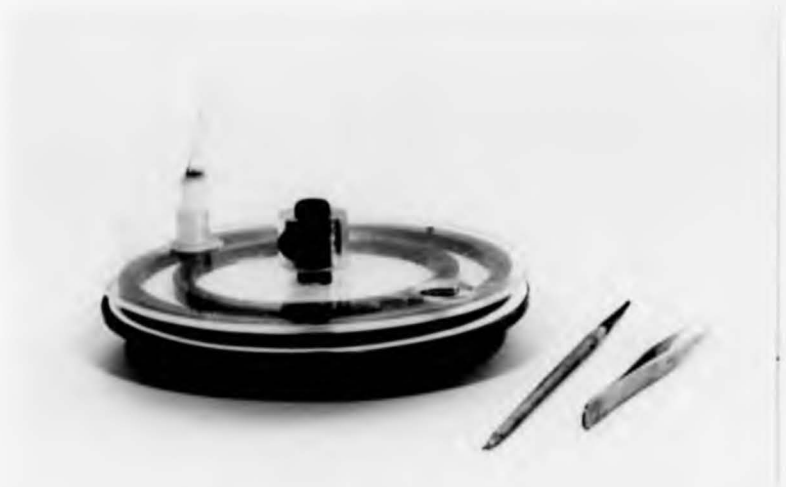

Figure 2.4. Turntable for sorting insects, fine forceps and fine brush

maximum survival time for the flies since a high percentage of L.longipalpis and P.papatasi die during or after oviposition. Pillbox cages without the damp cotton wool-filled plastic pot in the base were therefore used for engorged sandflies. This precaution was not necessary for either Culicoides species.

Both sandflies and Culicoides were stored at 25°C and in the dark as described in section 2.2.1. Between 1 and 20 insects were removed at daily intervals after infection in order to follow the course of viral development in the insects. All such samples were killed by freezing at -70°C and were stored at this temperature for processing at a future date.

2.2.3.2. Intrathoracic inoculation

The apparatus used for the inoculation of insects was designed by Boorman (1975). It is illustrated in Figures 2.5, 2.6, and 2.7. Needles with a diameter of between 45 and 60µm were prepared by the extrusion of fine glass capillary tubes on a Leitz needle puller. They were mounted with araldite on the tip of a 26 or 27 gauge hypodermic needle attached to a 1ml plastic syringe. Virus was drawn into the needle by operation of the syringe, the barrel of which was then connected with silicone rubber tubing to a source of compressed air at a pressure of 3 to 4 p.s.i. This provided the pressure to inject the inoculum. Release of the compressed air was controlled by a foot pedal. This left both hands free for the manipulation of the needle and the insects. A switch graduated from 0.1 to 1.0 seconds controlled the time interval during which pressure was applied. This factor together with the diameter of the needle determined the volume of the inoculum, which was approximately 0.0001 to 0.0002 ml.

Female sandflies and Culicoides were inoculated with virus between 2 and 10 days post emergence. The concentration of virus in the inoculum was not less than 6.0 $\log_{10}$ MLD_{50}/ml. Individual values

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Figure 2.5. Apparatus for the intrathoracic inoculation of insects with viruses (after Boorman, 1975)

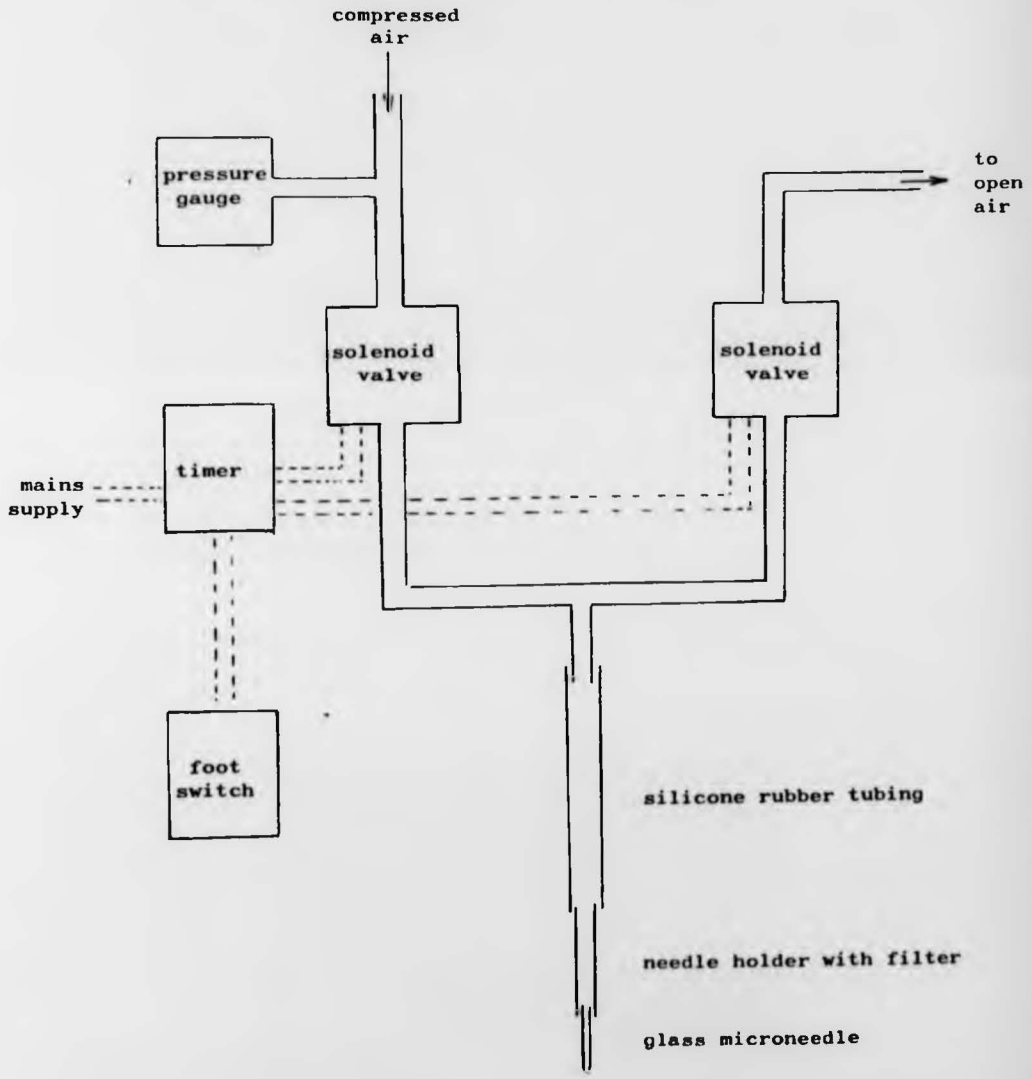

Figure 2.6. Inoculation apparatus and cabinet

Figure 2.7. Inoculation apparatus, pressure gauge, and timer

Figure 2.6. Inoculation apparatus and cabinet

Figure 2.7. Inoculation apparatus, pressure gauge, and timer

for each experiment are given in the results.

Insects were anaesthetised with CO_2 and were sprinkled onto the turntable under the dissecting microscope. The tip of the needle was inserted between the pleural sclerites of the thorax, just below the wing. The abdomen could be seen to distend slightly as the virus was injected. Five to 25 insects were killed immediately after inoculation so that the initial infection rate and the quantity of virus which had been inoculated, (the day 0 value), could be determined. The remaining insects were returned to clean cages and were stored at 25°C as described in section 2.2.1. Between 1 and 20 insects were removed at daily intervals after infection in order to follow the course of viral development in the insects. All samples were killed by freezing at -70°C and were stored at this temperature before processing.

2.2.4. Transmission experiments

Female insects were injected with virus and were then membrane fed on uninfected mouse blood several days later in order to determine whether they could transmit virus. The blood and membrane used in these experiments were each processed to test for the presence of transmitted virus. All females that ingested clean blood were titrated individually to determine the virus content of each fly, and the number of flies infected, so that an estimate of the amount of virus transmitted by each female could be made.

Ideally in transmission experiments insects should be infected as soon as possible after emergence to ensure that the maximum number will survive and feed after a 5 to 10 day incubation period. L.longipalpis and P.papatasi sandflies do not normally take a second blood meal in the laboratory, and therefore transmission experiments with these insects rely on the use of the intrathoracic inoculation

technique to provide the initial infection. Female sandflies above the age of 14 days were reluctant to feed and therefore the initial inoculation was best performed on flies less than 9 days old.

2.2.5. Thermal inactivation of virus

During investigation of the growth of virus in a system it is necessary to obtain a measure of the stability of the virus under the conditions of the experiment so that any persistence of stable virus can be distinguished from virus multiplication. The stability at 25°C of each of the viruses used in these experiments i.e. SFS, SFN, PAC, and BT was therefore investigated. Both the stock virus suspension in 1% BA or EMM, and mixture of virus and heparin-treated mouse blood as used in membrane feeding experiments, were incubated at 25°C. Samples were removed at daily intervals. The pH of each sample was tested before storage at -70°C prior to titration. This was the nearest possible approach to incubation of virus in the insect since mixtures of triturated insects and virus rapidly became contaminated when incubated at 25°C.

2.2.6. Estimation of virus

Infected insects were ground individually, or in small groups of up to 10 flies, in 1ml of cold diluent in Griffiths tubes. Serial tenfold dilutions of the resultant suspensions were prepared and assayed by one of the following methods.

All Phlebotomus fever group virus titrations were performed by the intracerebral (i.c.) inoculation of serial tenfold virus dilutions into groups of 3 to 5 day old suckling mice. Each dilution was inoculated into a group of 6 mice, each mouse receiving 0.02 ml. The diluent used was 0.1% BA with added antibiotics, (penicillin 1000 i.u./ml, streptomycin 1000 i.u./ml, (Glaxo)). The mice were examined

at 1,5,9, and 14 day intervals and the mortality was recorded. Phlebotomus fever virus stock suspensions, blood-virus mixtures used for membrane feeding, and the blood and membrane used in transmission experiments were titrated in the same way. Membranes were first ground in 1ml of 0.1% BA with antibiotics in Griffiths tubes. The resultant suspension was centrifuged at 2,500 r.p.m. for 10 minutes before titration of the supernatant. The method of Spearmann-Kärber (in Finney, 1964) was used to calculate the virus concentration (titre) of each sample. The results were expressed in terms of the 50% mouse lethal dose (MLD_{50}) i.e. as $\log_{10}MLD_{50}$ per fly or per ml.

Bluetongue virus titrations were performed by inoculation of virus suspensions onto BHK 21 cells and microscopical observation of viral cytopathic effect (CPE) in these cells. The diluent for virus suspensions was EMM. 0.1 ml of each serial dilution was inoculated into each of 5 wells in a microtitre plate containing BHK 21 cells. The plates were set up 24 hours previously using a sufficient concentration of BHK 21 cells in Eagles Growth medium, (EGM, consisting of EMM with 5% foetal bovine serum), to give a 60% cell monolayer at the bottom of each well by the following day.

The cells and virus were incubated together at 37°C for up to 7 days, although the titration end point could usually be observed after 5 days. Virus concentrations were calculated by the Spearmann-Kärber method and were expressed in terms of the 50% tissue culture infective dose as $\log_{10}TCID_{50}$ per fly or per ml.

Bluetongue virus stocks and the blood-virus mixtures used for membrane feeding were titrated in the same way. However the blood and membrane used in transmission experiments with this virus could not be titrated in BHK 21 cells since these preparations caused excessive contamination of the cell monolayers, even with antibiotics included in the diluent. The blood and membrane were therefore titrated by the i.c.

inoculation of suckling mice as described for the Phlebotomus fever group viruses. The two assay systems are of similar sensitivity with respect to BT virus.

The identity of virus recovered from infected insects, and from the blood and membrane used in transmission experiments, was confirmed by serum-neutralisation tests using specific antiserum produced in rabbits.

2.2.7. Virus distribution experiments

A preliminary investigation was made into the distribution of PAC and BT in sandflies and Culicoides on successive days after virus infection. Infected insects were dissected into the three major body parts: head, thorax and abdomen, at daily intervals after infection, and each part was then titrated separately in the manner described for entire insects.

Insects were dissected under a binocular microscope, using a magnification of x 11 or x 12. The instruments used for dissection were made from "Hagedorn" type surgical needles (Holborn Surgical Instrument Co.,London) mounted with araldite in 110 mm lengths of 4mm diameter glass tubing (Figure 3.2.). Several lengths and thicknesses of glass tubing were tested but the 110 x 4mm proved the easiest to manipulate.

Individual insects (previously killed by freezing at -70°C) were placed on a glass microscope slide on the microscope stage. Four insects were spaced out on each slide. The head of each was cut off with the dissecting needles, and was transferred to a Griffiths tissue grinder. It was important to ensure that the salivary glands remained in the thorax and were not extracted attached to the head. To avoid cross-contamination from virus in any haemolymph squeezed out during the first dissection, the thorax and abdomen were transferred to a second slide. These were carefully divided and

placed in separate tissue grinders. The division of the thorax and abdomen of both Culicoides and sandflies was extremely difficult, particularly if the dissection was carried out on insects which had been killed within 24 hours of ingesting blood-virus mixtures. Care had to be taken not to pull out the fore or midgut, nor to rupture the gut during these dissections.

The body sections were ground up in groups of from 1 to 10 heads, thoraces, or abdomens, in 1ml of 0.1% BA (PAC), or in 1ml of EMM (BT). The resultant suspensions were titrated in suckling mice or BHK 21 cells respectively, and the results were expressed as $\log_{10}MLD_{50}$ or $TCID_{50}$ of virus per head, thorax, or abdomen. The experiments were repeated at least twice to confirm the results.

The quantity of tissue in each of the three body parts was different, both the thorax and the abdomen being considerably larger than the head. Therefore, to enable a direct comparison of the virus concentration in the three parts to be made, the weight of each part was determined, and the ratio between the weights of the three parts was calculated. Batches of 20 separated heads, thoraces and abdomens of C.variipennis were weighed on days 0,1, and 6 after ingestion of blood. Unfed females, 6 days old, were also dissected and weighed. Both these, and the females weighed on day 6 post feeding, (by which time the blood meal had been digested and the eggs had been laid), were considered to represent insects used in inoculation experiments. The number of sandflies available for these experiments were limited, and only unfed L.longipalpis were dissected and weighed.

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2.3. Results

2.3.1. Artificial techniques for the infection of sandflies with viruses

Both L.longipalpis and P.papatasi sandflies proved much more fragile than the species of Culicoides and mosquitoes that have been used in this laboratory. They required extremely careful handling since the loss of even one leg would result in the death of the sandfly within 24 hours. Fine watchmakers forceps or a small camel hair brush were used to move anaesthetised flies. When forceps were used insects were picked up by a wing rather than a leg. All manipulation was kept to an absolute minimum throughout both membrane feeding and inoculation procedures.

2.3.1.1. Membrane feeding

This technique was extremely successful. During the course of the project over 800 L.longipalpis and 300 P.papatasi were membrane fed on blood and virus.

Females would feed immediately after they had been transferred from the gauze to the pillbox cages, thus aspiration did not adversely affect the feeding response. The optimum age for feeding was 3 to 8 days post emergence. Older flies would feed, but these would be of less use for experiments on virus multiplication since they could provide only a limited number of daily samples. Younger females were reluctant to feed at all.

In the majority of experiments using 3 to 8 day old females, 95 to 100% of those offered a bloodmeal through the membrane feeder were fully engorged within 30 minutes. Those that were reluctant to feed could often be stimulated to do so by breathing several times into their cage. Blood was visible in the fore and mid gut immediately after feeding. Provided only small numbers of sandflies were present in

each cage, (i.e. 6 to 10), turntable sorting was found to be unnecessary since fully engorged females could be sorted with the naked eye. An aspirator could be used to remove the occasional unfed sandfly. "Dry" pillbox cages, without the provision of damp cotton wool, were therefore used to hold sandflies during later feeding experiments so that they could be left in their cages after feeding, and handling was minimised.

The volume of the blood meal in a fully engorged sandfly was approximately 0.0005 ml (Killick-Kendrick et al., 1977). In preliminary experiments to test the reliability of the method for infecting sandflies, 50 individual females were titrated within 2 hours of feeding on blood and SFN virus. The concentration of virus in the feeding mixture was 7.3 $\log_{10}MLD_{50}/ml$. All blood fed females contained virus. The mean amount of virus ingested by each female was 3.8 $\log_{10}MLD_{50}$.

As the project continued it was found that occasionally, under apparently identical laboratory conditions and handling procedures, only a very small percentage of a batch of 100 sandflies would feed, even when offered blood meals on several consecutive days at the same or different times of day. Breathing on them did not stimulate them to feed, neither did raising the temperature of the blood meal to 42°C. This was an additional reason for only feeding flies in small batches. Flies that did not appear to contain blood may still have probed the membrane and become contaminated with virus. They were therefore "wasted" since they could not be reliably used a second time. This was of particular importance in later experiments on development of virus within the insect.

Viable eggs were obtained from membrane fed flies, an important consideration if transovarial transmission experiments are to be carried out. When egg laying was prevented the maximum survival time

post feeding was 15 days. Similar survival times were obtained when batches of 25 control sandflies were either membrane fed on blood alone, or were fed naturally on shaved adult mice placed on top of their cages.

2.3.1.2. Intrathoracic inoculation

This technique could be reliably used to infect sandflies with viruses. In initial experiments 4 separate batches of approximately 30 females and 20 males were inoculated with SFN virus (7.6 $\log_{10}MLD_{50}$/ml). Titration of 25 individual females killed within 2 hours of inoculation showed that 24 out of the 25 were infected, an initial infection rate of 96%. The mean virus concentration per fly at this time was 2.8 $\log_{10}MLD_{50}$ with a range in the results for individual infected flies of from 1.9 to 3.9 $\log_{10}MLD_{50}$. The volume of the inoculum in these experiments was estimated to be between 0.005 and 0.00005 ml. The survival rate of sandflies after inoculation was very low in these initial experiments. Only 50% of females and 20% of males survived the operation, a further 50% dying within 24 hours. However the survival of flies was greatly improved by modifying the procedure as follows.

(i) Moist rather than dry CO_2 was used as an anaesthetic, and the flow through the turntable was adjusted to the minimum necessary to maintain a very light anaesthesia (400cc/min). This also applied when sorting membrane fed flies.

(ii) Only 6 to 10 insects were placed on the turntable at any one time, and each insect was returned to the cage immediately after inoculation. It was extremely important at this stage to maintain a high humidity. Therefore as soon as a batch of sandflies had been returned to their cage, several breaths were exhaled into it to raise the humidity of the immediate surroundings. This both hastened recovery and improved the percentage recovery rate. A cotton wool pad wet with

30% sucrose was placed on top of the cage, which was then quickly returned to the plastic box. The whole operation could be performed in less than 5 minutes.

(iii) To ensure minimum mechanical damage to the sandfly, glass microneedles with an orifice diameter not exceeding 50 µm were employed. These sometimes became blocked with thoracic hairs and had to be changed more often than is necessary when inoculating midges or mosquitoes. Larger needles can be used for these insects despite the fact that midges are smaller than sandflies.

(iv) Internal damage to the insect could also be reduced by delivering the inoculum with two short pressure bursts instead of one longer one.

By adopting these procedures the survival rate was improved to 95% for females and 75% for males. The maximum survival time post inoculation was 15 days in the case of females. These could be successfully membrane fed during this time. Similar results were also obtained with P.papatasi females. Inoculated males of both species did not survive so well; 50% died within 48 hours and the maximum survival time was 8 days post inoculation. Only females were inoculated with virus in experiments investigating virus development in sandflies.

No difference was observed between the survival of flies inoculated with virus and that of control flies inoculated with 1% BA or with EMM.

With experience 100 flies per hour could be inoculated.

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2.3.2. Thermal inactivation of virus at 25°C

2.3.2.1. Phlebotomus fever group viruses

The results are shown in Table 2.1. All three viruses were fairly stable when mixed with mouse blood. The pH of blood-virus mixtures was 6.5 decreasing to 6.0 - 6.5 after 7 days incubation and remaining at this level for at least a further 7 days. Virus suspensions in 1% BA alone were less stable than the blood-virus mixtures. Pacui virus was denatured after 2 days at 25°C. The concentration of SFN and SFS viruses decreased more gradually over 6 and 13 days respectively. The pH of SFS and PAC viruses in 1% BA was 7.0 throughout the experiment. The pH of SFN virus decreased from 7.0 to 6.5 - 7.0 after 2 days at 25°C, and remained at this level until the experiment was terminated.

2.3.2.2. Bluetongue

The results are shown in Table 2.2. The stock BT virus in EMM was fairly stable for short periods at 25°C. The virus concentration decreased by 0.8 and 1.4 $\log_{10}TCID_{50}$/ml respectively over 9 and 14 days. The pH of this virus stock was 6.5 until day 6 of the experiment, after which results of 7.0 - 7.5 were recorded. Mixtures of blood and virus were less stable than the stock virus in EMM. The virus concentration decreased by 2.8 $\log_{10}TCID_{50}$/ml over 9 days, and by 3.0 $\log_{10}TCID_{50}$/ml over 14 days. The pH of blood-virus mixtures was 6.5 decreasing to 6.0 - 6.5 after 24 hours, and remaining between 6.0 and 6.5 for a further 12 days.

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Table 2.1. Thermal inactivation of Phlebotomus fever group viruses at 25°C

Virus	Virus content of samples ($log_{10}MLD_{50}$/ml)					
	SFN		SFS		PAC	
	in 1%BA	1:1 mixture blood:virus	in 1%BA	1:1 mixture blood:virus	in 1%BA	1:1 mixture blood:virus
Days at 25°C						
0	7.9	7.2	9.0	8.7	7.4	7.0
1	6.2	7.1	8.5	8.5	4.9	6.8
2	4.9	6.6	7.9	8.9	T	6.2
3	4.2	7.2	6.5	7.7	0	6.2
4	2.5	6.5	6.1	7.9	0	6.4
5	-*	-	-	-	-	-
6	2.3	6.9	4.9	7.4	-	5.9
7	0	6.2	4.5	7.9	-	5.7
8	0	-	-	-	-	4.9
9	-	5.5	3.4	6.9	-	4.1
10	-	-	-	-	-	-
11	-	-	-	-	-	-
12	-	-	-	-	-	-
13	-	4.7	T	-	-	-
14	-	4.4	T	6.7	-	3.7
↓						
27	-	-	-	3.4	-	-

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $log_{10}MLD_{50}$/ml)

-* Not tested

↓ Intermediate days not tested

Table 2.2. Thermal inactivation of BT virus at 25°C

Days at 25°C	Virus content of sample ($log_{10}TCID_{50}$/ ml)	
	Virus suspension in EMM	1:1 mixture blood:virus
0	7.9	7.9
1	8.1	7.9
2	7.5	6.3
3	7.3	5.3
4	7.1	5.7
5	-*	-
6	7.1	4.7
7	7.5	4.7
8	7.3	5.3
9	7.1	5.1
10	-	-
11	-	-
12	-	-
13	6.7	4.7
14	6.5	4.9

-* Not tested

2.3.3. Concentration of virus used for infecting insects

Unless otherwise stated the concentration (in $\log_{10}MLD_{50}$ or $\log_{10}TCID_{50}/ml$) of virus stocks or blood-virus mixtures used in intrathoracic inoculation and membrane feeding experiments were as follows :

Virus	Inoculum	Blood-virus mixture
SFS	9.0 to 9.3	8.7 to 9.0
SFN	7.6 to 7.9	7.2 to 7.3
PAC	7.4 to 7.5	7.0 to 7.2
BT	7.9 to 8.3	7.8 to 8.0

2.3.4. Infection of L.longipalpis with Phlebotomus fever group viruses Sicilian and Naples sandfly fever and Pacui

2.3.4.1. Infection by membrane feeding

Similar results were obtained with each of the three viruses (see Table 2.3. SFS; 2.4. SFN; 2.5. PAC). The initial infection rate ad determined by titration of individual flies killed immediately after feeding (i.e. on day 0) was 100%. The quantity of virus ingested by each female varied between 3.2 and 4.9 $\log_{10}MLD_{50}$ for SFS, 2.8 to 4.6 $\log_{10}MLD_{50}$ for SFN, and 2.6 to 3.9 $\log_{10}MLD_{50}$ for PAC. None of the viruses multiplied in L.longipalpis and all trace of virus had disappeared within 2 to 3 days after membrane feeding.

2.3.4.2. Infection by intrathoracic inoculation

Only PAC virus multiplied in L.longipalpis after infection by this technique. Both SFS and SFN however were detected in some sandflies

Table 2.3. Virus ($log_{10}MLD_{50}$/female) recovered from L.longipalpis at daily intervals after membrane feeding on Sicilian sandfly fever

Virus content of feeding mixture : 9.0 $log_{10}MLD_{50}$/ml		
Days post feeding	Mean virus concn. per fly	No. infected / No. tested
0	4.6	19/19
1	1.8	4/4
2	T	1/2
3	0	0/4
4	0	0/4
5	0	0/7
6	0	0/10
7	0	0/10
8	0	0/6
9	0	0/10
10	0	0/4

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $log_{10}MLD_{50}$/insect)

Table 2.4. Virus ($\log_{10}MLD_{50}$/female) recovered from L.longipalpis at daily intervals after membrane feeding on Naples sandfly fever

Virus content of feeding mixture : 7.3 $\log_{10}MLD_{50}$/ml		
Days post feeding	Mean virus concn. per fly	No. infected / No. tested
0	4.2	50/50
1	2.9	7[a]
2	0	0/4
3	0	0/8
4	0	0/10
5	0	0/10
6	0	0/8
7	0	0/10
8	0	0/7
9	0	0/8
10	0	0/6
11	0	0/6

a Total number of insects tested when titrated in groups of 2 to 4 flies

Table 2.5. Virus ($\log_{10}MLD_{50}$/female) recovered from L.longipalpis at daily intervals after membrane feeding on Pacui

Virus content of feeding mixture : 7.2 $\log_{10}MLD_{50}$/ml		
Days post feeding	Mean virus concn. per fly	No. infected / No. tested
0	3.5	20/20
1	1.8	4/4
2	0	0/4
3	0	0/6
4	0	0/6
5	0	0/6
6	0	0/10
7	0	0/8
8	0	0/5
9	0	0/8
10	0	0/8
11	0	0/6
12	0	0/6
13	0	0/6
14	0	0/8
15	0	0/5

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $\log_{10}MLD_{50}$/insect)

for several days after inoculation of each virus. The concentration of SFS and SFN virus stocks used in these experiments was high. A second series of experiments was therefore carried out using a lower concentration of SFS and SFN virus for each inoculum.

The initial infection rate as determined by titration of individual flies killed within 2 hours of inoculation (i.e. on day 0) was 96 to 100% in all experiments.

SFS : (Table 2.6) In the first series of experiments the inoculum contained 9.3 $\log_{10}MLD_{50}$virus/ml. The mean virus content per fly on day 0 was 3.5 $\log_{10}MLD_{50}$ (range, 2.4 to 4.2). This value fell to 2.3 (range, trace to 3.4)on day 2, and remained between a trace level (less than 0.5 $\log_{10}MLD_{50}$/fly) and 2.9 until day 13 when the experiment terminated. The maximum virus concentration recorded after day 0 was 3.9 recovered from a single female (out of the 102 tested) on day 9 post inoculation.

In the second experiment using an inoculum containing 7.3 $\log_{10}$ MLD_{50}virus/ml, a mean virus content of 1.0 $\log_{10}MLD_{50}$/fly (range, trace to 2.1) was recorded on day 0. Sixty-one females were titrated over the following 13 days, but virus was recovered only on days 1,6,8, and 10, at trace levels in 10 individual females.

SFN : (Table 2.7) The mean virus content per fly on day 0 was 2.8 $\log_{10}MLD_{50}$ (range, 1.5 to 3.9) and 2.3 $\log_{10}MLD_{50}$ (range, 1.9 to 2.9) at the higher and lower inoculum respectively. Virus was not detected later than 48 hours after inoculation except for occasional small amounts (between a trace and 2.1) in 43% of females given the higher virus dose.

PAC : (Table 2.8) A mean virus concentration of 1.5 $\log_{10}MLD_{50}$ (range, trace to 2.5) was recorded per fly on day 0. An initial

Table 2.6. Virus ($log_{10}MLD_{50}$/female) recovered from L.longipalpis at daily intervals after intrathoracic inoculation of Sicilian sandfly fever

Virus content of inoculum ($log_{10}MLD_{50}$/ml)	9.3			7.3	
Days post inoculation	Virus concn. per fly		No.infected / No.tested	Virus concn. per fly	No.infected / No.tested
	Mean	Range		Mean	
0	3.5	2.4 - 4.2	16/16	1.0	10[a]
1	3.1	2.0 - 3.9	14	T	2/4
2	2.3	T - 3.4	23	0	0/3
3	1.7	1.6 - 2.9	15/20	0	0/3
4	2.8	2.5 - 3.1	2/2	0	0/3
5	T		2/4	0	0/6
6	1.0	T - 3.2	3/3	T	3/6
7	1.6	T - 3.5	8/8	0	0/4
8	2.5		1/3	T	3/6
9	2.4	1.8 - 3.9	7/9	0	0/5
10	2.1	T - 3.4	3/3	T	2/9
11	2.4	2.0 - 2.9	5/6	0	0/5
12	2.0	T - 2.9	4/4	0	0/4
13	2.9	2.2 - 3.5	3/3	0	0/3

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $log_{10}MLD_{50}$/insect)

a Total number of insects tested when titrated in groups of 2 to 4 flies

Table 2.7. Virus ($log_{10}MLD_{50}$/female) recovered from L.longipalpis at daily intervals after intrathoracic inoculation of Naples sandfly fever

Virus content of inoculum ($log_{10}MLD_{50}$/ml)	7.6			6.6	
Days post inoculation	Virus concn.per fly		No.infected / No.tested	Virus concn. per fly	No.infected / No.tested
	Mean	Range		Mean	
0	2.8	1.5 - 3.9	17/17	2.3	5/5
1	2.9	2.1 - 3.9	11/11	0	0/7
2	2.7		2/7	0	0/4
3	1.0	T - 1.9	2/3	-*	-
4	0		0/4	-	-
5	1.4	T - 2.1	3/3	0	0/4
6	T		4/7	0	0/5
7	0		0/6	0	0/4
8	T	T - 1.7	4/6	0	0/8
9	T	T - 1.7	3/4	0	0/1
10	T	T - 1.8	2/9	0	0/9
11	T		3/4	0	0/3
12	0		0/3	0	0/3
13	T		1/1	-	-
14	-		-	-	-
15	0		0/1	-	-

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $log_{10}MLD_{50}$/insect)

-* Not tested

Table 2.8. Virus ($log_{10}MLD_{50}$/female) recovered from L.longipalpis at daily intervals after intrathoracic inoculation of Pacui

Virus content of inoculum :	7.5 $log_{10}MLD_{50}$/ml		
Days post inoculation	Virus concn. per fly		No.infected / No.tested
	Mean	Range	
0	1.5	T - 2.5	5/5
1	0.9	T - 2.4	9/9
2	2.6	T - 3.2	12/13
3	3.5	2.5 - 4.7	10/11
4	4.9	4.7 - 5.0	2/2
5	4.5	3.7 - 5.4	8/9
6	4.9	3.9 - 5.4	4/4
7	3.8	2.9 - 4.2	7/8
8	4.6	3.9 - 5.1	6/6
9	-*	-	-
10	5.1	4.9 - 5.2	2/2
11	3.9	T - 4.5	5/7
12	4.2	3.4 - 4.7	5/6
13	4.8	4.7 - 4.9	2/2
14	5.4		2/2
15	4.6	4.5 - 4.9	4/4

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $log_{10}MLD_{50}$/insect)

-* Not tested

Table 2.9. Transmission of Pacui by inoculated L.longipalpis

Age of ♀ on inoculation (days)	Days post inoc. membrane fed	Age when ♀ membrane fed (days)	No. ♀ fed	No. ♀ infected	Mean virus concn./fly ($log_{10}MLD_{50}$)	Virus content ($log_{10}MLD_{50}$/ml)		Virus transmitted ($log_{10}MLD_{50}$/♀)
						Membrane	Blood	
3,5,7	5	8,10,12	15	15	4.4	2.9	1.9	1.7
6,7	6	12,13	30	30	4.9	1.7	2.1	0.7
6,7	7	13,14	0	-	-	-	-	-
7,8	7	14,15	25	25	4.2	3.2	T	1.8
3,5,7	8	11,13,15	8	8	4.6	1.8	0	0.9

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $log_{10}MLD_{50}$/insect)

decrease in concentration after 24 hours was followed by a steady increase to a mean value of 4.9 by day 4 post inoculation. This level of concentration was maintained for at least 11 days in a high proportion of the flies sampled. The maximum concentration recorded from individual insects was 5.4 from flies tested on days 5,6, and 14 post inoculation. Of a total of 52 females assayed between day 4 and 14 post inoculation only 5 contained no detectable virus. Seven females contained less than 4.0 $\log_{10}MLD_{50}$virus, the remainder contained over 4.0 $\log_{10}MLD_{50}$virus.

2.3.4.3. Transmission experiments

Separate batches of L.longipalpis sandflies were membrane fed on uninfected mouse blood 5,6,7, and 8 days after their inoculation with PAC virus. Between 8 and 30 females fed on each occasion, and subsequent testing of individual flies showed that all of these contained virus. Pacui virus was recovered from the membrane used on each occasion and from the blood used on 3 out of 4 occasions. These results are summarised in Table 2.9.

The amount of virus transmitted by a single female in each separate experiment was calculated by dividing the total amount of virus recovered from the blood and the membrane, by the number of infected sandflies that had fed. Between 0.7 and 1.8 $\log_{10}MLD_{50}$virus was found to be transmitted by each female.

2.3.4.4. Use of insect cell culture passaged Pacui virus for oral infection of L.longipalpis

In view of the results obtained when L.longipalpis was inoculated with PAC, the possibility that the sandfly would be susceptible to the virus if membrane fed on insect cell culture passaged virus stock was considered.

Two cell lines derived from Ae.albopictus and Ae.aegypti mosquitoes were infected with PAC virus. A full description of the procedures for maintaining these cell lines, for their infection with virus and assay for evidence of virus replication, together with the results of these experiments, is given in appendix 2. Pacui only multiplied in the Ae.albopictus cell line, reaching a concentration of 5.1 $\log_{10}MLD_{50}$/ml after 7 days. A second virus passage in these cells yielded a virus concentration of only 3.1 $\log_{10}MLD_{50}$/ml.

L.longipalpis females were membrane fed on 1:1 mixtures of mouse blood and insect cell culture virus from both the first and second cell passage. The virus concentration of the blood meal was low, 4.4 and 2.9 $\log_{10}MLD_{50}$virus/ml respectively. The initial infection rate of sandflies was correspondingly low, 20% in each case. Between 0 and 2.7 $\log_{10}MLD_{50}$virus was ingested by each female (mean of infected samples 0.9 $\log_{10}MLD_{50}$virus). One hundred and twelve females were subsequently titrated between days 1 and 10 post feeding. Virus was detected in only 9 females, at trace levels on days 5 and 6 post infection. The remaining 103 females contained no detectable virus.

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2.3.5. Infection of P.papatasi with Phlebotomus fever group viruses

2.3.5.1. Infection by membrane feeding

Pacui virus did not multiply in P.papatasi after infection by this method. The results are shown in Table 2.10. The virus content of the blood meal was 7.2 $\log_{10}MLD_{50}$/ml. The initial infection rate was 100%. The mean amount of virus ingested by each sandfly was 2.1 $\log_{10}MLD_{50}$ (range, 1.7 to 2.5). Virus was not detected in females

Table 2.10. Virus ($log_{10}MLD_{50}$/female) recovered from P.papatasi at daily intervals after membrane feeding on Pacui

Virus content of feeding mixture : 7.2 $log_{10}MLD_{50}$/ml		
Days post feeding	Mean virus concn. per fly	No.infected / No.tested
0	2.1	9/9
1	1.9	4[a]
2	-*	-
3	0	2
4	T	3/4
5	1.4	7
6	T	1/8
7	0	0/17
8	0	0/23
9	-	-
10	0	0/11
11	0	0/2
12	T	5/22
13	0	0/10
14	0	0/6

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $log_{10}MLD_{50}$/insect)

-* Not tested

a Total number of insects tested when titrated in groups of between 2 and 4 flies

tested after day 2 post infection, except for occasional low levels (i.e. trace to 1.4 $\log_{10}MLD_{50}$/fly) recovered from not more than 16 out of the 112 flies tested.

No membrane feeding experiments were conducted with SFS and SFN viruses since insufficient insects were available.

2.3.5.2. Infection by intrathoracic inoculation

PAC : (Table 2.11) Pacui virus multiplied in P.papatasi after infection by intrathoracic inoculation. Inoculated females contained between a trace and 1.7 (mean 0.5) $\log_{10}MLD_{50}$virus on day 0. The mean virus concentration per fly increased rapidly to a maximum of 5.4 $\log_{10}MLD_{50}$ by day 4 post infection. All individual females titrated between day 3 and 14 post inoculation were infected, and contained between 2.9 and 5.4 $\log_{10}MLD_{50}$ of virus. The mean virus concentration per fly over this period was consistently greater than 4.0 $\log_{10}MLD_{50}$.

SFS : (Table 2.12) Only a limited number of P.papatasi females were available for infection with this virus and with SFN, and the results are therefore less detailed. SFS virus appeared to multiply in these insects, although to a lower level than did PAC virus. Between 2.4 and 3.7 $\log_{10}MLD_{50}$ (mean 2.9) was present in each sandfly on day 0. A 20-fold increase in the mean virus content per fly was recorded over the following 8 days. Between day 1 and day 14 post inoculation, when the experiment terminated, the mean results were higher than the day 0 value in 10 out of the 14 determinations. Most of the sandflies were titrated in pools of 2 to 4 flies and therefore it is not known how many individual insects were infected. The highest result obtained was 4.4 $\log_{10}MLD_{50}$/fly from a pool of 3 flies titrated on day 8 post inoculation.

Table 2.11. Virus ($log_{10}MLD_{50}$/female) recovered from P.papatasi at daily intervals after intrathoracic inoculation of Pacui

Virus content of inoculum : 7.5 $log_{10}MLD_{50}$/ml			
Days post inoculation	Virus concn. per fly		No.infected / No.tested
	Mean	Range	
0	0.5	T - 1.7	5/6
1	2.3	1.8 - 3.2	6/7
2	3.8		5[a]
3	4.5	2.9 - 5.1	7
4	5.4		3
5	4.5	3.1 - 5.2	8
6	4.7	4.2 - 5.1	7/7
7	4.6	4.2 - 5.4	7/7
8	4.7	4.5 - 4.9	3/3
9	4.6	4.2 - 4.9	10
10	4.4		2
11	-*		-
12	4.7	4.5 - 4.9	4
13	4.5		1/1
14	4.1		3

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $log_{10}MLD_{50}$/insect)

-* Not tested

a Total number of insects tested when titrated in groups of 2 to 4 flies

Table 2.12. Virus ($\log_{10}MLD_{50}$/female) recovered from P.papatasi at daily intervals after intrathoracic inoculation of Sicilian sandfly fever

Virus content of inoculum : 9.0 $\log_{10}MLD_{50}$/ml			
Days post inoculation	Virus concn. per fly		No.infected / No.tested
	Mean	Range	
0	2.9	2.4 - 3.7	8/8
1	3.2		1/3
2	3.2		2[a]
3	0		0/6
4	4.0	3.7 - 4.2	6
5	3.5	2.9 - 3.9	12
6	3.7	3.5 - 3.8	15
7	3.3	1.6 - 4.1	20
8	4.2	3.9 - 4.4	7
9	2.5	1.3 - 3.6	8
10	T		2
11	3.4	2.9 - 4.2	5
12	3.8		9
13	2.6	T - 3.9	4
14	3.7	3.2 - 4.2	1

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $\log_{10}MLD_{50}$/insect)

-* Not tested

a Total number of insects tested when titrated in groups of 2 to 4 flies

Table 2.13. Virus ($log_{10}MLD_{50}$/female) recovered from P.papatasi at daily intervals after intrathoracic inoculation of Naples sandfly fever

Virus content of inoculum : 7.9 $log_{10}MLD_{50}$/ml			
Days post inoculation	Virus concn. per fly		No.infected / No.tested
	Mean	Range	
0	1.7	T - 2.5	8/9
1	1.1		6[a]
2	1.0	T - 1.9	7
3	-*		-
4	1.7	1.4 - 1.9	7
5	0.8	T - 1.6	9
6	1.1	T - 2.9	15
7	1.9	T - 3.8	12
8	1.7	1.4 - 2.0	8
9	2.0	1.7 - 2.2	16
10	-		-
11	T		8
12	1.6		2
13	T		3/3
14	3.2		1/1

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $log_{10}MLD_{50}$/insect)

-* Not tested

a Total number of insects tested when titrated in groups of 2 to 4 flies

Table 2.14. Transmission experiments with P.papatasi inoculated with Pacui virus

Age of ♀ on inoc. (days)	Day post inoc. membrane fed	Age when ♀ membrane fed (days)	No. ♀ fed	No. ♀ infected	Mean virus concn./fly ($log_{10}MLD_{50}$)	Virus content ($log_{10}MLD_{50}$/ml)		Virus transmitted ($log_{10}MLD_{50}$/♀)
						Membrane	Blood	
2 to 7	6	8 to 13	5	5	4.7	0	0	0
2 to 7	7	9 to 14	2	2	4.8	0	0	0
2 to 7	8	10 to 15	2	2	4.6	0	0	0
2 to 7	9	11 to 16	1	1	4.4	0	0	0

SFN : (Table 2.13) The mean virus content of inoculated sandflies on day 0 was 1.7 $\log_{10}MLD_{50}$/fly. This was exceeded only 3 times over a period of 14 days. The highest mean value recorded was 3.2 $\log_{10}MLD_{50}$/fly on day 14 post inoculation, representing a 32-fold increase in virus concentration per fly. Results for individual females are not available since insects were titrated in small pools of 2 to 4 flies. The highest result out of a total of 34 determinations was 3.8 $\log_{10}MLD_{50}$/fly obtained from a pool of 5 females on day 7 post infection. Only 7 pools yielded virus concentrations greater than 2.0 $\log_{10}MLD_{50}$/fly. This represented 24 out of the total of 94 females tested after day 0.

2.3.5.3. Transmission experiments

The details of these experiments are shown in Table 2.14. Separate batches of P.papatasi sandflies were membrane fed on uninfected mouse blood 6,7,8, and 9 days after their inoculation with PAC virus. Females were reluctant to feed and only 1 to 5 ingested blood on each occasion. Titration of individual blood fed flies proved that each of these contained between 4.4 and 4.9 $\log_{10}MLD_{50}$ virus. Virus was not recovered from the blood or membrane used in any of these experiments.

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2.3.6. Infection of C.variipennis and C.nubeculosus with Phlebotomus fever group viruses, Sicilian and Naples sandfly fever, and Pacui

Unless stated otherwise, in all experiments with Culicoides and the Phlebotomus fever group viruses insects were titrated in pools of between 2 and 6 females. The mean virus concentrations recorded in the

tables are therefore the means of results from one or more pools of females and not from individual flies.

2.3.6.1. Infection by membrane feeding

None of these viruses multiplied in C.variipennis or C.nubeculosus after infection by membrane feeding. The results were similar to those obtained after oral infection of L.longipalpis with the same viruses. In a preliminary series of experiments, 50 females of both Culicoides species were titrated individually immediately after membrane feeding on each virus, to ascertain the initial infection rate. This was 100% in each case. Tables 2.15 (C.variipennis) and 2.16 (C.nubeculosus) illustrate the results obtained when insects were titrated at daily intervals after ingesting virus. The mean amount of virus ingested by each female was, for C.variipennis (C.vp) and C.nubeculosus (C.nb) respectively, 4.6 and 4.5 $\log_{10}MLD_{50}$ of SFS; 3.9 and 3.7 $\log_{10}MLD_{50}$ of SFN; and 2.3 and 3.7 $\log_{10}MLD_{50}$ of PAC. Virus was not detected in either species after day 2 post feeding except for trace levels recovered on day 4 after infection of insects with SFS.

Table 2.17 shows the results obtained when Culicoides were titrated at hourly intervals after oral infection with SFN virus. The virus concentration per fly decreased gradually over 7 hours from 4.6 to 3.9 $\log_{10}MLD_{50}$ (C.vp); and from 4.0 to 3.2 $\log_{10}MLD_{50}$ (C.nb). Only traces of virus were detected in insects tested 24 hours after feeding.

2.3.6.2. Infection by intrathoracic inoculation

None of the three Phlebotomus fever group viruses multiplied in either species of Culicoides after infection by inoculation. The results are shown in Table 2.18 (C.vp) and 2.19 (C.nb). The initial infection rate for each species-virus combination was 95 to 100%. The mean virus

Table 2.15. Virus ($log_{10}MLD_{50}$/insect) recovered from C.variipennis at daily intervals after membrane feeding on Phlebotomus fever group viruses

Virus	SFN		SFS		PAC	
Virus content of feeding mixture ($log_{10}MLD_{50}$/ml)	7.3		9.0		7.2	
Days post feeding	Mean virus concn. per fly	No.tested (in pools)	Mean virus concn. per fly	No.tested (in pools)	Mean virus concn. per fly	No.tested (in pools)
0	3.9	16	4.6	11	2.3	14
1	T	13	T	4	0	14
2	0	13	0	4	0	12
3	0	8	0	4	0	15
4	0	12	T	4	0	4
5	-*	-	-	-	0	4
6	-	-	-	-	0	10
7	0	16	0	13	0	16
8	0	4	0	11	0	14
9	0	4	0	15	0	12
10	-	-	0	11	0	16
11	-	-	-	-	-	-
12	-	-	-	-	0	4

Cont'd.....

Table 2.15. Continued.....

Virus	SFN		SFS		PAC	
Virus content of feeding mixture ($log_{10}MLD_{50}$/ml)	7.3		9.0		7.2	
Days post feeding	Mean virus concn. per fly	No.tested (in pools)	Mean virus concn. per fly	No.tested (in pools)	Mean virus concn. per fly	No.tested (in pools)
13	-	-	-	-	0	4
14	0	4	-	-	0	4
15	-	-	-	-	0	4
16	-	-	-	-	0	4
17	-	-	-	-	0	4

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $log_{10}MLD_{50}$/insect)

-* Not tested

Table 2.16. Virus ($log_{10}MLD_{50}$/female) recovered from C.nubeculosus at daily intervals after membrane feeding on Phlebotomus fever group viruses

Virus	SFN		SFS		PAC	
Virus content of feeding mixture ($log_{10}MLD_{50}$/ml)	7.3		9.0		7.2	
Days post feeding	Mean virus concn. per female	No.tested (in pools)	Mean virus concn. per female	No.tested (in pools)	Mean virus concn. per female	No.tested (in pools)
0	3.7	20	4.5	11	3.7	18
1	T	12	T	4	0	13
2	0	16	0	4	0	12
3	0	12	0	4	0	11
4	0	8	T	4	0	4
5	0	8	-*	-	0	17
6	-	-	0	13	0	16
7	0	8	0	11	0	16
8	0	8	0	1	0	12
9	0	4	-	-	0	17
10	-	-	-	-	0	14

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $log_{10}MLD_{50}$/insect)

-* Not tested

Table 2.17. Virus ($log_{10}MLD_{50}$/female) recovered from C.nubeculosus and C.variipennis at hourly intervals after membrane feeding on Naples sandfly fever

Species	C.nubeculosus		C.variipennis	
Hours post feeding	Mean virus concn. per fly	No.tested (in pools)	Mean virus concn. per fly	No.tested (in pools)
0	4.0	8	4.6	4
1	3.8	3	4.2	4
2	4.4	4	4.6	4
3	4.1	4	4.2	4
4	3.1	4	4.2	4
5	4.3	4	4.1	4
6	3.6	4	4.4	4
7	3.2	4	3.9	4
↓				
24	T	4	T	4

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $log_{10}MLD_{50}$/insect)

↓ Intermediate samples not tested

Table 2.18. Virus ($log_{10}MLD_{50}$/female) recovered from C.variipennis at daily intervals after intrathoracic inoculation of Phlebotomus fever group viruses

Virus	SFN		SFS		PAC	
Virus content of inoculum ($log_{10}MLD_{50}$/ml)	7.9		9.0		7.5	
Days post inoculation	Mean virus concn. per fly	No.tested (in pools)	Mean virus concn. per fly	No.tested (in pools)	Mean virus concn. per fly	No.tested (in pools)
0	2.7	16	3.5	12	2.2	11
1	2.2	7	2.5	5	1.3	14
2	T	13	1.9	13	1.2	11
3	T	12	T	12	1.1	14
4	T	8	-*	-	-	-
5	T	31	1.6	4	-	-
6	T	10	1.1	21	0	14
7	T	20	1.3	17	T	14
8	0	4	1.7	13	T	4
9	T	6	1.0	18	-	-
10	0	4	1.7	14	1.0	11

Cont'd.....

Table 2.18 Continued.....

Virus	SFN		SFS		PAC	
Virus content of inoculum ($log_{10}MLD_{50}$/ml)	7.9		9.0		7.5	
Days post inoculation	Mean virus concn. per fly	No.tested (in pools)	Mean virus concn. per fly	No.tested (in pools)	Mean virus concn. per fly	No.tested (in pools)
11	-	-	-	-	T	5
12	0	27	-	-	T	14
13	-	-	T	4	-	-
14	0	12	-	-	T	17
15	T	4	T	4	0	8
16	-	-	-	-	0	9

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $log_{10}MLD_{50}$/insect)

-* Not tested

Table 2.19. Virus ($log_{10}MLD_{50}$/female recovered from C.nubeculosus at daily intervals after intrathoracic inoculation of Phlebotomus fever group viruses

Virus	SFN		SFS		PAC	
Virus content of inoculum ($log_{10}MLD_{50}$/ml)	7.9		9.0		7.5	
Days post inoculation	Mean virus concn. per fly	No.tested (in pools)	Mean virus concn. per fly	No.tested (in pools)	Mean virus concn. per fly	No.tested (in pools)
0	3.4	16	3.5	9	2.3	11
1	2.4	8	1.9	5	1.4	4
2	1.7	12	1.2	4	T	15
3	1.5	7	T	4	1.4	13
4	-*	-	-	-	T	17
5	T	3	1.4	3	0	21
6	T	10	1.3	15	T	4
7	T	8	0.8	12	0	8
8	-	-	1.4	18	1.0	10
9	T	4	T	2	T	4
10	T	8	T	3	0	3
11	0	4	T	8	-	-

Cont'd.....

Table 2.19 Continued .....

Virus	SFN		SFS		PAC	
Virus content of inoculum ($log_{10}MLD_{50}$/ml)	7.9		9.0		7.5	
Days post inoculation	Mean virus concn. per fly	No.tested (in pools)	Mean virus concn. per fly	No.tested (in pools)	Mean virus concn. per fly	No.tested (in pools)
12	T	4	1.6	2	T	3
13	0	4	0	8	0	4
14	0	8	1.6	4	0	4
15	0	4	-	-	0	4
16	0	4	-	-	0	4
17	-	-	-	-	-	-
18	-	-	-	-	-	-
19	-	-	-	-	-	-
20	0	4	-	-	-	-
21	0	4	-	-	-	-
22	0	4	-	-	-	-

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $log_{10}MLD_{50}$/insect)

-* Not tested

content ($log_{10}MLD_{50}$) per female on day 0 was as follows :

SFS : 3.5 (C.vp) and 3.5 (C.nb)

SFN : 2.7 (C.vp) and 3.4 (C.nb)

PAC : 2.2 (C.vp) and 2.3 (C.nb)

The virus content of infected insects decreased to a trace within 4 to 6 days of inoculation of SFN and PAC. Both these viruses were then detected at levels of between a trace and 1.0 $log_{10}MLD_{50}$/fly in both species of Culicoides for up to 15 days post inoculation. SFS virus persisted in concentrations of between a trace and 1.7 log_{10} MLD_{50}/fly for at least 14 days post inoculation in C.nubeculosus, and 15 days in C.variipennis, after which time the experiment terminated. After day 2 post inoculation the highest concentrations of virus recovered from individual pools of insects were 2.1 $log_{10}MLD_{50}$/fly on days 9 and 10 (C.vp), and 2.2 $log_{10}MLD_{50}$/fly on days 6 and 8 (C.nb). The original day 0 value was never exceeded and it was concluded that virus multiplication had not occurred. Transmission experiments were therefore not performed.

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2.3.7. Infection of L.longipalpis and P.papatasi sandflies with Bluetongue virus

The results were similar for both species and are presented in Table 2.20 for L.longipalpis (L.l) and 2.21 for P.papatasi (P.p). All females were titrated individually in both membrane feeding and inoculation experiments.

2.3.7.1. Infection by membrane feeding

Bluetongue virus did not multiply in L.longipalpis or P.papatasi after infection via the oral route. The initial infection rate of females on day 0 was 80% and 100% for the two species respectively.

The mean amount of virus ingested was the same for both species, i.e. 2.1 $\log_{10}TCID_{50}$/fly. The range of values was from a trace to 2.9 $\log_{10}TCID_{50}$/fly (L.l) and from a trace to 3.1 $\log_{10}TCID_{50}$/fly (P.p).

Within 3 days after membrane feeding the amount of virus recovered from sandflies had decreased to between a trace value and nil. Eighty four L.longipalpis females were tested between days 3 and 11 post feeding; 68 of these contained no virus, and only traces of virus were detected in the remainder. None of the 166 P.papatasi females tested after day 3 post feeding contained virus.

2.3.7.2. Infection by intrathoracic inoculation

Bluetongue virus multiplied in both L.longipalpis and P.papatasi females after infection by this method, however the level of virus multiplication was not high. The initial infection rate was 100% for both species. The mean virus concentrations per fly on day 0 were 2.5 $\log_{10}TCID_{50}$ (range, trace to 3.5) for L.longipalpis, and 1.5 $\log_{10}TCID_{50}$ (range, trace to 2.1) for P.papatasi.

In L.longipalpis there was an initial decrease in the mean virus concentration per fly over 24 hours to 1.9 $\log_{10}TCID_{50}$. Virus multiplication then took place. Between day 5 and day 14 post inoculation the mean virus concentration per fly varied from 3.1 to 4.1 $\log_{10}TCID_{50}$. Eighteen of the 100 females tested after day 5 contained more than 4.0 $\log_{10}TCID_{50}$ virus. The maximum level recorded from an individual female was 4.9 on day 11.

In P.papatasi there was an overall increase in the virus content of inoculated insects from day 0 to day 5 post inoculation. The mean amount of virus per fly by day 5 was 3.0 $\log_{10}TCID_{50}$ (range 2.5 to 3.5). Similar levels were maintained for a further 8 days by which time all the flies had been used. The maximum concentration recorded from a single

Table 2.20. Virus ($log_{10}TCID_{50}$/female) recovered from L.longipalpis at daily intervals after infection with Bluetongue

Method of infection	Membrane feeding		Inoculation		
Virus content, blood/inoculum	8.0 $log_{10}TCID_{50}$/ml		8.3 $log_{10}TCID_{50}$/ml		
Days post infection	Virus concn. per fly	No.infected / No.tested	Virus concn.per fly		No.infected / No.tested
	Mean		Mean	Range	
0	2.1	12/15	2.5	T - 3.5	13/13
1	1.5	6/10	1.9	T - 2.9	10/13
2	0.7	6/10	2.1	1.5 - 3.3	9/12
3	0	0/3	3.2	2.7 - 3.7	10/10
4	T	7/13	2.8	1.3 - 3.7	7/7
5	T	2/16	3.4	2.9 - 3.9	10/10
6	T	3/12	3.3	2.7 - 4.3	21/21
7	T	1/10	3.3	2.7 - 4.7	16/16
8	T	1/16	3.5	2.5 - 4.1	17/17
9	0	0/6	3.6	2.5 - 4.3	12/12
10	T	1/5	3.1	2.4 - 4.1	7/8
11	T	1/3	3.6	2.1 - 4.9	6/6
12	-*	-	3.3	1.9 - 4.2	9/9
13	-	-	3.3	T - 4.7	7/7
14	-	-	4.1	3.7 - 4.3	4/4

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $log_{10}TCID_{50}$/insect)

-* Not tested

Table 2.21. Virus ($log_{10}TCID_{50}$/female) recovered from P.papatasi at daily intervals after infection with Bluetongue

Method of infection	Membrane feeding		Inoculation		
Virus content, blood/inoculum	8.0 $log_{10}TCID_{50}$/ml		8.3 $log_{10}TCID_{50}$/ml		
Days post infection	Virus concn. per fly	No.infected / No.tested	Virus concn.per fly		No.infected / No.tested
	Mean		Mean	Range	
0	2.1	6/6	1.5	T - 2.1	10/10
1	1.7	3/4	1.6	1.1 - 1.9	3/3
2	1.4	5/6	2.3		1/1
3	-*	-	0		0/1
4	0	0/11	1.9	1.3 - 2.3	3/3
5	0	0/5	3.0	2.5 - 3.5	4/4
6	0	0/5	3.1		1/1
7	0	0/21	2.0	1.7 - 2.3	2/2
8	0	0/19	2.3	1.7 - 2.7	5/5
9	0	0/24	3.6	2.5 - 4.3	4/4
10	-	-	1.8	1.7 - 1.9	2/2
11	0	0/17	2.6		3/3
12	0	0/22	-		-
13	0	0/15	2.3		1/1
14	0	0/11	-		-
15	0	0/14	-		-
↓					
22	0	0/2	-		-

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $log_{10}TCID_{50}$/insect)

-* Not tested

↓ Intermediate days not tested

Table 2.22 Experiments on the transmission of Bluetongue virus by inoculated L.longipalpis

Age of ♀ on inoculation (days)	Day post inoc. membrane fed	Age when ♀ membrane fed (days)	No. ♀ fed	No. ♀ infected	Mean virus concn./fly ($log_{10}TCID_{50}$)	Virus content ($log_{10}TCID_{50}$/ml)	
						Membrane	Blood
5,6	7	12,13	5	5	3.4	0	0
5,6	8	13,14	20	20	3.5	0	0
6,7	8	14,15	13	Cell culture titration contaminated		0	0
2,4,6	6	8,10,12	10	10	3.3.	0	0
2,4,6	9	11,13,15	11	11	3.6	0	0
2 - 6	7	9 - 13	28	28	3.3	0	0
2 - 6	8	10 - 14	26	26	3.5	0	0
2 - 6	9	11 - 15	8	8	3.6	0	0

female was 4.3 $\log_{10}TCID_{50}$ on day 9.

With the exception of a single L.longipalpis female all the insects tested after day 3 contained BT virus.

2.3.7.3. Transmission experiments

The results are shown in Table 2.22. Eight batches of L.longipalpis sandflies were inoculated with BT virus and were membrane fed on uninfected blood 6 to 9 days later. Between 5 and 28 females fed on each occasion, but no virus was recovered from either the membrane or the blood used in these experiments. Titration of individual females showed that all those that fed had contained virus. The mean virus content per fly was between 3.3 and 3.6 $\log_{10}TCID_{50}$.

Transmission experiments were not performed with P.papatasi since insufficient insects were available.

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2.3.8. Infection of C.variipennis and C.nubeculosus with Bluetongue virus

The membrane feeding and intrathoracic inoculation results are combined in Table 2.23 for C.variipennis and Table 2.24 for C.nubeculosus. The Culicoides used in these experiments were usually titrated in pools containing from 2 to 6 insects. The mean results quoted are therefore means of several such determinations rather than the mean results of titrations of individual flies.

2.3.8.1. Infection by membrane feeding

Bluetongue virus multiplied in C.variipennis but not in C.nubeculosus after infection by this method. The initial infection rate for both species was 100%.

The mean amount of virus ingested by C.nubeculosus females was

Table 2.23. Multiplication of Bluetongue virus in C.variipennis females after infection by membrane feeding and intrathoracic inoculation

Method of infection	Membrane feeding		Inoculation	
Virus concn.of blood/inoculum ($log_{10}TCID_{50}$/ml)	7.8		7.9	
Days post infection	Virus concn. per fly ($log_{10}TCID_{50}$)	No.infected / No.tested	Virus concn. per fly ($log_{10}TCID_{50}$)	Total no. tested (in pools)
0	2.4	10/10	1.3	14
1	T	5/5	0	10
2	0	0/5	3.9	19
3	3.8	8[a]	4.9	14
4	3.4	5	5.3	6
5	2.9	14	5.6	11
6	4.3	6	5.1	29
7	3.3	24	4.9	20
8	4.1	28	5.4	10
9	3.7	14	4.9	10
10	3.3	36	-*	-
11	-	-	-	-
12	-	-	4.6	6
13	-	-	-	-
14	4.3	6	-	-

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $log_{10}TCID_{50}$/insect)

-* Not tested

a Total number of insects tested when titrated in groups of 2 to 6 flies

Table 2.24 Virus ($log_{10}TCID_{50}$/female) recovered from C.nubeculosus at daily intervals after infection with Bluetongue

Method of infection	Membrane feeding		Inoculation	
Virus concn. of blood/inoculum ($log_{10}TCID_{50}$/ml)	7.8		7.9	
Days post infection	Virus concn. per fly	No.infected / No.tested	Virus concn. per fly	No.tested (in pools)
0	3.5	10/10	1.7	15
1	2.1	9/9	0.8	13
2	-*	-	3.9	6
3	T	2/7	-	-
4	0	0/7	4.3	17
5	0	0/7	5.0	12
6	0	0/20	5.2	12
7	0	0/13	4.8	10
8	0	0/12	4.7	16
9	0	0/20	4.6	10
10	-	-	4.8	16
11	0	0/10	-	-
12	0	0/17	-	-

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $log_{10}TCID_{50}$/insect)

-* Not tested

3.5 $\log_{10}TCID_{50}$/fly. The virus concentration per fly decreased to a trace within 3 days of the initial infection and thereafter virus was not detected in any of the 106 flies tested.

C.variipennis females ingested 2.4 $\log_{10}TCID_{50}$virus/fly. An initial decrease in the virus content of infected females was observed over the following 2 days. Virus multiplication then became apparent, and a peak mean concentration of 4.3 $\log_{10}TCID_{50}$/fly was reached 6 days after feeding. This level of concentration was maintained until day 14 when the experiment terminated. The range in results from individual pools titrated between days 3 and 14 post infection was 1.4 to 5.1 $\log_{10}TCID_{50}$/fly.

2.3.8.2. Infection by intrathoracic inoculation

Bluetongue virus multiplied in both species of Culicoides after infection by this route. The results obtained were similar in both species. Between a trace and 1.7 $\log_{10}TCID_{50}$ (mean 1.3) of virus was inoculated into C.variipennis females. The corresponding figures for C.nubeculosus were 1.5 to 2.2 $\log_{10}TCID_{50}$ (mean 1.7). There was an initial decrease in the virus concentration of inoculated females in both species over 24 hours. Virus multiplication then took place, and by day 3 post inoculation some pools of inoculated C.variipennis contained as much as 5.5 $\log_{10}TCID_{50}$/fly. The highest mean value for this species was 5.6 $\log_{10}TCID_{50}$/fly on day 5 post feeding. The highest individual result was 5.9 $\log_{10}TCID_{50}$/fly recorded from a pool of 6 insects on day 5. Only 3 of the 16 pools of flies tested after day 3 post inoculation contained less than 5.0 $\log_{10}TCID_{50}$virus/fly.

The virus concentrations obtained after inoculation of C.nubeculosus with BT were lower. Twelve out of 15 pools tested between day 3 and 10 post feeding contained less than 5.0 $\log_{10}TCID_{50}$/fly.

The highest mean result for C.nubeculosus was 5.2 $\log_{10}TCID_{50}$/fly on day 6 post feeding. The highest individual result was 5.6 $\log_{10}TCID_{50}$/fly from a pool of 4 insects tested 5 days after feeding.

2.3.8.3. Transmission experiments

No transmission experiments were conducted with BT virus and Culicoides in this project since this subject was already being investigated in our laboratory by Dr. P. Mellor. The results obtained so far are summarised below.

C.variipennis: BT virus was recovered from the membrane used in transmission experiments when 10 infected females were membrane fed 6 days after they had been inoculated with the virus. Orally infected C.variipennis transmitted BT virus 12 days after their initial infection. Virus was not detected in the membrane or blood used in transmission experiments when orally infected females were fed on clean blood 4,7 and 10 days after their initial infection. The numbers of females which fed on each occasion were 7,8 and 5, respectively.

C.nubeculosus: BT virus was transmitted by this species 6 and 7 days after infection of females by inoculation, when 9 and 10 females respectively were membrane fed on clean blood. Virus was not recovered from the membrane or blood used in transmission studies when between 8 and 9 infected females fed on clean blood 3,4 and 10 days after their inoculation with BT virus.

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2.3.9. Distribution experiments

2.3.9.1. Weights of dissected C.variipennis and L.longipalpis

The weights of the head, thorax, and abdomen of C.variipennis and L.longipalpis females, together with the ratio of the weights of the three parts are shown in Table 2.25. The results were limited in their accuracy by two factors; some haemolymph was lost during dissections, particularly when the thorax and abdomen were separated, and the balance available was not entirely suitable for weighing such small quantities of tissue. However, although the results were not sufficiently accurate to enable the virus concentration per unit weight of tissue in each body part to be determined, they were considered to provide a guide to the relative weights of the three parts. They therefore enabled a more direct comparison to be made of the virus concentrations per head, thorax and abdomen.

The results for C.variipennis were variable, and therefore a mean head : thorax : abdomen weight ratio was calculated using the three sets of results obtained from the 6 day old unfed females and the results from females dissected and weighed 6 days after feeding. The mean head: thorax : abdomen ratio was 1:5:8. The mean of the two weight ratios obtained with L.longipalpis was also determined and was found to be 1:6:5. These ratios were used in distribution experiments when comparing the virus concentrations in each of the three body parts.

2.3.9.2. Distribution of Pacui virus in inoculated L.longipalpis

The results are shown in Table 2.26. Virus was detected in both the thorax and the abdomen immediately after intrathoracic inoculation. There was an initial decrease in the virus content of these parts over 24 hours. Virus multiplication then took place and maximum virus concentrations were recorded between day 4 and day 11

Table 2.25. Weight ratios of body parts of C.variipennis and L.longipalpis

Days post feeding	Weight x 10^{-5} gm				Weight ratio Hd : Th : Ab
	Whole insect	Head	Thorax	Abdomen	
C.variipennis					
0	48	2.0	6.0	38	1 : 3 : 19
0	50	1.4	5.0	37	1 : 3.6 : 26
1	47	1.0	3.2	24	1 : 3.2 : 24
2	-*	2.0	8.0	30	1 : 4.0 : 15
6	-*	2.0	12.5	17.5	1 : 6.2 : 8.8
Unfed	23	2.5	9.3	10	1 : 3.6 : 4
Unfed	-	1.5	7.3	12.3	1 : 4.8 : 8.2
Unfed	-	1.5	8.5	10	1 : 5.6 : 6.6
L.longipalpis					
Unfed	29.6	2.5	15.9	12.5	1 : 6.2 : 5
Unfed	-	5.0	13.7	12.2	1 : 5.5 : 4.9

-* Not done

Table 2.26 Distribution of Pacui virus in L.longipalpis females at daily intervals after infection by intrathoracic inoculation

Body part	Head		Thorax		Abdomen	
Days post inoculation	Virus concn. per head ($\log_{10}MLD_{50}$)	No.tested (in pools)	Virus concn. per thorax ($\log_{10}MLD_{50}$)	No.tested (in pools)	Virus concn. per abdomen ($\log_{10}MLD_{50}$)	No.tested (in pools)
0	0	10	1.0	9	0.8	10
1	0	15	T	14	T	14
2	1.5	10	2.9	10	3.5	10
3	1.9	3	3.6	3	3.5	3
4	3.5	12	4.6	11	3.8	11
5	3.0	8	3.9	8	4.5	8
6	2.9	10	3.9	10	4.2	10
7	3.5	10	4.2	10	4.1	10
8	3.2	3	4.1	3	3.9	3
9	-*	-	-	-	-	-
10	-	-	-	-	-	-
11	4.1	4	4.3	4	2.9	4

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $\log_{10}MLD_{50}$/insect part)

-* Not tested

post inoculation. This parallelled the results when whole insects were titrated. The levels of virus in the thorax were similar to those in the abdomen throughout the experiment except on day 11, when the virus content of the abdomen was considerably lower than that of the thorax.

Virus was detected in the head in increasing concentrations from day 2 onwards. If the results are corrected to allow for the difference in the amount of tissue in the three body parts, (i.e. the head: thorax : abdomen weight ratio, 1:6:5), then the virus concentration of the head equalled that of the abdomen and thorax by day 4 and 7 post inoculation respectively. By day 11 post inoculation the virus concentration of the head exceeded the virus concentration of each of the other two parts.

In membrane fed flies, virus was found only in the abdomen on day 0, and was not detectable later than 1 day after feeding.

2.3.9.3. Distribution of Bluetongue virus in inoculated L.longipalpis

Bluetongue virus was detected in the thorax and the abdomen immediately after inoculation, and from day 2 post infection onwards there was a general increase in the amount of virus recovered from both parts (Table 2.27). The proportionate concentrations of virus in the thorax and in the abdomen during these experiments were similar to those described after sandflies had been inoculated with PAC virus. Between day 11 and day 12 post inoculation the virus content of the abdomen was considerably lower than that of the thorax.

Virus was not detected in the head until day 6 post inoculation. Between day 6 and day 8 trace levels were recorded from the heads of 11 out of the 30 insects used. Heads taken from 2 individual females on day 8 contained 1.1 and 1.4 $\log_{10}TCID_{50}$virus. The remaining 17 heads contained no detectable virus. From day 9 to day 12 all the pools of heads tested contained more than 1.2 $\log_{10}TCID_{50}$virus/head. When the

Table 2.27. Distribution of Bluetongue virus in L.longipalpis females at daily intervals after infection by intrathoracic inoculation

Body part	Head		Thorax		Abdomen	
Days post inoculation	Virus concn. per head ($log_{10}TCID_{50}$)	No.tested (in pools)	Virus concn. per thorax ($log_{10}TCID_{50}$)	No.tested (in pools)	Virus concn. per abdomen ($log_{10}TCID_{50}$)	No.tested (in pools)
0	0	14	1.2	12	1.1	12
1	0	8	1.2	7	T	7
2	0	11	T	11	T	11
3	0	4	1.5	4	1.5	4
4	0	3	1.6	3	1.6	3
5	0	8	2.4	8	T	8
6	T	11	2.4	7	2.1	7
7	T	9	2.6	8	2.0	8
8	T	10	2.8	10	2.9	10
9	1.2	6	3.2	9	2.1	9
10	1.7	4	3.3	4	2.9	4
11	1.2	3	3.0	3	1.4	3
12	1.4	3	3.0	3	1.8	3

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $log_{10}TCID_{50}$/insect part)

results were considered in terms of the weight ratios between the three body parts, the virus concentration of the head never equalled or exceeded that of the thorax during the course of the experiment, and exceeded the concentration of the abdomen only on day 11 and day 12 post inoculation.

2.3.9.4. Distribution of Bluetongue virus in inoculated C.variipennis and C.nubeculosus

The results obtained from both species of Culicoides were almost identical, and therefore only those for C.variipennis are included (Table 2.28). 1.1, 2.8, and 3.0 $\log_{10}TCID_{50}$ of virus was present in the head, thorax and abdomen, respectively on day 0. The virus concentration in each body section increased over the following 13 days reaching maximum levels of 5.0 to 5.8 $\log_{10}TCID_{50}$/part during this time. A slight decrease in the mean values was observed between day 13 and day 17 post inoculation when the experiment terminated, although the range of values over this period included virus concentrations of up to 5.5 $\log_{10}TCID_{50}$/part. When the results were adjusted for the weight ratios of the three body parts (i.e. 1:5:8), then the virus content of the head was found to equal or exceed that of the thorax or abdomen during the latter part of the experiment. For example the results on day 9 become, 5.0 head : 4.8 thorax : 4.2 abdomen ; and on day 12, 5.1 head : 4.4 thorax : 3.7 abdomen.

2.3.9.5. Distribution of Bluetongue virus in membrane fed C.variipennis and C.nubeculosus

Bluetongue virus multiplies in C.variipennis but not in C.nubeculosus after infection by membrane feeding. When membrane fed C.nubeculosus were dissected and titrated on day 0, virus was only found in the abdomen. The concentration decreased to a trace by day 3 post feeding and thereafter no virus was recovered from any

Table 2.28. Distribution of Bluetongue virus in C.variipennis females at daily intervals after infection by intrathoracic inoculation

Body part	Head		Thorax		Abdomen	
Days post inoculation	Virus concn. per head ($log_{10}TCID_{50}$)	No.tested (in pools)	Virus concn. per thorax ($log_{10}TCID_{50}$)	No.tested (in pools)	Virus concn. per abdomen ($log_{10}TCID_{50}$)	No.tested (in pools)
0	1.1	24	2.8	23	3.0	23
1	1.2	17	2.1	17	1.9	16
2	3.2	23	3.8	18	3.8	19
3	2.6	10	4.4	10	3.8	10
4	4.2	27	4.5	19	5.1	11
5	3.5	16	4.5	12	4.7	11
6 (1:5:8)[a]	3.1	21	5.1	15	5.5	11
7	4.1	4	4.2	3	2.8	3
8	4.8	11	5.7	10	5.2	9
9	5.0	7	5.5	8	5.1	6
10	4.5	9	5.1	9	4.7	9
11	4.9	9	5.2	10	4.9	9
12	5.1	8	5.1	6	4.6	4
13	5.2	7	4.8	6	5.8	4

Cont'd.....

Table 2.28 Continued .....

Body part	Head		Thorax		Abdomen	
Days post inoculation	Virus concn. per head ($log_{10}TCID_{50}$)	No.tested (in pools)	Virus concn. per thorax ($log_{10}TCID_{50}$)	No.tested (in pools)	Virus concn. per abdomen ($log_{10}TCID_{50}$)	No.tested (in pools)
14	-*	-	-	-	-	-
15	-	-	-	-	-	-
16	4.1	6	3.9	7	4.5	7
17	4.4	5	4.4	5	4.1	5

-* Not tested

a Weight ratio of head : thorax : abdomen

Table 2.29 Distribution of Bluetongue virus in C.nubeculosus females at daily intervals after infection by membrane feeding

Body part	Head		Thorax		Abdomen	
Days post feeding	Virus concn. per head ($log_{10}TCID_{50}$)	No.tested (in pools)	Virus concn. per thorax ($log_{10}TCID_{50}$)	No.tested (in pools)	Virus concn. per abdomen ($log_{10}TCID_{50}$)	No.tested (in pools)
0	0	13	0	11	3.5	10
1	0	17	0	9	2.1	9
2	-*	-	-	-	-	-
3	0	7	0	8	T	7
4	0	8	0	8	0	8
5	0	9	0	7	0	7
6	0	5	0	5	0	5
7	-	-	-	-	-	-
8	0	12	0	9	0	8
9	-	-	-	-	-	-
10	-	-	-	-	-	-
11	-	-	-	-	-	-
12	0	3	0	3	0	3

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $log_{10}TCID_{50}$/insect part).

-* Not tested

Table 2.30. Distribution of Bluetongue virus in C.variipennis females at daily intervals after infection by membrane feeding

Body part	Head		Thorax		Abdomen	
Days post feeding	Virus concn. per head ($log_{10}TCID_{50}$)	No.tested (in pools)	Virus concn. per thorax ($log_{10}TCID_{50}$)	No.tested (in pools)	Virus concn. per abdomen ($log_{10}TCID_{50}$)	No.tested (in pools)
0 (1:3:23)[a]	T	8	T	6	2.6	6
1 (1:3:24)	0	9	T	9	0	8
2	0	9	0	9	0.9	9
3	1.6	14	2.2	14	3.2	16
4	1.1	11	3.1	11	3.8	11
5	2.5	12	3.4	12	4.0	12
6 (1:5:8)	2.7	21	2.4	25	3.5	25
7	3.0	11	3.7	11	3.0	11
8	3.7	19	4.0	19	3.8	19
9	3.8	10	4.5	10	4.8	10
10	3.7	15	3.9	7	3.9	6
11	4.0	12	3.5	12	4.6	12
12	3.9	6	4.4	5	4.0	5
13	3.7	6	3.5	7	3.6	8

Cont'd.....

Table 2.30. Continued.....

Body part	Head		Thorax		Abdomen	
Days post feeding	Virus concn. per head ($log_{10}TCID_{50}$)	No.tested (in pools)	Virus concn. per thorax ($log_{10}TCID_{50}$)	No.tested (in pools)	Virus concn. per abdomen ($log_{10}TCID_{50}$)	No.tested (in pools)
14	4.4	13	4.4	13	3.6	13
15	4.0	10	5.0	11	5.4	9
16	4.2	9	4.6	9	3.9	9
17	4.9	11	5.1	10	4.7	11

T Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $log_{10}TCID_{50}$/insect)

a Weight ratio of head : thorax : abdomen

part of these insects (Table 2.29).

Similarly, in C.variipennis most virus was found in the abdomen on day 0, and only a trace was detected in the head and thorax at this time (Table 2.30). By day 5 post infection increased virus concentrations were present in all three parts of the body, and maximum levels were reached around day 15 post infection. From day 6 until the end of the experiment on day 17, the virus content of the head equalled or exceeded that of the thorax or abdomen if the results were corrected to allow for the weight ratio of the three parts. For example, the corrected values on day 7 are, head 3.0 : thorax 3.0 : abdomen 2.1, and on day 17, head 4.9 : thorax 4.4 : abdomen 3.8.

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2.4. Discussion

2.4.1. Artificial techniques for the infection of sandflies with viruses

Both the membrane feeding and the intrathoracic inoculation technique described in this thesis can be used to infect L.longipalpis and P.papatasi sandflies with viruses. The membrane feeding technique is both quick and simple and has obvious advantages over the use of vertebrate hosts for arbovirus studies. P.papatasi membrane fed slightly more readily than L.longipalpis although both species usually ingested blood within 10 minutes of the attachment of cages to the membrane feeder. The occasional reluctance of a whole batch of L.longipalpis to feed has also been observed when these insects are

fed on hamsters (Killick-Kendrick et al., 1977). It has been suggested that this may be comparable to the situation observed in nature where there are fluctuations in the numbers of sandflies attracted to bait animals during apparently identical environmental conditions (Ward in Killick-Kendrick et al., 1977).

Although membrane feeding techniques have not previously been used to infect sandflies with viruses, a few species have been artificially fed on Leishmania parasites. Most of the methods employed however have had considerable limitations if large numbers of insects are to be used. Adler and Theodor (1927) were able to feed P.papatasi on suspensions of Leishmania through membranesof shaved rabbit skin. Experiments with P.sergenti Parrot were less successfull (Adler and Theodor, 1929). Sandflies were fed in large glass tubes into which a smaller tube containing the blood meal was inserted. There was no provision for controlling the temperature of the feeding solutions, and since the insects were in closed tubes a breathing stimulus could not be applied to reluctant feeders. Hertig and Hertig (1927) developed a technique for force feeding individual Phlebotomus sandflies on suspensions of Leishmania. This involved placing a fine glass capillary pipette over the mouthparts of a single female. Fluid containing suspensions of Leishmania was then dripped into each pipette, and was automatically ingested by each sandfly. This is rather a laborious and time-consuming procedure. The technique was later successfully used to feed five Lutzomyia species on blood containing Leishmania flagellates. These species were : L.sanguinaria, L.gomezi, L.panamensis Shannon, L.trapidoi and L.ylephilator (Hertig and McConnell, 1963). Johnson (in Hertig and McConnell, 1963) reported little success with attempts to feed Lutzomyia species through membranes prepared from guinea pig mesentery and large intestine, human kidney capsule, human peritoneum and human skin. Gemetchu (1976) was equally unsuccessful in feeding

P.longipes. No details of the apparatus was given but membranes tried were chick skin, Baudruche, sausage skin, and the belly skin of one day old white mice. Blood meals offered were of defibrinated rabbit blood or human blood. Schmidt (1964) was able to feed P.papatasi and P.orientalis on heparin-treated mouse blood through mouse skin and Baudruche membranes. Again no further experimental details are available and the numbers of flies that were successfully fed is not stated.

Before the start of this project P.D. Ready at Imperial College had been trying unsuccessfully for some time to membrane feed sandflies from their L.longipalpis colony using various different types of membrane. When my experiments with insects from the same colony proved successful, he adopted the apparatus and methods described in this thesis for his work on the feeding habits of L.longipalpis (Ready, 1978). Ward (1977), in Brazil, used a similar technique for feeding colonised L.flaviscutellata. A feeding rate of only 20% was however obtained, with 18 out of 90 females ingesting rabbit blood through a chick skin membrane.

The inoculation technique has not previously been applied to sandflies, either to infect them with viruses or with other organisms. Following the procedures described in this thesis such treatment does not affect the subsequent longevity or the apparent behaviour of female sandflies. The survival of males was less satisfactory but these would not normally be used in vector studies.

Although intrathoracic inoculation of virus does not constitute a natural route of infection, it may be considered to represent the situation occurring in the insect once virus has been ingested and has passed through the gut wall into the haemocoele. As such it has three main applications.

(1) The technique can be used to infect a known vector with virus if the vector will not blood feed under laboratory conditions.

(2) It can be used to provide the initial infection in transmission experiments when dealing with insects that do not normally take a second blood meal under laboratory conditions.

(3) Many viruses will multiply in insects after infection by intrathoracic inoculation but not after oral ingestion since the former method bypasses any existing "gut barrier" to infection. If virus does not multiply after inoculation then it will almost certainly not do so after oral ingestion. Since ingestion of virus may result in a very low infection rate even in susceptible species, large batches of insects must be screened for evidence of virus multiplication when testing the susceptibility of a species. Inoculation of virus into a susceptible species usually gives rise to a subsequent infection rate of 95 to 100%. Thus, when only small numbers of insects are available for study the inoculation technique can provide a useful indication of whether an insect species will be susceptible to oral infection with a virus.

One of the primary criteria for classification of a virus as an arbovirus is its ability to multiply in insects after inoculation or ingestion. In the past mosquitoes have usually been the test insects chosen for such work. Since mosquitoes may be insusceptible to infection with many of the Phlebotomus fever group viruses (Tesh, 1975), viruses suspected of being sandfly-borne could be tested by inoculation into sandflies. The technique of intrathoracic inoculation has furthermore been found to be extremely sensitive for the primary isolation and propagation of Dengue (DEN) viruses (Rosen and Gubler, 1974). It is possible that this could apply to some of the Phlebotomus fever group viruses and sandflies.

2.4.2. Phlebotomus fever group viruses

The colony of L.longipalpis used in this project was not found to be suitable for use as a model in sandfly fever studies since neither SFS nor SFN virus multiplied in these insects following infection by either membrane feeding or intrathoracic inoculation. The results obtained after inoculation of L.longipalpis with SFS virus were equivocal since using the more highly concentrated inoculum (i.e. 9.3 $\log_{10}MLD_{50}$/ml) 81% of the females tested between days 3 and 13 were infected, with a mean virus concentration of up to 2.9 $\log_{10}MLD_{50}$/ fly. However the original day 0 value of 3.5 was never exceeded. In view of the virtual disappearance of virus after day 1 in the second experiment, when a lower dose of virus was used for the inoculum, and considering the stability of SFS virus at 25°C, the result was interpreted as the persistence of virus due to its stability in the buffered environment of the insects' tissues. It was concluded that no multiplication of SFS virus had occurred.

A limited number of inoculation experiments with P.papatasi and the two sandfly fever viruses indicated that SFS would multiply in this sandfly species. The results with SFN virus and P.papatasi were equivocal. The virus persisted in these sandflies for a longer period than in L.longipalpis but in only 3 out of 34 determinations was the day 0 virus concentration exceeded. The vector status of P.papatasi for SFN virus has not been confirmed and these results may be a further indication that this species is not the natural vector; however many more experiments are necessary before such positive conclusions can be drawn.

Pacui virus, a third member of the Phlebotomus fever group, multiplied both in L.longipalpis and P.papatasi after infection by intrathoracic inoculation although multiplication of the virus did not occur in these insects after oral infection. Both sandfly species may

therefore be of use for studying the development of this virus in an insect host. None of the Phlebotomus fever group viruses multiplied in C.variipennis and C.nubeculosus after infection by either method.

All three viruses disappeared from L.longipalpis sandflies and from Culicoides within 48 hours of oral ingestion. Similar results were obtained with P.papatasi after oral infection of this species with PAC virus. Mixtures of blood and virus incubated at the same temperature as infected insects, i.e. $25^{o}C \pm 1^{o}C$, were relatively stable over the same period of time. The more rapid disappearance of virus from the insect suggests that the environment of the gut may be unsuitable for the survival of virus and denaturation may take place due to an adverse pH of the gut contents, or as the result of an enzyme or other chemical reaction.

For biting flies such as midges, mosquitoes, and sandflies to act as biological vectors of virus, ingested virus must penetrate into the insect's cells and tissues from the lumen of the gut. Multiplication of virus must take place in the insect's cells and a sufficiently high concentration must eventually be attained in the salivary glands to render the saliva infective when injected into a vertebrate host. Murphy et al. (1975) suggested that there are at least two pathways by which virus may reach the salivary glands from the gut. One is by way of the haemolymph, and the other by progressive sequential infection of contiguous organs. Since the haemolymph bathes all the cells and tissues it will be involved at some stage in the spread of infection by both routes. It is not known how arboviruses pass from the immediate environment of the gut into the insect's tissues. Virus is ingested with a blood meal and passes through the foregut

directly to the midgut, bypassing the diverticulum into which sugar meals are taken. The front part of the foregut is lined with cuticle which provides an impermeable barrier to virus infection. Virus probably penetrates the cells of the gut wall in the midgut region where the epithelium is exposed. It is not known how this process takes place. Most other viruses have fairly restricted patterns of infectivity and it has been proposed that these attach to special receptor sites on cell surfaces, the receptor sites being specific for each virus (e.g. Holland, 1964 on enteroviruses; Kohn, 1979). The susceptibility of an insect to oral infection with an arbovirus might therefore depend on the presence of the appropriate receptor sites on the cells of the gut epithelium. This concept, as applied to the development of BT virus in C.variipennis, is currently being investigated in our laboratory. Since arboviruses exhibit such a diverse range of host and tissue specificity, application of the concept of virus attachment to specific receptor sites would necessitate the presence of common receptors on many different types of cells of both vertebrate and invertebrate origin (Wise, 1975).

It is possible that restrictions on the susceptibility of insects to arbovirus infection occur during the processes of penetration and uncoating of virus at an intracellular level after the initial attachment of virus to the cell. Arboviruses may be able to enter a variety of different cell types in a variety of insect species, but they may be capable of replicating in only a limited range of cells.

Failure of virus to pass from the gut into the tissues of the insect by whatever means renders that insect insusceptible to oral infection with the virus in question. However, many viruses will multiply in insects which have proved insusceptible to infection by

the oral route if the virus is inoculated directly into the haemocoele, i.e. by bypassing the gut. Hurlbut and Thomas (1960,1969) demonstrated that even non-haematophagous insects, such as the meadow grass-hopper and the Indian meal moth, could support the replication of viruses including Semliki forest (SF), Sindbis (SIN), Eastern equine encephalomyelitis (EEE), Western equine encephalomyelitis (WEE), and Chikungunya (CHIK) after inoculation of virus into the haemocoele. This ability of arboviruses to multiply in a wider range of insects if the initial infection is produced by direct inoculation of virus into the haemocoele rather than orally, can be seen to a varying degree in most of the arbovirus groups. In insects which are orally susceptible to a particular virus an equivalent infection can usually be produced with a reduced dose of virus if this is given by intrahaemocoelic inoculation. This also applies to the insect pathogenic viruses, where considerably lower doses can prove fatal when inoculated into the haemocoele rather than when given orally (Tinsley, 1975). Infection of insects by inoculation may also reduce the length of the extrinsic incubation period. For example, transmission of Ukauwa (= Bunyamwera) virus by Ae.aegypti was demonstrated within 5 days of the initial inoculation of virus, whereas after virus ingestion the extrinsic incubation period was 12 days (Ogunbi, 1968). Similar results were obtained with C.variipennis and African horse sickness (AHS) virus in this laboratory. C.variipennis were able to transmit AHS 4 days after inoculation but not until 13 days after ingestion of the virus (Mellor et al., 1975). Observations such as these on the effect of intrahaemocoelic as opposed to oral infection on subsequent virus development in insects led to the development of the concept of the invertebrate gut forming a "barrier" to virus infection. The various hypotheses suggested by Chamberlain and

Sudia (1961) as an attempt to explain the nature of such a "gut barrier" were listed in part 1.3. but are restated below.

1. Threshold level of infectivity.
2. Impermeability of a peritrophic membrane.
3. Inactivation of virus by digestive fluids.
4. Absence of specific receptor sites on the gut cells.
5. Variations in permeability of gut cell membranes.
6. Presence of a surface type defence mechanism.

The concepts of susceptibility and the gut as a barrier to infection have been reviewed more recently by Varma (1972) and Tinsley (1975). Varma suggested that limited susceptibility might result from : destruction of virus by antiviral substances within the cells; the induction of interferon-like substances produced as a result of cellular infection; or death of virus particles due to the absence of specific metabolic requirements in the cells.

All such hypotheses have been based on the study of arbovirus infections in mosquitoes since there is little information on the development of these viruses in other groups of insects.

L.longipalpis and P.papatasi sandflies were susceptible to infection with PAC after inoculation of this virus but not after ingestion of virus, and therefore a "gut barrier" could be involved in limiting the susceptibility of these insects to infection with PAC via the oral route. Since neither SFS nor SFN virus multiplied in L.longipalpis after infection by inoculation, and similar results were obtained after inoculation of C.variipennis and C.nubeculosus with SFS, SFN, and PAC,it is unlikely that a "gut barrier" is the only mechanism restricting the development of these viruses in these insects. The results suggest that, with the exception of L.longipalpis and PAC virus, none of the cells and tissues of L.longipalpis, C.variipennis, and

C.nubeculosus, can support the replication of the three Phlebotomus fever virus stocks used in these experiments.

With the exception of the peritrophic membrane as a gut barrier to arbovirus infection, the hypotheses of Chamberlain and Sudia can be equally applied to insects' cells and tissues other than those of the gut to explain the total refractoriness of some species to certain virus infections. Both the haemocoelic fluid and the digestive fluids could denature virus when it is extracellular in the haemocoele and gut respectively. However denaturation would have to be rapid to prevent virus coming into contact with and infecting the cells. In the insect-virus combinations used in this project virus was detectable in individual infected insects for up to 24 hours after ingestion and for several days after inoculation. Titration of infected C.variipennis at hourly intervals after ingestion of SFN virus showed that there was no appreciable drop in the virus concentration of infected flies over 7 hours. This ought to be long enough to allow virus to attach to, or penetrate, the gut cells if this were possible. LaMotte (1960) however has shown that in mosquitoes digestion commences on the periphery of the blood meal. This could result in the primary destruction of the virus nearest to the gut epithelium. If this took place rapidly virus might be prevented from infecting the gut cells.

Virus persisted in insects for longer periods after inoculation. The concentration of virus declined slowly indicating that no rapid denaturation was taking place. The concepts of specific virus receptor sites on the cell surfaces, of the permeability of cell membranes limiting virus penetration into the cell, and of intracellular defence mechanisms such as physiological and biochemical incompatability of the

cell with the virus, can all be applied to tissues other than those of the gut. If susceptibility to a virus infection is a function of the cells themselves, then it would be of interest to discover why, when virus multiplies in an insect after inoculation but not after membrane feeding, the gut cells should be refractory whereas other cells in the insect are susceptible to infection. The use of insect cell cultures to study the mechanisms of arbovirus attachment to, and replication in insect cells might advance our knowledge of the interactions between these viruses and their hosts. Unfortunately there are as yet no Culicoides or sandfly cell lines available for such studies.

The threshold level of infectivity is a quantitative "gut barrier" mechanism and is defined as the concentration of virus in a blood meal which is sufficient to infect a significant number (e.g. 1% to 5%) of susceptible insects. The concept can also be applied to infection by inoculation, although the threshold level in this case is usually lower. The threshold level varies with the insect species and the virus. For example Chamberlain and Sudia (1961) found that a blood-virus concentration of 10^3 per 0.03ml of EEE was sufficient to infect Ae.aegypti mosquitoes, whereas a concentration of 10^8 per 0.03ml of the same virus was required to infect Cx.salinarius Coquillet. Usually the higher the concentration of the virus in a blood meal the higher the subsequent infection rate. The example of C.variipennis orally infected with AHS virus was given in part 1.3. (i.e. virus content of the blood meal below 5.7 $\log_{10}TCID_{50}$/ml, infection rate = 0%; virus content of the blood meal 6.7 $\log_{10}TCID_{50}$/ml, infection rate = 35%). A similar threshold level was observed when the same colony of C.variipennis was infected with BT virus (Dr.P.Mellor personal communication, 1979) and similar results have been obtained with many

other insect-virus combinations. LaMotte (1960) obtained infection rates of 23%, 88%, and 100% when Cx.quinquefasciatus Say mosquitoes were offered blood meals containing Japanese encephalitis (JE) virus at concentrations of 1.4, 1.8 - 2.8, and 3.5, $\log_{10}LD_{50}$ per 0.003ml chick blood respectively. Thomas (1963) reported the infection rates of Cx.tarsalis after ingestion of WEE virus to increase from 7% (virus content of blood meal 1.5 $\log_{10}$Chick LD_{50}) to 96% (virus content of blood meal 3.3 $\log_{10}$Chick LD_{50}) and 100% (virus content of blood meal 5.1 $\log_{10}$Chick LD_{50}). Watts et al., (1973) found that using a blood meal containing 3.5 $\log_{10}$Mouse LD_{50} per 0.03ml of La Crosse (LAC) virus to infect Ae.vexans Meigen the infection rate was 23%. When the blood virus content was increased to 5.4 $\log_{10}$Mouse LD_{50} per 0.03ml the infection rate increased to 72%. Such results indicate that in initial experiments to determine the susceptibility of an insect species to oral infection with a virus it is advisable to use as high a virus concentration in the blood meal as possible, in order to overcome any threshold effect. The Phlebotomus fever group viruses used in this project were considered to be of sufficiently high concentration to infect at least a small percentage of the insects tested, if they were to prove susceptible to infection by the oral route.

The threshold level may represent the concentration of virus required to neutralise any inhibitory substances secreted by the gut epithelium, or to neutralise the action of digestive enzymes or adverse pH factors. In the case of infection by inoculation it may similarly be explained as the concentration of virus necessary to neutralise adverse factors in the haemocoele. The threshold level may also be a statistical effect, i.e. a single virus particle in the centre of the blood meal in the gut would have less chance of penetrating the gut wall

than would a number of virus particles distributed throughout the blood meal (Boorman, unpublished).

The only potential "gut barrier" that might be visually demonstrated by histological techniques is a peritrophic membrane. This structure is usually defined as one or more membranes secreted by the midgut epithelium during or after the intake of food, and forming a tube or sack around the food bolus (Richards and Richards, 1977). A peritrophic membrane is found in both larval and adult stages of many but not all insects. It is considered at least in some species to protect the midgut epithelium from mechanical damage caused by food particles (Wigglesworth, 1972). It may act as a barrier to parasitic infections (Lewis, 1953; Stohler, 1957; Orihel, 1975), and possibly to viruses also. The effectiveness of the peritrophic membrane as a gut barrier to virus infection would depend upon factors such as the speed with which it forms and thus separates the blood meal and the virus this contains from the gut wall; the structure, including the pore size and completeness of the membrane; and the length of time it persists as an entire impermeable sack around the food. Chamberlain and Sudia (1961) suggested that if the permeability of the membrane were only a function of the size of the pores then viruses of similar size would be expected to pass through with equal facility. Tinsley (1975) postulated that the permeability of the membrane might be determined by the nature of the electrical charge on the membrane and on the virus particle. There is little information available on these aspects.

No definitive peritrophic membrane was observed when C.variipennis and C.nubeculosus were examined up to 48 hours after ingestion of blood (Mellor and Jennings, unpublished results). Detailed examination of P.longipes by Gemetchu (1974) showed that the peritrophic membrane in this sandfly species began to form with the intake of a blood meal and became firm and stable after 48 hours. The formation of a peritrophic

membrane in P.papatasi and L.longipalpis has not been studied in such detail. The membrane, if present, does not prevent P.papatasi acting as a vector of SFS virus in nature.

Care must be exercised when interpreting negative results such as obtained in this project since they may be a function of the particular colonies of insects, or of the virus stocks used, and thus may not accurately represent the absolute vector potential of the species for the viruses in question under natural conditions. Recent work has shown that both field and colonised populations of insects can be polymorphic in their reaction to a virus infection and that the polymorphism may be genetically determined. For example, Jones and Foster (1974) working with BT virus and C.variipennis found that the infection rate of orally infected flies originating from different field populations varied from 0 to 39%. The virus content of the blood meal used in these experiments was sufficient to infect 33% of their colonised population. Both in this C.variipennis colony and in the colony maintained at A.V.R.I. the infection rate after oral ingestion of BT virus is only 30-35% despite an initial infection rate of 100%. Thus approximately 60-70% of these insects are refractory to the virus infection. Jones and Foster (1974) were able to develop both highly BT virus susceptible (82 - 100% susceptible) and highly BT virus resistant (0 - 3% susceptible) insect lines by genetic selection from the parent colony. Similar results have been obtained by other authors. Gubler and Rosen (1976) for example observed that colonised strains of Ae.albopictus, which had initially been collected from different geographical locations, varied as much as 100-fold in their susceptibility to oral infection with four serotypes of DEN. If polymorphism to infection is genetically determined then it is likely that changes in susceptibility will arise in different colonies due to selection pressures during colonisation.

It is possible that the mouse adapted strains of virus used in these experiments were no longer infective for insects. This was more likely to apply to SFS and SFN than to PAC since the mouse brain passage level of the former two viruses was much higher than that of PAC. Although the latter virus disappeared rapidly from infected Culicoides it multiplied to a high level in both sandfly species after infection by inoculation and was therefore infective for an arthropod host.

Reports on the effect of continued passage of arboviruses in laboratory animals and cell cultures on the infectivity of these viruses for insects are few and conflicting. Hurlbut and Thomas (1960) found that the 460th mouse brain passage of EEE virus produced similar results when used to infect arthropods as the original 3rd and 4th passage level stocks. Similarly, mouse brain passage levels 3,4, and 111 of St.Louis encephalitis (SLE) virus did not vary in their infectivity for arthropods. In this laboratory BHK 21 cell passage levels 4 to 15 of BT virus have all been used to infect Culicoides with similar results. Foster et al. (1968) however found a difference in the infection rate of C.variipennis when these insects were fed on sheep inoculated with a vaccine strain of BT virus (attenuated by chick-embryo passage), as compared to the infection rate obtained when insects were fed on sheep inoculated with virulent virus. Using the vaccine virus the infection rate was reduced from 30% to 9%. A single passage of this virus through the insect rendered it virulent for sheep. There have been a few other reports of altered characteristics of virus with respect to the development in the insect host. Saliba et al. (1973) for example report that the infectivity of a Cache-Valley-like virus for mosquitoes was reduced between the 4th and 10th mouse brain passage. Earlier work which is sometimes quoted in this context, for example that

of Whitman (1939), and Bates and Roca Garcia (1946), on the susceptibility of Ae.aegypti and Haemagogus mosquitoes to Yellow fever (YF), and of Sabin and Schlesinger (1945) on Ae.aegypti and DEN, implicates low virus concentrations in the blood of the infected animal (i.e. in the blood meal of the mosquito) as the main factor affecting the results, rather than any modification of the virus from continued vertebrate passage. Such work requires clarification by controlled experiments, preferably using membrane feeding techniques, to provide known virus concentrations in all blood meals.

The experiments included at the end of part 2 were intended as a positive control to check the infectivity for insects of the two Sandfly fever virus stocks by observation of the behaviour of these viruses in P.papatasi, an established vector - at least of SFS. The colony of P.papatasi used for these experiments was supposedly susceptible to infection with Sandfly fever virus, probably SFS (Modi personal communication, 1979), although no details of the multiplication rate of either virus in this colony,or in wild-caught sandflies, have been published. The sandflies were infected by inoculation to overcome any problems caused by low infection rates or reluctance of females to blood feed - thus illustrating the value of this technique.

Both SFS and SFN viruses behaved differently after inoculation into P.papatasi than after inoculation into L.longipalpis. The results for SFS indicated that the stock virus would multiply in P.papatasi rather than merely persisting in the buffered environment of the insect's tissues as it was concluded to do in L.longipalpis. However, the level of virus multiplication was lower than expected from the results obtained after inoculation of each species with PAC virus. The latter virus increased in concentration by 4.9 $\log_{10}MLD_{50}$/fly over 4 days, the maximum concentration recorded from a single P.papatasi female being 5.4 $\log_{10}MLD_{50}$/fly. The concentration of SFS virus increased by only 1.3

$\log_{10}$MLD$_{50}$/fly over a period of 8 days, the maximum amount of virus recovered from a single female being 4.4 $\log_{10}$MLD$_{50}$. Similar results were obtained by Tesh (1975) when 8 Phlebotomus fever group viruses were inoculated into Ae.albopictus and Cx.fatigans mosquitoes. Increases in mean virus concentrations of between 1.1 and 3.3 $\log_{10}$ plaque forming units per mosquito were recorded, the highest levels of multiplication occurring with Arumowot (AMT) and PAC in Ae.albopictus, and AMT and Karimabad (KAR) in Cx.fatigans. As stated previously, details of the rate or level of multiplication of SFS virus, either in wild populations of P.papatasi or in the colony used in these experiments, were not available for comparison with my results. It was concluded however that the SFS virus used in my experiments was infective for insects - at least to some extent - and therefore the L.longipalpis colony was not susceptible to the virus.

The results obtained with P.papatasi and SFN virus were inconsistent. Although a similar increase in virus concentration to that observed with SFS in P.papatasi was obtained, this was only apparent in 3 out of the 34 groups of sandflies titrated after day 0. Insufficient data was obtained to draw any positive conclusions on the infectivity of this virus for insects, particularly on consideration of the fact that the vector status of P.papatasi for SFN is unconfirmed, and this sandfly may not support virus multiplication in nature.

Pacui virus multiplied in an Ae.albopictus cell line and the possibility that insect cell passaged virus might produce different results when used instead of the mouse brain passaged stock to orally infect L.longipalpis was investigated. The insect cell passaged virus did not multiply in these sandflies. However the virus content of the blood meal was low, and the initial infection rate was correspondingly low. More than 100 sandflies were titrated over a period of 14 days after the initial ingestion of virus. This should have been a sufficient

number to produce a significant virus concentration in at least one fly if virus multiplication was likely to occur. It would be interesting to concentrate the insect cell passaged virus used in these experiments, or to use virus passaged by intrathoracic inoculation of insects. This line of research was not pursued at this time due to the large numbers of sandflies required and the numbers of mice necessary for titration of samples. It is significant that PAC virus multiplied in the Ae.albopictus cell line used in these experiments since Buckley (1972) obtained conflicting results with Singh's Ae.albopictus cell line. The origin of the Ae.albopictus cells used in this project is uncertain, but it is probable that they are also from Singh's cell line. This provides further evidence that not only may different colonies of an insect species differ in their susceptibility to infection with a particular virus, but also different cell lines or passage levels of cell lines from the same species of insect may differ in their response to a virus infection.

Ideally in experiments to test the susceptibility of insects to infection with arboviruses, the lowest passage levels of both the virus stock and the insect colony generation should be used in order to duplicate the field situation as closely as possible. Because of the low infection rates often obtained it is also important to use as large a number of insects as possible in such experiments. In the field the enormous populations of biting insects present enable viral and parasitic diseases to circulate efficiently between the invertebrate and vertebrate hosts even when vector infection rates of less than 1% are involved (Buckley, 1938; Mellor, 1975). Unfortunately it is not often practically possible to satisfy the three criteria of low passage levels of virus, low generation levels of the insect colony, and large numbers of insects for each experiment. In the experiments reported in this thesis a minimum number of 100 insects per infection was used wherever possible. The generation level of the insect colonies and the passage

level of seed virus stocks were not under my control.

The failure of SFS and SFN viruses to multiply in C.variipennis and C.nubeculosus after infection by membrane feeding or intrathoracic inoculation, is less unexpected than the failure of these two viruses to multiply in L.longipalpis sandflies. With the exception of Rift Valley fever (RVF) virus, none of the Phlebotomus fever group of viruses have been isolated from Culicoides. Several members of the group have been isolated from Lutzomyia species (see Table 1.3.). These include PAC (as used in these experiments) isolated from L.flaviscutellata in Brazil (Aitken et al., 1975); Aguacate (AGU), Cacao (CAC) and Punta Toro (PT) isolated from both L.trapidoi and L.ylephilator; and CAC isolated from L.trapidoi in Panama (Tesh et al., 1974; Tesh et al., 1975). The disappearance of SFS and SFN viruses after inoculation into Lutzomyia sandflies and into Culicoides, together with the results obtained with Culicoides and PAC virus, suggests that these viruses may have a more restricted host range than many mosquito-borne arboviruses. The experimental host range of many of the latter viruses has been found to encompass a variety of mosquito species and genera and may even include insects from different families (see section 1.3.). It is of interest to mention here that other sandfly-borne viruses such as Vesicular stomatitis Indiana (VSI) and Chandipura (CHP)(both rhabdoviruses), and also members of the Changuinola subgroup of the orbiviruses, have been shown to multiply in mosquitoes after infection by inoculation, and in mosquito cell lines (e.g. Main et al., 1977; Singh and Paul, 1968; Buckley, 1972).

Tesh (1975) found that only 8 out of 22 Phlebotomus fever group viruses would multiply in one or both of the mosquito species Ae.albopictus and Cx.fatigans after infection by inoculation. However these results were obtained from experiments using male mosquitoes. Whether this can be taken as a true representation of the behaviour of

these viruses in female insects is questionable. Virus multiplication was assumed to occur if more than 10 plaque forming units of virus per mosquito were present 10 days after the initial inoculation. A negative result was recorded when less than 10 plaque forming units per mosquito were present at this time. The positive results were confirmed by inoculation of each virus into female mosquitoes, however no such check was made of the negative results. These require confirmation since only small numbers of males, i.e. 2 to 21, were used for each sample. The virus concentration of the inoculum of some of the viruses used was very low, and insects were tested on only one day (day 10) after infection. Experience in this laboratory has shown that large numbers of insects should be tested over a period of several days before a negative result can be concluded. This is particularly important when insects are infected orally because of the possibility of low infection rates. However the results presented in this thesis demonstrate that even after infection of insects by inoculation the range of virus concentrations recovered from individual insects at intervals after inoculation can include very low values (cf.experiments with sandflies, BT and SFS viruses). Thus it is advisable to use as many insects as possible in such experiments before concluding that virus multiplication does not occur.

The 8 viruses which Tesh found to multiply in mosquitoes included both New and Old World members of the Phlebotomus fever group, as did the 14 viruses which did not multiply. Two of the 8 viruses, AMT and Itaporanga (ITP), had originally been isolated from mosquitoes and not from sandflies. Neither SFS nor SFN virus were found to multiply in either mosquito species. Ae.aegypti has also proved refractory to SFS virus (Whitman in Buckley, 1972), and cell lines derived from Ae.aegypti and Ae.albopictus mosquitoes did not support the replication of either SFS or SFN viruses (Buckley, 1969). In contrast to these results, Hurlbut and Thomas (1960) found that SFS virus multiplied both in

Cx.tarsalis and Cx.univittatus mosquitoes after inoculation into the haemocoele.

Pacui was the only Phlebotomus fever group virus used in these experiments which multiplied in L.longipalpis sandflies, and this was only after infection of insects by inoculation. This virus also multiplied after inoculation into P.papatasi. Pacui virus has previously been shown to multiply in Cx.fatigans and Ae.albopictus mosquitoes after intrathoracic inoculation (Tesh, 1975), but Whitman (in Aitken, 1975) was unable to maintain an infection through two salivary gland passages in three other mosquito species, Ae.aegypti, Cx.pipiens quinquefasciatus Say and An.quadrimaculatus Say, after intrathoracic inoculation of the virus.

Pacui virus was present in the salivary glands of L.longipalpis within 5 days of inoculation since transmission of virus could be demonstrated at this time. A similar extrinsic incubation period has been observed after inoculation of C.variipennis and C.nubeculosus with BT virus. This virus was transmitted by both Culicoides species 6 days after its initial inoculation into these insects. Transmission was not demonstrated before this time (Dr.P.Mellor personal communication, 1980).

The quantity of PAC virus transmitted by each sandfly was approximately twice that transmitted by C.variipennis infected with Eubenangee (EUB) (Mellor and Jennings, 1980). The virus content of individual sandflies and Culicoides in each case was the same. The size of the blood meal of sandflies is approximately twice that of Culicoides and possibly the volume of saliva injected whilst feeding is relatively greater.

P.papatasi was not shown to transmit PAC virus. This was considered to be due to the small numbers of females, i.e. 1 to 5, that fed during

transmission experiments since the techniques used for the detection of transmitted virus have only limited sensitivity. In experiments with BT virus infected Culicoides in this laboratory, transmitted virus has usually been recovered from the membrane when 9 or more females ingested blood. The smallest number of L.longipalpis sandflies which transmitted detectable amounts of PAC virus was 8. This was the smallest number of this species used. A more sensitive test system is really required for transmission experiments with viruses, such as those of the Phlebotomus fever group, for which susceptible vertebrate hosts cannot be used.

4.6.2. Bluetongue virus

Bluetongue virus multiplied in L.longipalpis and P.papatasi sandflies after infection by intrathoracic inoculation but not after membrane feeding. The virus multiplied in both species of Culicoides after infection by the former method but only C.variipennis was susceptible to infection by membrane feeding. The results obtained with C.variipennis were in accordance with those obtained by other workers e.g. Jochim and Jones (1966), Foster and Jones (1979), Mellor personal communication (1979).

Bluetongue has not yet been isolated from any insect other than Culicoides and few laboratory infections of genera other than Culicoides have previously been reported. Luedke et al. (1965) obtained transmission of BT virus by the sheep ked Melophagus ovinus (Linn.) although this was thought to be mechanical rather than biological transmission. Two reports of virus multiplication in mosquitoes have also been made. Nieschultz et al. (1934) reported BT virus multiplication in Ae.lineatopennis Ludlow with one possible incidence of virus transmission, and St.George and McCaughan (1979) were able to infect sheep with BT type 20 by injecting them with homogenates prepared from Cx.annulirostris Skuse and Ae.aegypti mosquitoes that had been inoculated with the virus 6 days previously. Bluetongue virus has also been found to multiply in

cell lines derived from Ae.albopictus and Ae.pseudoscutellaris Theobald mosquitoes, although an Ae.aegypti cell line proved refractory to infection (Jennings and Boorman, 1979).

Since BT virus multiplied in sandflies and C.nubeculosus only after inoculation, a gut barrier such as described in section 2.4.2. may operate to limit the susceptibility of these insect to oral infection with this virus. Mellor and Boorman (1980) have recently shown that BT virus will multiply in C.nubeculosus after oral infection if the insects are membrane fed on mixtures of blood, virus, and microfilariae. The latter pass through the gut wall and in so doing "inoculate" virus from the interior of the gut into the insect's tissues. It is considered that the virus particles stick to the parasite and are carried through the gut wall in this way.

The extent of BT virus replication in the two species of sandfly after infection by inoculation was less than expected, since this method of infection which bypasses the gut often results in high concentrations of virus developing in insects that do not become infected by the more natural oral route (see section 2.4.2.). Pacui virus, which multiplied in L.longipalpis and P.papatasi only after intrathoracic inoculation, reached a much higher concentration in these insects than did BT virus and the overall increase in concentration was much greater. In sandflies inoculated with PAC the maximum mean increase in virus concentration per fly was 3.9 (L.l.) and 4.9 (P.p.) $\log_{10}MLD_{50}$, whereas with BT virus inoculated sandflies the maximum mean increase was 1.6 (L.l.) and 2.1 (P.p.) $\log_{10}MLD_{50}$. This level of multiplication was similar to that of SFS virus after inoculation into P.papatasi, the maximum mean increase in this case being 1.3 $\log_{10}MLD_{50}$ per fly.

The level of multiplication of BT virus in sandflies is lower than the level of multiplication of this virus after inoculation into the two Culicoides species. Virus concentrations of at least 5.0 $\log_{10}TCID_{50}$

per fly were obtained within 5 days of inoculation of either C.variipennis or C.nubeculosus. This represented a maximum mean increase in concentration of 3.5 to 4.3 $\log_{10}TCID_{50}$ per fly. After oral infection of C.variipennis with BT virus the maximum mean increase from the day 0 value was only 1.9. However, in membrane feeding experiments the day 0 value represents the concentration of virus in the blood meal and this bears little relationship to the amount of virus which penetrates into the insect. The extent of virus multiplication after the two methods of infection can be compared more critically if the concentration of virus per insect on day 1 to 2 post feeding is taken to represent the initial virus content of membrane fed flies. The maximum mean increase after this method of infection then becomes 4.3 $\log_{10}TCID_{50}$per fly. Maximum virus concentrations are reached about 10 days after ingestion as opposed to 5 days after inoculation of virus. The difference is probably due to the time required for virus to pass from the gut lumen into the gut cells and haemolymph.

The infection rate of this colony of C.variipennis after oral infection with BT virus is only 30 - 35% (Dr.P.Mellor personal communication, 1977) and therefore approximately 65 - 70% of these insects are totally refractory to infection with this virus by this route. There is some variation in the level of virus multiplication even in susceptible flies. Titration of individual insects has shown that the maximum virus concentrations obtained after 10 days incubation at 25°C can vary from 2.4 to 6.2 $\log_{10}TCID_{50}$per fly (Jennings and Mellor, unpublished results). This illustrates that there may be a range in susceptibility of individual insects to oral infection with a virus even in a population of the vector species. This should always be considered when performing experiments to determine virus multiplication rates in insect populations since if the infection rate is less than 100%, and insects are processed in pools, the results per fly will be subject to

error because it is uncertain how many flies per pool are infected. Ideally insects should be titrated individually, but this is not always practicable. If insects must be pooled then it is best to use as small a pool as possible to minimise errors. For example, if the infection rate is 35%, then in a pool of 3 flies only 1 would be expected to be infected, although all 3 could contain virus. Adjusting the virus concentration per pool to per fly, i.e. dividing the result by 3, could give rise to an error of 0.3 $\log_{10}TCID_{50}$/fly if only 1 fly of the 3 were infected. Using a pool of 9 flies, 3 would be expected to be infected, although any number between 1 and 9 could contain virus. Thus when the results are corrected from virus concentration per pool to virus concentration per fly, i.e. divided by 9, there is a potential error of 0.8 $\log_{10}TCID_{50}$ in the concentration of virus recorded per fly, should only 1 of the 9 be infected. It was not always possible in this project to titrate insects individually, but the number per pool was limited to 6.

Experiments are in progress to determine the range of oral susceptibility of C.variipennis with respect to BT virus. Such work is time-consuming since it involves the infection and titration of large numbers of individual insects. As stated previously the level of BT virus multiplication in sandflies was low, being from 40 to 100-fold. In insects which are susceptible to a virus infection after intrathoracic inoculation the more usual pattern of virus multiplication is for an initial fall in virus concentration during the first 24 to 48 hours to be followed by an increase in virus concentration of at least 1000-fold. Lower levels of multiplication have been observed in other arbovirus-insect combinations, usually when a virus is inoculated into an unatural host. For example, similar results to those obtained from sandflies inoculated with BT virus were observed when several arboviruses,

Koongal (KOO), Wongal (WON) and Trubanaman (TRU) originally isolated from mosquitoes in Australia, were experimentally inoculated into Cx.fatigans during tests to establish their status as arboviruses (Carley et al., 1973).

The transmission of BT virus by sandflies was not demonstrated within the survival time of insects used in these experiments. The Pirbright colony of C.variipennis is able to transmit BT virus 6 days after infection by inoculation and 10-12 days after infection by membrane feeding (Dr.P.Mellor personal communication, 1979). Similar results with this species have been obtained by other workers such as Foster et al. (1963), Jones and Foster (1966), Luedke et al. (1967), and Foster and Jones (1979). All the sandflies used in my transmission experiments were found to contain virus. The mean virus concentration per sandfly was 3.4 $\log_{10}TCID_{50}$, with a range of 2.9 to 4.5. It was considered that sufficient numbers of females had fed on at least 6 out of 8 occasions for virus to have been detected if it had been transmitted.(In similar experiments using PAC, virus was recovered from the membrane after 8 females had taken a blood meal). It seems unlikely that BT virus was present in the salivary glands, although it may have been present at only a very low concentration. These results suggest that neither L.longipalpis nor P.papatasi would be likely to act as vectors of BT virus, although as stated in section 2.4.2. the possible refractory nature of the individual colonies should be considered before these conclusions are extended to wild populations.

4.6.3. Distribution of viruses in insects

Dissection of infected L.longipalpis sandflies on successive days after inoculation of PAC or BT virus and titration of the separate body parts, (i.e. head, thorax, and abdomen), showed that not only did the level of multiplication of these two viruses in sandflies differ,

but the distribution of virus in the insect differed also. The major difference in distribution was evident from the concentration of each virus recovered from the heads of infected insects. Each virus could be detected in the thorax and abdomen immediately after inoculation. The concentration of virus in these two body sections increased over several days and maximum levels were obtained within 5 days (PAC), and 8 to 10 days (BT), after the initial infection. The concentration of PAC virus in the head of infected insects was similar to that present in the thorax and in the abdomen by day 4 post inoculation, and equivalent concentrations of virus were maintained in all three parts of the insect until the experiment terminated on day 11. Bluetongue virus however was not detected in the head of infected sandflies until day 6 post inoculation. It was present in the head at trace levels only until day 9, and did not reach the levels recorded for the thorax within the survival time of the insects used in these experiments (i.e. within 12 days).

The fate of PAC and BT viruses in L.longipalpis was compared to that of BT after inoculation of this virus into C.variipennis and C.nubeculosus. Both the level of virus multiplication and the distribution of BT virus in the head, thorax and abdomen, on successive days after inoculation of Culicoides was similar to that obtained when L.longipalpis was inoculated with PAC. The maximum virus concentration of each body section was slightly higher in BT infected Culicoides than in PAC infected sandflies but the proportions of virus recovered from the head, thorax, and abdomen were similar in the two insect-virus combinations. Bluetongue virus was present in the head of infected Culicoides in concentrations equal to or greater than the concentrations present in the thorax or abdomen from day 7 to day 17 when the experiment was terminated.

Titration of dissected insects that had been orally infected with virus and which proved refractory to infection by this route, i.e. L.longipalpis membrane fed on PAC virus, and C.nubeculosus membrane fed on BT virus, showed that immediately after infection virus was present only in the abdomen, where it persisted in gradually decreasing amounts for 1 to 3 days.

C.variipennis is susceptible both to oral infection with BT virus and to infection by the intrathoracic route. The distribution of virus in the three body parts after the two different methods of infection could therefore be compared. It was found that in membrane fed insects virus was initially only present in the abdomen, and the thorax and head did not become infected until day 3 post infection. Maximum virus concentrations were then attained in all three body sections between 9 and 17 days after ingestion of virus. The maximum virus content of each body part was equivalent to that obtained after inoculation of virus, but in the latter case virus reached the head more quickly and peak concentrations were recorded within 4 to 9 days of inoculation of virus.

The amount of virus recovered from the three body parts of insects in these distribution experiments was expressed as the virus concentration per head, thorax, or abdomen. Since the thorax and abdomen each weighed at least 5 times as much as the head in both C.variipennis and L.longipalpis, the concentration of virus in the head was greater in relation to that of the other two parts than these results initially suggested.

It is interesting that high concentrations of virus were present in the head of BT infected Culicoides and of PAC infected L.longipalpis.

Similar results to these have been obtained with a number of other insect-virus combinations. Dengue-2 for example, was found to reach high concentrations in the heads of infected Ae.albopictus (Kuberski and Rosen, 1977; Kuberski,1979), and high concentrations of WEE virus were found in the heads of infected Cx.tarsalis (Thomas, 1963). The head consists mainly of nervous tissue and this suggests that these viruses may be neurotropic in the insect host. Supportive evidence for this theory can be seen in the electron microscope studies of Larsen and Ashley (1971) who demonstrated the presence of Venezuelan equine encephalomyelitis (VEE) in the brain of Ae.aegypti within 48 hours of ingestion of virus. LaMotte (1960) found high levels of JE virus in both the cephalic and thoracic ganglia of infected Cx.tarsalis, often at concentrations 100 to 1000 times greater than in larger organs. Dengue-2 has been shown to multiply in the encephalon of Ae.albopictus (Sriurairatna and Bhamarapravati, 1977), and similar results were obtained by Doi (1970) who located JE virus in the nervous tissue of Cx.tritaeniorhynchus summorosus Dyar. The concentration of virus present in the heads of BT infected L.longipalpis was low compared to that present in the heads of PAC infected L.longipalpis, and similarly the maximum concentration of whole BT infected sandflies was lower than that of PAC infected insects. It would be interesting to discover whether this represented a lower level of multiplication of BT virus in all tissues and organs including the nervous tissue; or whether BT virus multiplication was restricted to a more limited range of tissues in this insect, the virus in the head being due to the presence of infected haemolymph.

Most of the information available concerning the distribution of arboviruses in an infected host during the course of viral development refers to mosquito species. Studies by authors such as those mentioned above indicate that the organs of these vectors are infected sequentially as follows. After oral ingestion of virus the primary site of infection is thought to be in the epithelial cells of the midgut. Multiplication of virus then takes place in the cells along the whole of the midgut wall. Fat cells and haemolymph become infected, and secondary foci of infection are established in other organs such as the salivary glands, the brain and other nervous tissue, malpighian tubules, and ovaries. The precise sequence of organ infection varies with the virus and insect species involved. For example, Boorman (1960) found Uganda S (UGS) virus in the haemolymph of Ae.aegypti 10 minutes after oral ingestion of virus by this mosquito,and suggested that virus passed directly through the gut wall without first multiplying in the epithelial cells. Miles et al. (1973) obtained similar results with Whataroa (WHA) virus and Ae.australis mosquitoes. Virus was detected in the haemolymph of 11% of mosquitoes 30 minutes after blood feeding.

There have been no equivalent studies on the sequential stages of arbovirus infections in Culicoides or sandflies. The information concerning mosquitoes has been obtained by dissection and titration of organs, by immunological staining methods such as the immunofluorescence and immunoperoxidase techniques, and by electron microscope studies. The dissection of organs from Culicoides was attempted during this project. The gut, salivary glands, malpighian tubules and ovaries, were dissected out. However the virus content of individual organs could not be accurately determined because during dissections the organs were contaminated with virus contained in the haemolymph. This was difficult to remove since the individual tissues were

too small to manipulate for efficient washing.

In the following section the possibility of using an immunological process to locate virus in different internal organs of the insect, thus overcoming the problems of dissection at this level of size, is considered.

Part 3

Use of the fluorescent antibody test (FAT) for the detection of viral antigen in insect tissue

3.1. Introduction

The methods most commonly used for the detection of arboviruses in insects involve titration of suspensions of insects in assay systems such as cell cultures or suckling mice. Such methods are time-consuming and expensive to perform, especially if experiments require the processing of large numbers of insects, for example in transmission experiments or infection rate studies. In addition there is usually a period of several days or even weeks before the results of such experiments are obtained.

When titration methods are applied to studies of the distribution of virus within the insect the results are limited in accuracy by the size of the insects involved. This is because virus is usually present in the haemolymph and thus contaminates the surface of all tissues and organs during dissection procedures. The washing of individual organs to remove any such surface contaminating virus is difficult when working with insects the size of *Culicoides* or sandflies.

Methods of dissection and titration clearly have disadvantages, and alternative procedures both for the more rapid detection of virus in infected insects, and for a more accurate description of the distribution of virus within the insect, for example by the demonstration of virus in serial sections of insects, would be valuable.

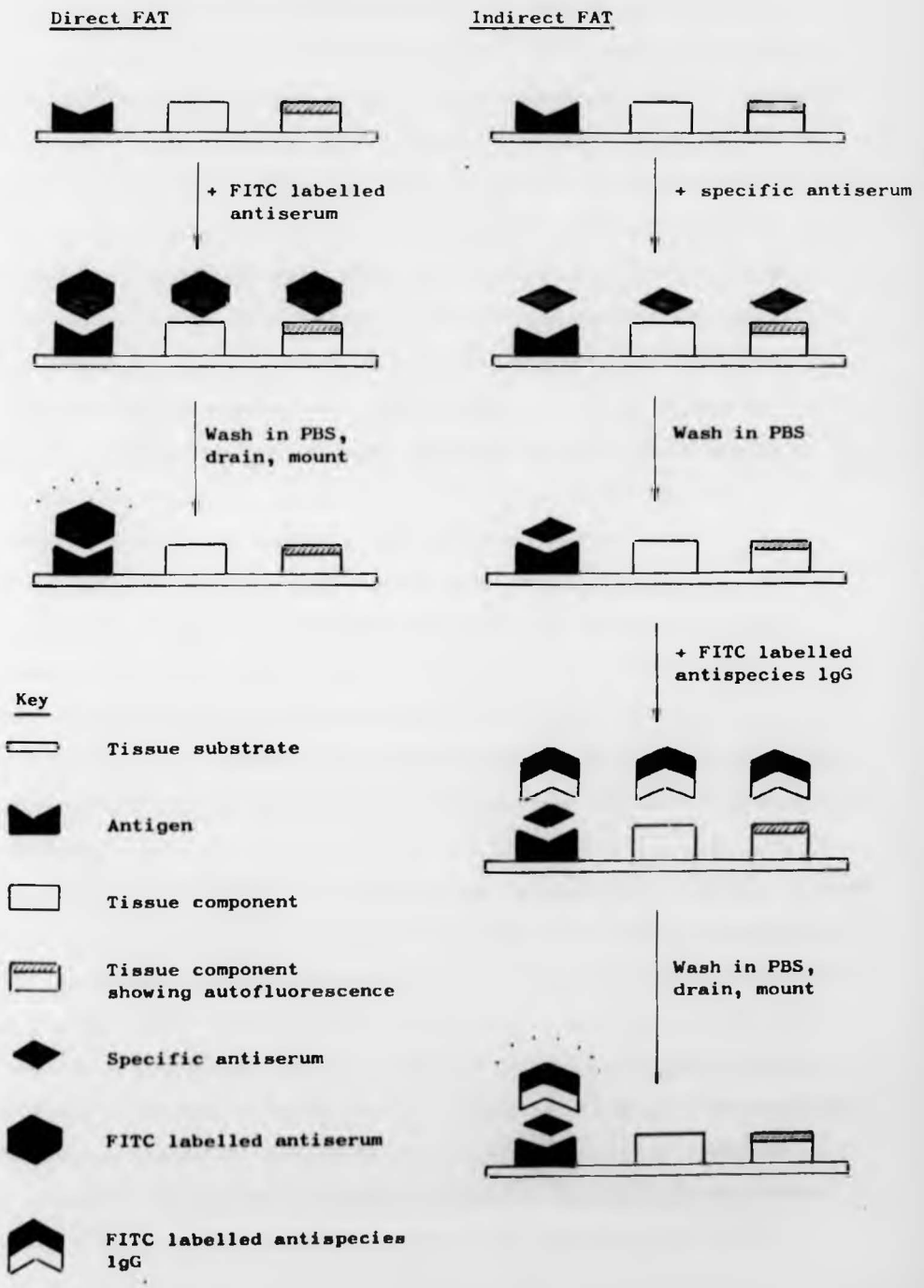
Unfortunately the conventional histological techniques of sectioning and staining developed for light microscopical observation of biological preparations cannot be used to study viruses directly. However modern immunological techniques such as the fluorescent antibody technique (FAT) or the immunoperoxidase technique (IPT) can be used to demonstrate viral antigens in animal and plant tissue. The FAT was first used by Coons *et al.* (1942) to demonstrate pneumococcal antigen in organs taken from infected mice. Since that time the technique has been used to detect

a wide variety of antigens in many different types of cells and tissues. It has been used in the diagnosis of diseases caused by protozoa (e.g. Boonpucknavig and Nairn, 1967), bacteria (e.g. Cherry and Moody, 1965; Deacon et al., 1959), rickettsia (e.g. Walker and Cain, 1978), and helminths such as Schistosoma (Wilson et al., 1977). The technique has also been applied to the tracing of tissue antigens, and of antibodies such as enzymes, hormones, plasma proteins and immunoglobulins, in vertebrate tissue (see Nairn, 1969 for a review).

The IPT is a more recently developed technique (Avrameas, 1971; Kurstak, 1971) which has also been used to demonstrate a variety of antigens in vertebrate tissues (Herrmann et al.,1974; Burns et al., 1974; Jones and Lewert, 1975; Okuno et al., 1977). When this project was begun the reagents necessary for the IPT were carcinogenic and therefore the development of the FAT was preferred.

The principle of the FAT is illustrated in Figure 3.1. The application of a fluorescent "marker", usually fluorescein isothiocyanate (FITC), to the test antigen enables the latter to be visualised under a fluorescence microscope using an ultraviolet or short wave length-blue light source. The success of the test depends primarily upon the specific combination of an antigen with the homologous antibody. The FAT can be carried out in one or two stages i.e. as a direct or indirect test. In the former the antigen is combined directly with FITC conjugated specific antibody. The indirect FAT involves an initial reaction between antigen and homologous antibody. The resultant antigen-antibody complex is then treated with FITC conjugated antispecies antibody. The latter method is considered to be more sensitive and has the advantage that a single FITC conjugated antispecies antibody can be used for many different antigens, provided that the

Figure 3.1. Diagrammatic illustration of the principle of the fluorescent antibody test (FAT)



homologous antisera are all produced in the same animal species.

The experimental conditions required to bring about the optimum specific reaction, and therefore maximum fluorescence, may be different for each antigen-antibody system. These conditions which include the specificity of the antibody and other immunological reagents, the nature of the fixative used, the dilution of reagents, and the time and temperature of the reaction, must therefore be determined for every application before the technique is adopted for routine laboratory use.

Most of the applications of the FAT mentioned above have been concerned with the detection of antigens in vertebrate cells and tissues, or for the screening of vertebrate sera for the presence of specific antibody. These applications usually involve the examination of microscopical preparations of cell monolayers, tissue smears, smears of micro-organisms, and frozen sections. Such preparations require the minimum of histological processing, often limited to fixation only. When used in this way the FAT can be performed quickly and easily and, since the results are obtained immediately, it can save a considerable amount of time when used in place of routine titration methods for the detection of antigen or specific antibody.

The use of the FAT to study antigens in sections of tissues prepared from paraffin wax embedded material has had a more limited application. This is because the reagents employed in the preparation of such sections may cause denaturation of the antigen so that the basic immunological reaction cannot occur. This is possibly the case with viral antigens although few appear to have been thoroughly investigated. Sainte-Marie (1962) was successful in applying the immunofluorescence technique to paraffin wax sections of mammalian tissue. The antigen was bovine serum albumin. 95% ethanol was employed as a fixative and all pre-

embedding stages were conducted at 4°C. This method proved satisfactory for the preservation of diptheria, tetanus toxoids, and influenza A virus antigens. Similarly, Burns et al. (1974) were able to use the FAT to study immunoglobulins in sections of mammalian tissue that had been fixed in formalin and then embedded routinely in paraffin wax. The effect of such procedures on viral antigen was not tested.

Many biological tissues exhibit a degree of autofluorescence when exposed to ultraviolet or blue light. The intensity and colour of this "natural" fluorescence varies with the type of tissue and its preparation. It can be accentuated by paraffin wax embedding procedures. If the FAT is used in combination with such procedures the natural autofluorescence may result in the production of brightly fluorescent artefacts.

The FAT has had limited use in the study of arbovirus infections in insects and other arthropods. Most of the available information refers to the study of viruses in tissue smears or frozen sections prepared from ticks or mosquitoes (see discussion 3.6). The test does not appear to have been used to study virus in smears or sections of Culicoides or sandflies. These insects are much smaller than mosquitoes or ticks, and whereas tissue smears could be easily prepared, the production of frozen sections from Culicoides and sandflies might prove to be more difficult. Sections of these small insects would normally be obtained using the double-embedding technique; specimens are embedded in celloidin and the resultant block is then embedded in paraffin wax. The celloidin embedding provides support for the insect and helps prevent the distortion and fragmentation which may result when cutting sections of such small brittle arthropods after single wax-embedding

techniques. Double-embedding procedures however are time-consuming and require a greater variety of reagents, any of which may have an adverse effect on viral antigenicity.

The research undertaken in this section of the project was intended to establish whether the FAT could be used to study arboviruses, such as BT or PAC, in Culicoides and sandflies. Emphasis was placed on the study of BT virus in C.variipennis, the North American vector of the disease (Foster et al., 1963), since both virus and insects were readily available. The work was divided into three sections as described below.

1. The immunofluorescence test was first applied to tissue smears from BT virus infected C.variipennis to determine whether the basic test could be used to detect the viral antigen in insect tissue. The suitability of the FAT for use in the detection of BT and PAC virus infected sandflies was also assessed.
2. Methods of preparing satisfactory serial sections of Culicoides for possible use in immunofluorescence experiments were investigated. Embedding procedures involving the minimum of histological preparation were used. The morphological preservation of the resultant sections were compared to determine if simple preparative techniques could be used to produce good sections.
3. The possibility of using the FAT to detect viral antigens in sections of infected insects prepared as in 2. above, was assessed by preparing smears of infected tissue and determining the effect of reagents used in routine histological embedding procedures on FATs subsequently performed on these smears. Smears were tested by immunofluorescence after each treatment to determine which reagents, if any, destroyed viral antigenicity.

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3.2 Use of the fluorescent antibody test to detect Bluetongue viral antigen in tissue smears prepared from laboratory infected C.variipennis

3.2.1. Materials

3.2.1.1. Virus

The virus used was BT type 4, strain A/SOT 1 (see section 2.2.2.). The stock virus was stored in suspension in EMM. BHK 21 cell passage levels 9,12, and 13, were used in these experiments.

3.2.1.2. Insects

C.variipennis adults were obtained from the colony at A.V.R.I. Pirbright (Boorman, 1974) and were maintained in the laboratory as described in section 2.2.1. During development of the test it was necessary to ensure that all females were infected with BT virus. Oral ingestion of this virus by C.variipennis females from the Pirbright colony results in a maximum infection rate of only 35% (Dr.P.Mellor personal communication , 1977). Insects were therefore infected by intrathoracic inoculation which produced final infection rates of 96 to 100%.

In all initial experiments inoculated flies were held at 25°C for between 7 and 9 days. By this time the virus had multiplied to the maximum concentration expected (5.0 to 5.9 $\log_{10}TCID_{50}$/insect) and was distributed throughout the body of the fly (see distribution experiments in part 2.3.9.). Insects were killed by freezing at -70°C, and were then stored at this temperature. Control insects were inoculated with EMM only and were killed 7 to 9 days post inoculation by freezing at -70°C. Additional control insects were obtained from the colony and were stored untreated at the same temperature.

3.2.1.3. Production of antiserum and conjugates

The specificity of the antibody used in the FAT is of greater importance than the antibody concentration. A short course of immunisation with purified antigen usually produces the most suitable specific antibody (Nairn, 1969). Previous work in the Entomology department at A.V.R.I. had indicated that antiserum to BT virus of a sufficiently high concentration for use in serum neutralisation tests was difficult to produce in rabbits or sheep. In case similar problems were encountered when producing antiserum suitable for immunofluorescence work several batches of antiserum were initially prepared from several animals. Rabbits were used as these were the most conveniently available laboratory animals.

Four rabbits were immunised. The schedule for the production of antiserum was as follows.

One ml of an emulsion containing equal volumes of BT virus and Freund's complete adjuvant was injected intramuscularly into each hind leg. Twenty-one days later each rabbit was given a subcutaneous inoculation of 1ml of BT virus alone. Two further subcutaneous injections were given at 14 day intervals. Two of the rabbits, R20 and R23, were treated throughout with partially purified virus (6.9 $\log_{10}TCID_{50}$/ml). The remaining two animals, R22 and R24, were inoculated with unpurified stock virus (BHK 21 cell passage 9, 7.3 $\log_{10}TCID_{50}$/ml). The details of the virus purification procedure and the rationale for using such virus is described in section 3.2.5.1. The rabbits were bled from the ear 10 days after the last injection of virus. A sufficient volume of blood, i.e. approximately 40ml, was collected from each rabbit to provide enough antiserum to last throughout the project should it prove satisfactory for use in the FAT.

The four batches of antiserum obtained had neutralising end-point titres of between 1:256 and 1:2000. All four batches gave satisfactory

results in preliminary screening experiments to assess their suitability for use in immunofluorescence tests. A comparison of the results obtained with antisera produced using partially purified and unpurified virus for immunisation is made later in section 3.2.5.1. The 1:2000 antiserum, from R20, gave the most consistent results with the brightest fluorescence. This was therefore used in all further routine tests. The best results were obtained using the R20 antiserum at a dilution of 1:20 in PBS. The pH of the PBS used throughout these experiments was 7.0.

Normal rabbit serum for use as a control was prepared after bleeding a naieve rabbit.

Attempts to conjugate BT antiserum with FITC (Becton Dickinson U.K. Ltd., Runcorn) were unsuccessful. An indirect FAT was therefore used in all experiments employing a commercially prepared FITC conjugated goat anti-rabbit immunoglobulin (1gG) (Miles Laboratories, Slough)at a standard dilution of 1:40 in PBS.

The antisera and the FITC conjugate were stored in 0.5 ml aliquots at -20°C, unless in daily use when both reagents were stored at 4°C to avoid frequent freezing and thawing. The conjugate was stored for 2 months at 4°C without adversly affecting the FAT results. Antiserum could be stored at this temperature for at least 2 weeks. Longer storage times at 4°C were not tested. Neither the antiserum nor the conjugate were affected by 3 years storage at -20°C.

3.2.2. Experimental Procedure

3.2.2.1. Preparation of slides and tissue smears

Smears of insect tissues were made on clear spots on 76 x 26 mm Polytetrafluoroethylene (PTFE) coated microscope slides (Figure 3.2.). The slides were first prepared by placing drops of glycerol in rows on the cleaned surface of the glass. Three coats of Klingerflon spray

(Richard Klinger Ltd., Sidcup,Kent) were applied, allowing each coat time to dry before applying the next. After the final application the glycerol was washed off with hot water and the slides were dried and polished.

Smears were prepared separately from the head, thorax and the abdomen. Insects were dissected under a binocular microscope using "Hagedorn" type optical surgical needles mounted in narrow glass tubing (Figure 3.2.). First the head was cut off and squashed on one spot on a slide, either by using the flat surface of a dissecting needle, or by applying pressure over a PTFE - coated coverslip placed on top of the head. The thorax and abdomen were divided and separate smears were made from each, either by dabbing the cut ends onto the slide, or by squashing each part with the needles. The dissecting needles were changed after each operation to prevent cross contamination of samples.

To enable unbiased observations to be made smears from infected and uninfected insects were prepared on each slide. (Between 2 and 4 infected, and 1 and 4 uninfected per slide). The slides were coded and mixed at random before examination.

3.2.2.2. Staining Procedure

The procedure described below was developed during the course of the experiments and was adopted for routine use. Variations in this procedure which were used in individual experiments will be discussed in the results section. These include variation of the timing and temperature of each stage of the FAT, and of the dilutions of reagents.

Tissue smears were prepared as described and dried at room temperature for 30 minutes.

They were fixed in acetone at $4^{\circ}C$ by immersion of the slides in cold acetone in Coplin jars. Smears were routinely fixed for 3 to 5 minutes, although periods of up to 20 minutes could be used without

affecting the subsequent results.

After fixation the acetone was allowed to evaporate. The experiments detailed in section 3.2.6. showed that fixed smears could be stored at $4^{o}C$ for several days before staining.

The slides were arranged, smears uppermost, on PTFE coated glass rods mounted in a glass container (Figure 3.3.). The rods lifted the slides off the bottom of the container so that they were easier to handle and the tracking of fluids from one slide to another was prevented.

A drop of diluted antiserum was placed on each smear. Disposable 1 ml glass pipettes were used to dispense both the antiserum and conjugate. These gave better volume control than either pasteur pipettes or wire loops. Two slides were treated with normal serum as an additional control. A pad of damp cotton wool was placed in the glass container, and the lid was put on to maintain the humidity. The slides were incubated with the antiserum for 1 hour at $37^{o}C$.

After this time the slides were removed from the container and were suspended in PBS in a perspex bath set on a magnetic stirrer (Figure 3.4.). They were given 3 moderately vigorous 5 minute washes in 3 changes of PBS, to remove all unbound antiserum. They were then drained and blotted to remove excess PBS, and were returned to the humidified glass container.

A drop of diluted conjugate was added to each smear and the slides were incubated for 1 hour at room temperature. Excess conjugate was then removed with a 10 minute wash of the slides in the PBS bath. The slides were then rinsed in distilled water, drained, and wiped of excess moisture. They were mounted using cleaned glass microscope coverslips with a 90% solution of glycerol in PBS as a mountant.

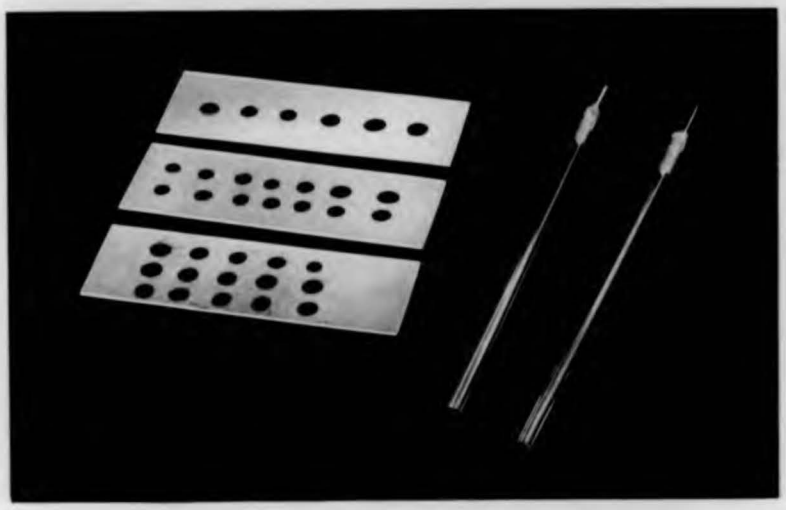

Figure 3.2. PTFE-coated slides and dissecting needles

Figure 3.3. Incubation tray

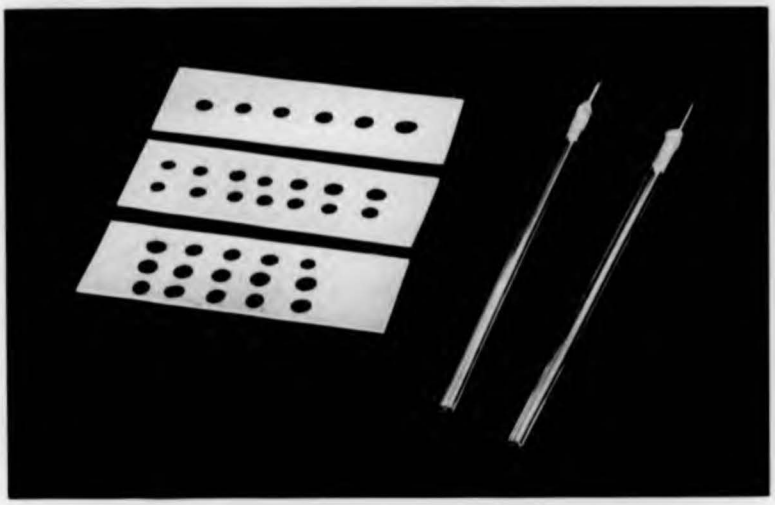

Figure 3.2. PTFE-coated slides and dissecting needles

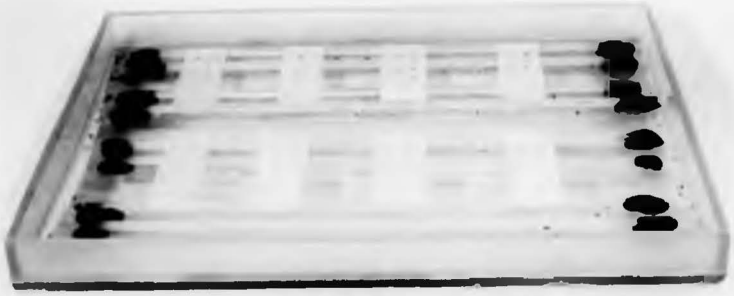

Figure 3.3. Incubation tray

Figure 3.4. Magnetic stirrer with perspex bath and slides

Figure 3.4. Magnetic stirrer with perspex bath and slides

3.2.2.3. Microscopy

Smears were examined with a Leitz orthoplan microscope, using either incident or transmitted light. The incident light source was provided by a 50W HBO high pressure mercury lamp. A BG 12 3mm filter was inserted in the lamp housing and selective excitation was provided by a built in 2 x KP490 filter. Transmitted light was provided by a 200W HBO super pressure mercury lamp. A 1mm UG1 transmission filter was used in combination with a BG 38 red suppression filter and a K460 barrier filter. Slides were examined within one day of staining. The initial examination was made using a x 10 eyepiece and a x 10 objective. Good positive smears were easily detected at this magnification. A higher magnification (x16 and x 40 objective) was sometimes employed to distinguish a less obviously positive sample.

Each batch of slides was usually examined once, then recoded and examined a second and third time. Occasional batches were assessed by a totally impartial observer. The microscopy could be tiring to the eyes and therefore the number of slides to be examined in one session was limited to 15, approximately 100 smears. The incident light illumination caused least eye strain and was therefore used in nearly all experiments.

3.2.2.4. Photography

Photographs were taken on Kodak high speed Ektachrome film using a Leitz automatic combiphot system camera. The incident light source was used for all photography. Initially exposure times were estimated to be between 2 and 5 minutes. An exposure of 3 minutes was later used as a standard and gave good results with all samples.

3.2.3. Results

Using the routine procedure described above, specific fluorescence was observed in all tissue smears prepared from BT virus infected

C.variipennis between 7 and 9 days after inoculation of virus. There was some background staining in all smears but the fluorescence of positive samples was granular in appearance and much brighter than the controls. Positive smears from the head, thorax and abdomen respectively are illustrated in Figures 3.5, 3.7 and 3.9. Uninfected controls from each body section are shown in Figures 3.6, 3.8 and 3.10.

Tissue fragments that had adhered to the slide during processing were ignored as they stained very brightly in all the samples from both infected and uninfected insects. The fragments of malpighian tubule and the lenses from the compound eye, which appear in Figures 3.10 and 3.11 respectively, illustrate this. No specific fluorescence was observed in infected smears treated with normal rabbit serum. Such samples were similar in appearance to those shown in Figures 3.6, 3.8 and 3.10.

Smears from the head were easiest to prepare to a consistent standard of thickness and were thus easiest to assess when stained. All further experiments, with the exception of 3.2.4. below, were therefore carried out on smears of infected head tissue.

3.2.4. Sensitivity of the technique

The sensitivity of the FAT was examined by comparison of a parallel series of infectivity titrations in BHK 21 cells, and immunofluorescence tests on pools of separated heads, thoraces and abdomens, of insects killed and dissected at daily intervals after virus inoculation. The methods for the dissection and titration of insects are described in section 2.2.7. The results of this parallel series of experiments are shown in Table 3.1. The specific fluorescence observed in the tissue smears was measured on an arbitrary scale of brightness from + (just distinguishable from a control smear) to +++ (very bright). Virus

Figure 3.5 FAT: C.variipennis head smear, Bluetongue virus infected tissue, 4 min. exposure, x 720

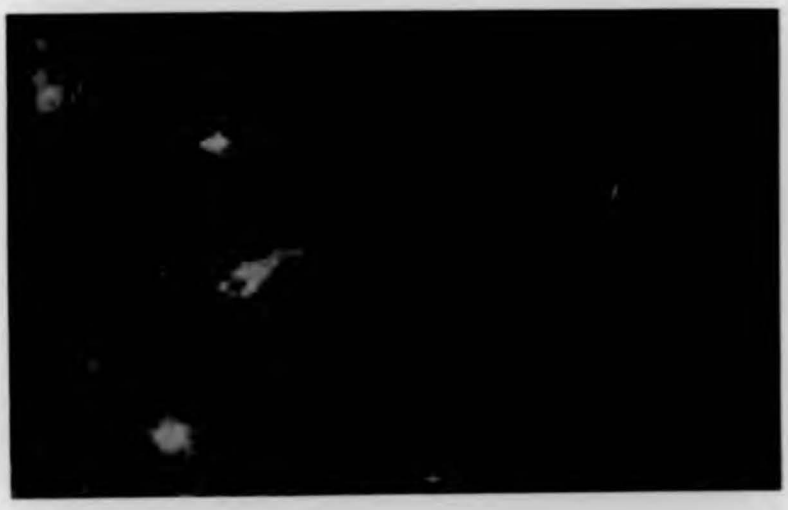

Figure 3.6 FAT: C.variipennis head smear, uninfected control, 4 min exposure, x 720

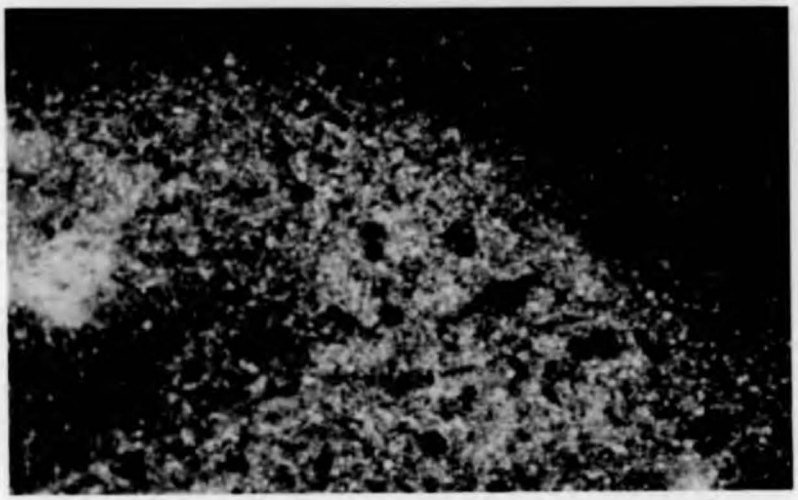

Figure 3.5 FAT: C.variipennis head smear, Bluetongue virus infected tissue, 4 min. exposure, x 720

Figure 3.6 FAT: C.variipennis head smear, uninfected control, 4 min exposure, x 720

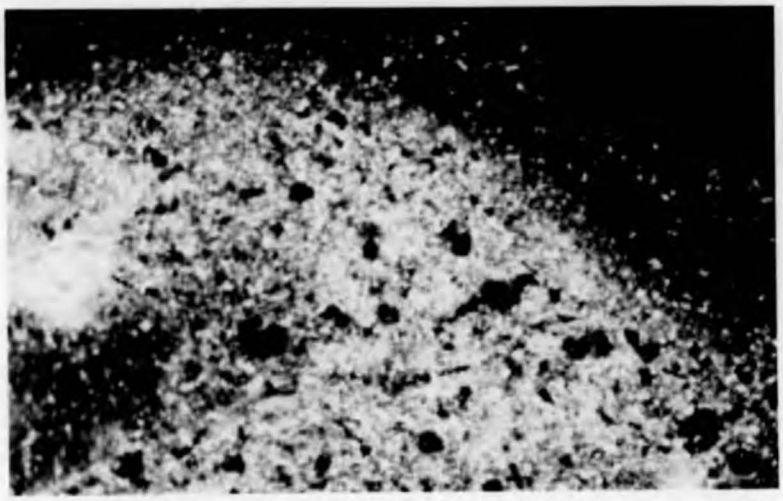

Figure 3.5 FAT: C.variipennis head smear, Bluetongue virus infected tissue, 4 min. exposure, x 720

Figure 3.6 FAT: C.variipennis head smear, uninfected control, 4 min exposure, x 720

Figure 3.7 FAT: C.variipennis thorax smear, Bluetongue virus infected tissue 3 min. exposure, x 720

Figure 3.8 FAT: C.variipennis thorax smear, uninfected control, 4 min. exposure, x 720

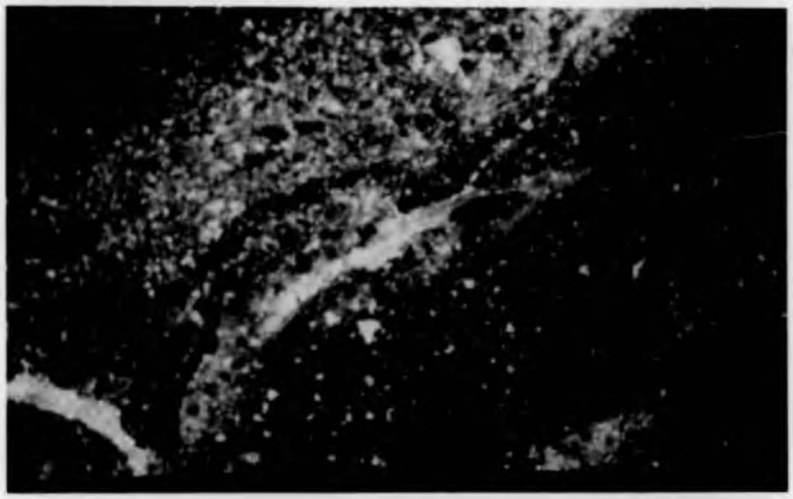

Figure 3.7 FAT: C.variipennis thorax smear, Bluetongue virus infected tissue 3 min. exposure, x 720

Figure 3.8 FAT: C.variipennis thorax smear, uninfected control, 4 min. exposure, x 720

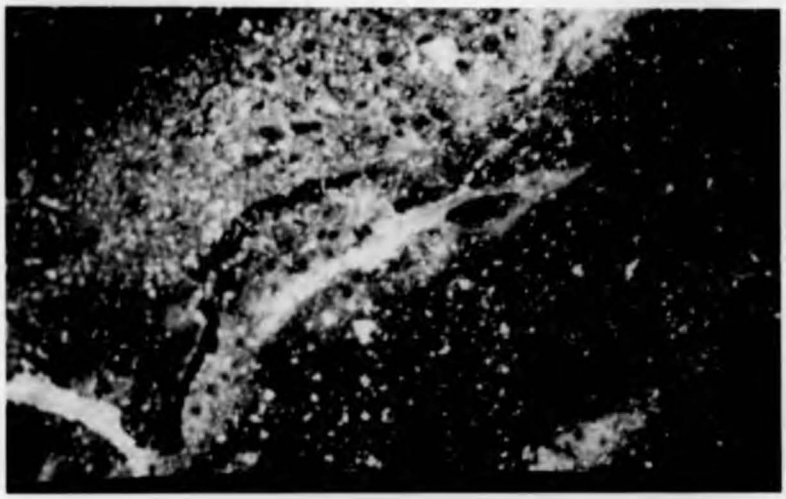

Figure 3.7 FAT: C.variipennis thorax smear, Bluetongue virus infected tissue 3 min. exposure, x 720

Figure 3.8 FAT: C.variipennis thorax smear, uninfected control, 4 min. exposure, x 720

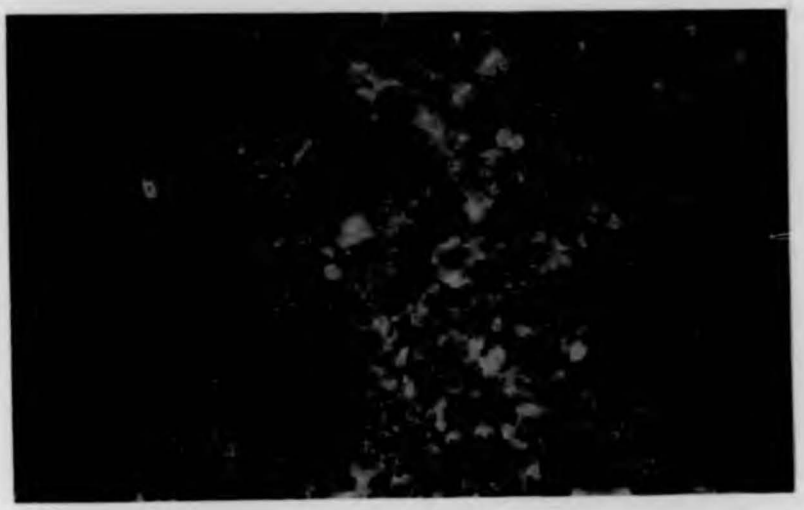

Figure 3.9 FAT: C.variipennis abdomen smear, Bluetongue virus infected tissue, 3.5 min. exposure, x 720

Figure 3.10 FAT: C.variipennis abdomen smear, uninfected control, 3.5 min. exposure, x 720

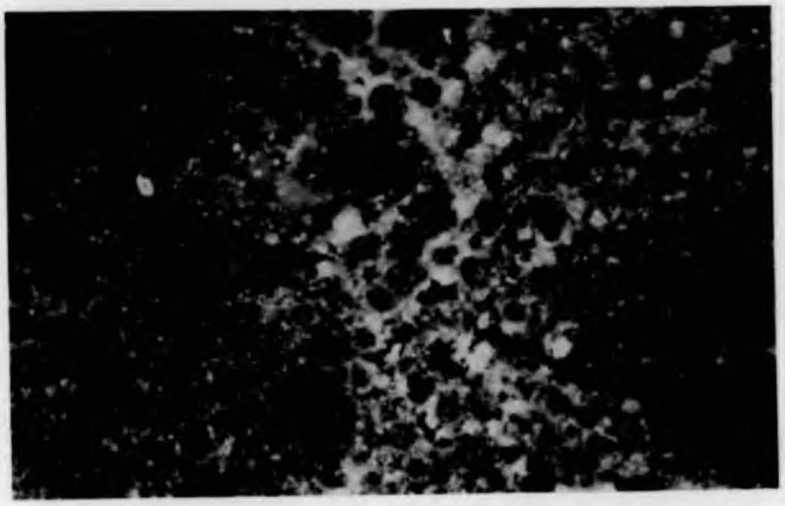

Figure 3.9 FAT: C.variipennis abdomen smear, Bluetongue virus infected tissue, 3.5 min. exposure, x 720

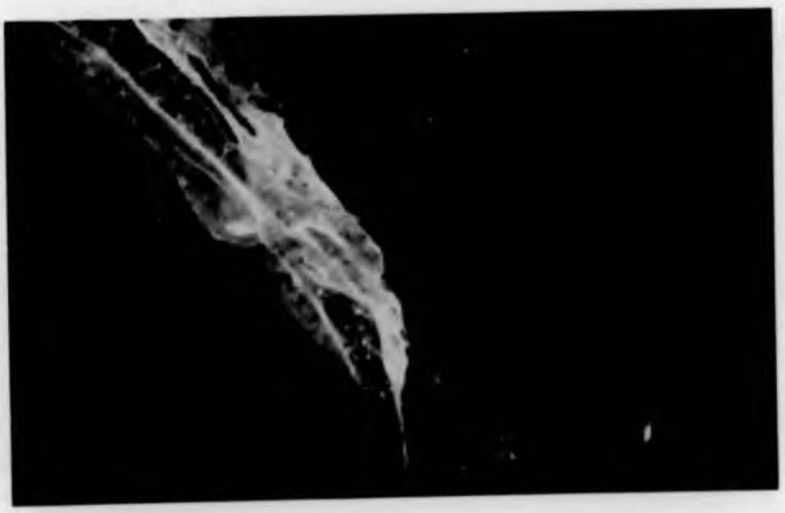

Figure 3.10 FAT: C.variipennis abdomen smear, uninfected control, 3.5 min. exposure, x 720

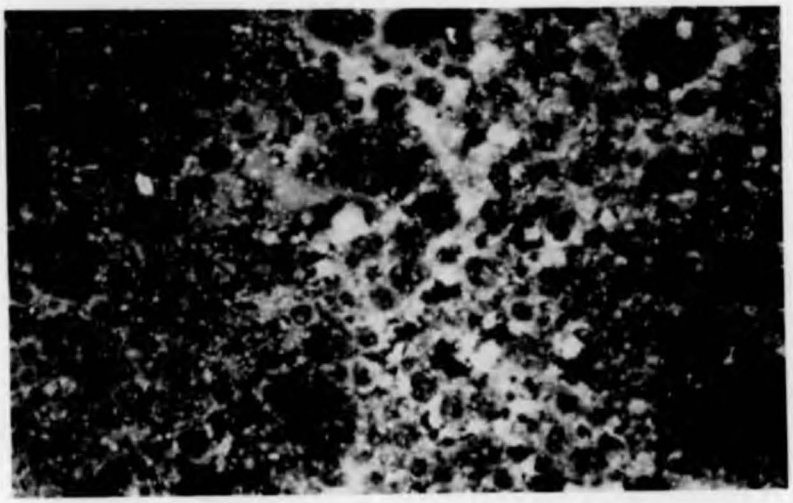

Figure 3.9 FAT: C.variipennis abdomen smear, Bluetongue virus infected tissue, 3.5 min. exposure,x 720

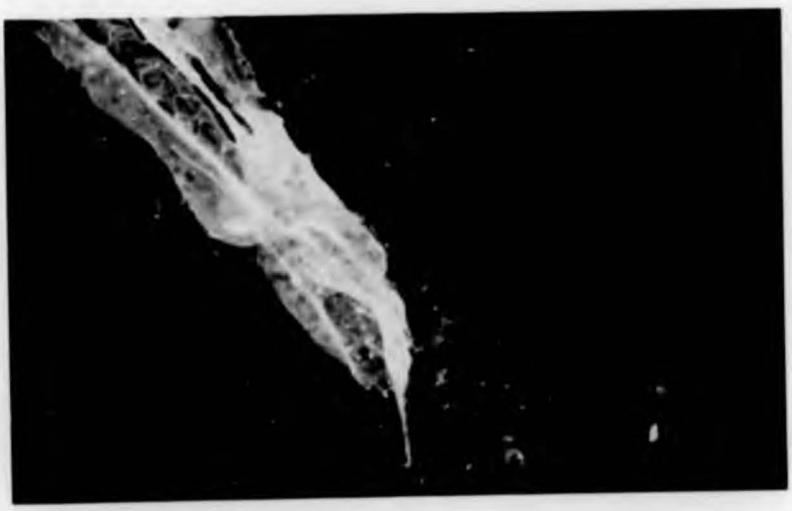

Figure 3.10 FAT: C.variipennis abdomen smear, uninfected control, 3.5 min. exposure,x 720

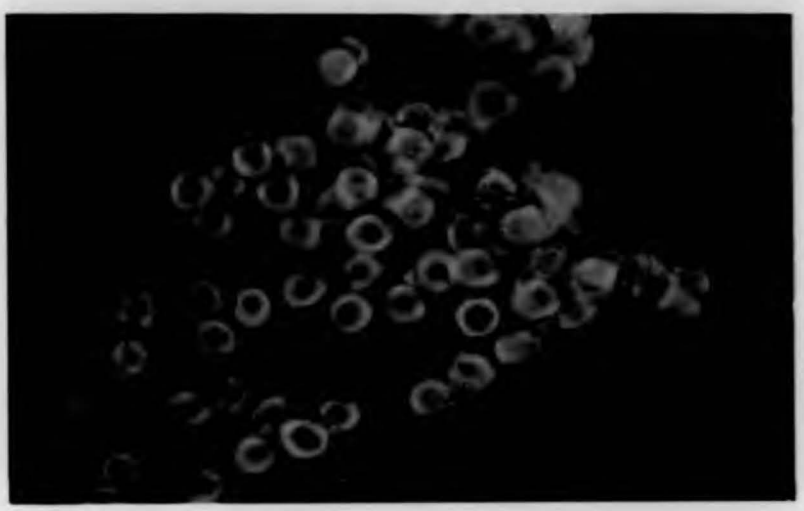

Figure 3.11 FAT: C.variipennis head smear, uninfected control showing lenses of the eye as an artefact
1.5 min. exposure, x 720

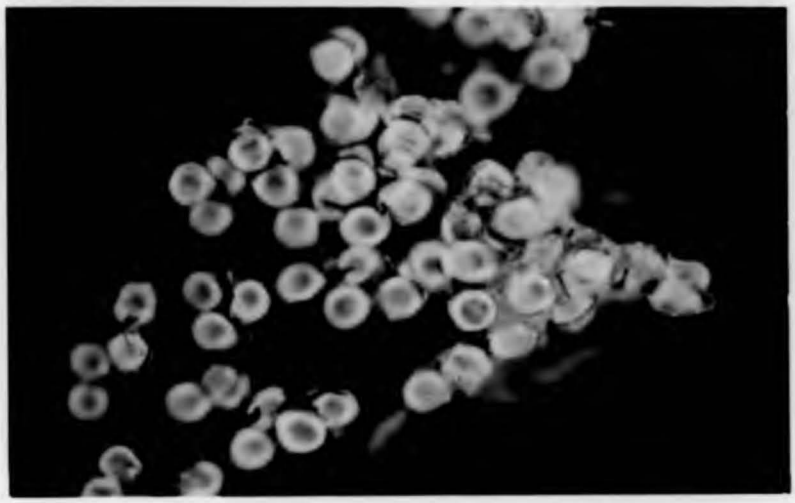

Figure 3.11 FAT: C.variipennis head smear, uninfected control showing lenses of the eye as an artefact
1.5 min. exposure, x 720

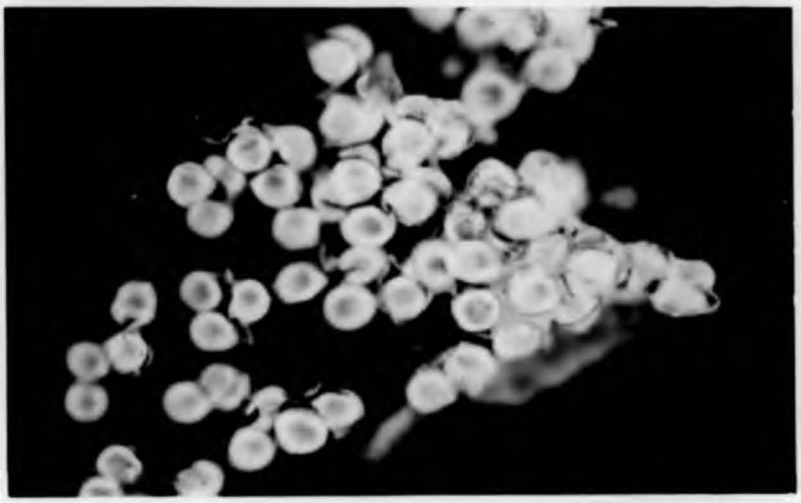

Figure 3.11 FAT: C.variipennis head smear, uninfected control showing lenses of the eye as an artefact
1.5 min. exposure, x 720

Table 3.1. Results of BHK cell titrations and immunofluorescence tests on C.variipennis after inoculation of Bluetongue virus

Days post-inoculation		0	1	2	3	4	5	6	7	8	9
BHK cell titration	Head	1.0	1.2	1.5	1.5	3.1	3.2	4.0	4.1	4.8	5.0
($\log_{10}TCID_{50}$ per head,	Thorax	2.8	2.1	2.9	4.3	4.3	4.5	4.6	4.2	5.7	5.5
thorax or abdomen)*	Abdomen	3.0	2.0	3.2	3.3	3.9	4.6	4.6	4.8	5.2	5.1
Fluorescence test	Head	1/11	8/11	6/10	5/11	8/11	10/10	10/10	24/24	25/25	10/10
(Number detected positive / Number tested)	Thorax	4/11	8/11	7/10	7/11	11/11	10/10	10/10	22/22	25/25	10/10
	Abdomen	3/6	3/5	2/6	0/5	5/6	2/2	5/5	5/5	22/22	5/5
Brightness of smears		+	+	+	+	++	++	++	+++	+++	+++

* Between 4 and 16 insects titrated per day

present in the insect on day 0, and possibly on day 1, was presumed to be from the inoculum. Multiplication of BT virus took place after day 1 and continued until day 9 when the experiment was terminated. From day 5 high concentrations of virus (3.2 to 5.7 $\log_{10}$ $TCID_{50}$/body part) were present throughout the insect. Specific fluorescence was detected in all infected smears prepared from insects at least 5 days after inoculation of virus. Only a proportion of infected flies were detected before day 5 post inoculation. The fluorescence was brightest in the latest samples.

3.2.5 Variations in experimental procedure used in attempts to improve the contrast in brightness between infected and uninfected tissue smears

The contrast in appearance between infected and uninfected tissue smears was good, provided the infected smears were prepared from insects with a virus content of more than 4.0 $\log_{10}TCID_{50}$/fly. The control smears never had the granular appearance of infected samples. However, they could appear quite bright, and this could sometimes be confusing when they were compared with infected smears prepared from insects containing only small amounts of virus. With practice such samples could be differentiated.

A series of experiments was performed to determine the effect of various procedures on the non-specific background fluorescence. These experiments are listed below.

1. Comparison of the effect of using unpurified and partially purified virus antigen for the production of antiserum.
2. Use of the IgG fraction of the whole BT antiserum in the first stage of the test.
3. Absorption of the BT antiserum with Culicoides tissue powders.
4. Dilution of reagents.

5. Variation of the times and temperature of the reaction.

3.2.5.1. Comparison of the effect of using unpurified and partially purified virus stock for the production of antiserum

Antiserum produced by immunisation of animals with virus grown in cell culture will have a wider range of specificity than antiserum produced by immunisation of animals with purified antigen. This is because animals react immunologically to the cell proteins as well as to the virus. Use of such antiserum in immunofluorescence tests may result in a certain amount of background fluorescence caused by reactions between non-specific serum proteins and the tissue substrate. Attempts were therefore made to purify the BT virus used in the production of the specific antiserum. The methods used are described in appendix 3.

The virus could only be partially purified. Similar unsatisfactory results have been obtained by other workers using BT virus (Dr.J.Newman, personal communication,1979). The virus appears to be difficult to purify because of its strong affinity for cell membranes and its instability in purified form (Verwoerd, 1969). Verwoerd (1969) and Verwoerd et al. (1972) report some success with the purification of BT types 10 and 3 using a Fluorocarbon extraction technique, however the yields of virus were low. Similar results have been obtained with type 10 by Martin and Zweerink (1972). This part of the project is now being investigated further in the Biochemistry department at A.V.R.I.

The partially purified virus was used to immunise rabbits R20 and R23. Rabbits R22 and R24 were immunised with unpurified virus. The four separate batches of antiserum produced were compared in several immunofluorescence tests. The brightest specific fluorescence was obtained using the R20 antiserum (partially purified virus). However there was no apparent difference in the brightness and specificity of

the fluorescence observed using antiserum from R23, the second rabbit immunised with partially purified virus, and antisera from rabbits R22 and R24 which were both treated with unpurified virus. It was therefore assumed that the use of the partially purified BT virus for the production of specific antiserum had had no absolute beneficial effect on the suitability of the antiserum for use in the FAT.

3.2.5.2. Use of IgG

IgG was prepared from the R20 antiserum by the caprilic acid separation method (Steinbuch and Audran, 1969) described in appendix 4. The IgG was used in place of the whole antiserum to react with the virus antigen in the first stage of the test. The nature of the background fluorescence was not affected and the results were less consistent than when the whole antiserum was used.

3.2.5.3. Absorption of antiserum

Non-specific fluorescence can be caused by reactions between homologous substances in the specific antiserum and in the tissue preparation (Nairn,1969). Absorption of the antiserum with the appropriate tissue powder or homogenate, before its use in immunofluorescence tests, should diminish any background fluorescence caused by such reactions. The tissue preparations used in these experiments were from Culicoides. Two batches of antiserum (from R20 and R23) were therefore absorbed with tissue powders prepared from Culicoides as described in appendix 5. No improvement in the degree of background fluorescence was observed when the absorbed antiserum was substituted for the whole antiserum in the first stage of the FAT.

3.2.5.4. Dilution of reagents

Dilution of antiserum, and or conjugate, may reduce non-specific protein interactions and hence the non-specific background fluorescence.

A series of dilutions of the R20 antiserum and of the commercial FITC conjugated IgG were prepared. A series of FATs were then performed in each of which a different dilution was substituted for the standard routine dilution of the antiserum or conjugate. Dilutions of from 1:10 to 1:1000 were tested. The results are shown in Table 3.2. In this table the brightness of the fluorescence in the smears is represented by the graded scale from <+ to ++++. These symbols do not refer to positive samples but are used to indicate the contrast in brightness between infected and uninfected tissue smears.

No improvement in contrast between infected and uninfected samples was observed when either the antiserum, or the conjugate, or both, were diluted to 1:100. Infected flies could however still be distinguished at these dilutions although greater care was needed in assessing the slides. Results were also just satisfactory using the conjugate at a dilution of 1:1000.

Combinations of the lower dilutions of reagents, i.e. 1:10 antiserum and 1:10 conjugate, did not give good results. Both infected and uninfected smears appeared very bright green using the 1:10 dilution of reagents, and this brightness masked the granular appearance of infected samples. The dilutions of antiserum and conjugate of 1:20 and 1:40 respectively gave the most consistent results which were most easily assessed. These dilutions were also economical of reagents.

3.2.5.5. Variation of times and temperature of the reaction

A series of experiments on the effect of varying the times and temperature of the two main stages of the technique (i.e. incubation of smears plus antiserum, and of smears plus conjugate) was carried out. In each experiment the temperature and time allowed for absorption

Table 3.2. Effect of varying dilution of reagents on contrast in brightness of samples

Antiserum dilution	Conjugate dilution	Contrast of smears	
		Infected	Uninfected
1:10	1:10	+ + + +	+ + +
	1:40	+ + +	+ +
	1:100	+ +	+
	1:1000	+	<+
1:20	1:10	+ + + +	+ + +
	1:40	+ + +	+
	1:100	+ +	<+
	1:1000	+	<+
1:100	1:10	+ +	<+
	1:40	+ +	<+
	1:100	+	<+
	1:1000	+	<+

of one of the reagents was the same as for the routine procedure described in section 3.2.2.2. The results are represented in Table 3.3.

No improvement in the test was apparent with any of these variations. The most reliable results were obtained using the standard procedure of incubation of antiserum plus smears for 1 hour at 37°C, and of conjugate plus smears for 1 hour at room temperature. However infected and uninfected samples could be distinguished when the antiserum and smears were incubated for as little as 10 minutes at 37°C, or when incubated at 4°C overnight. Similarly, infected samples could be detected after incubation of the conjugate and smears for 5 minutes at room temperature, or at 4°C overnight.

3.2.6. Storage of smears

Large batches of smears, i.e. from more than 100 insects, could not be prepared and stained on the same day. The length of time that smears could be stored between fixation and immunofluorescence testing was therefore investigated. The results are shown in Table 3.4.

A batch of 20 slides was prepared and the smears were fixed in acetone at 4°C. A fluorescence test was performed immediately on 2 of the slides. The remainder were stored unstained at 4°C. Two sample slides were removed and assessed by immunofluorescence after 24 hours. Two more slides were each stained at weekly intervals until all 20 had been used, 8 weeks after their original preparation.

Good results were obtained in all fluorescence tests on fixed smears stored at 4°C for between 0 and 8 weeks. There was no obvious decrease in the brightness of the specific fluorescence obtained in the smears over this period.

Good results were also obtained when unfixed smears were stored at 4°C for at least 7 days before fixation and staining. These were the maximum storage times tested. After 7 days of storage of fixed smears

Table 3.3 Effect of varying the times and temperature of the antiserum and conjugation incubation stages of the fluorescent antibody test on the contrast in brightness of infected and uninfected smears

Antiserum incubation		Conjugate incubation		Contrast of smears	
Temperature	Time	Temperature	Time	Infected	Uninfected
37°C	10 min.	20°C	60 min.	+ +	+
	20 min.			+ +	+
	30 min.			+ +	+
	40 min.			+ + +	+
	60 min.			+ + +	+
	90 min			+ + +	+
4°C	24 hr.	20°C	60 min.	+ +	+
37°C	60 min.	20°C	5 min.	+	<+
			10 min.	+	<+
			15 min.	+	<+
			30 min.	+ +	+
			60 min.	+ + +	+
			90 min.	+ + +	+
37°C	60 min.	4°C	24 hr.	+ +	+

Table 3.4. Effect of storage of smears on the brightness of specific fluorescence obtained in subsequent fluorescent antibody tests

Pre-storage treatment	Storage temperature	Storage time (days)	Contrast in brightness of smears	
			Infected	Uninfected
Acetone fixation at 4°C	4°C	0	+ + +	+
		1	+ + +	+
		7	+ + +	+
		14	+ + +	+
		21	+ + +	+
		28	+ + +	+
		56	+ + +	+
Acetone fixation at 4°C	-20°C	0	+ +	+
		7	+	+
		22	+	+
Unfixed	4°C	7	+ +	<+

at -20°C positive samples were just detectable by the FAT. However, after 22 days storage at this temperature no specific fluorescence was observed. Howell et al. (1967) report that BT virus is unstable at -20°C. Inactivation of the virus may have occurred during the storage period.

3.2.7. Persistence of fluorescence

The fluorescence of FITC conjugates fades with time. The degree of fading varies with the type of tissue preparation, the mounting conditions for example the pH of the mounting medium, and the nature of the conjugate. It is thus usually considered advisable to examine samples within 2 days of preparation.

To determine the length of time specific fluorescence was detectable in BT virus infected C.variipennis tissue smears, the FAT was routinely performed on a set of 6 prepared slides. These were examined immediately after staining and then at weekly intervals for 17 weeks. During this time the slides were stored at 4°C in the dark. They were recoded and randomised after each examination to prevent memorisation of the position of positive samples. The results are summarised in Table 3.5.

It was found that positive samples could still be accurately detected after 119 days (17 weeks) storage at 4°C in the dark. By this time the brightness of the fluorescence was estimated to have decreased by about 33%. Prolonged exposure to microscope illumination, i.e. during photography of slides, increased the speed of fading of the fluorescence.

3.2.8. Storage of insects in hederol

During field collections of insects for virus isolation tests they are sometimes stored for several days in solutions of between 0.1% and 1% detergent in buffered saline. The detergent acts as a wetting agent.

Table 3.5. Brightness of fluorescence in stained smears examined at intervals after the fluorescent antibody test

Time post FAT (days)	Contrast in brightness of smears	
	Infected	Uninfected
0	+ + +	+
4	+ + +	+
7	+ + +	+
22	+ + +	+
28	+ +	< +
36	+ +	< +
39	+ +	< +
119	+ +	< +

The effect of such storage on the FAT was investigated.

Bluetongue virus infected C.variipennis and control uninfected insects were placed separately in 1% detergent (hederol) in PBS. They were stored in this way for 1.5 hours, 24 hours and 6 days at room temperature. After each time interval 10 insects were removed from both the infected and uninfected pools. They were rinsed separately several times in PBS; 4 insects were titrated in BHK 21 cells to check the infectivity, and the remainder were tested by a routine FAT.

Specific fluorescence was observed in smears prepared from infected insects both after 1.5 and 24 hours storage in 1% hederol. No fluorescence was observed after 6 days storage in 1% hederol, however at this time only low concentrations of virus below the threshold level of the FAT were detected by titration of insects in BHK 21 cells.

3.2.9. Immunofluorescence tests on dissected organs

Gut tissues, salivary glands, malpighian tubules and ovaries, were dissected from infected and uninfected C.variipennis. The indirect FAT was conducted on whole organs or on smears prepared on microscope slides by teasing out the tissues with fine needles.

No conclusive results with respect to the virus content of the separate organs was obtained from these experiments. The smears were contaminated with virus contained in the haemolymph, and the whole or squashed organ preparations were rather thick. Both infected and uninfected samples therefore stained very brightly, masking any specific fluorescence.

• • •

3.3. Application of the indirect fluorescent antibody test to other insect-virus combinations

The suitability of the indirect FAT for detecting viral antigen in tissue smears from L.longipalpis sandflies was investigated.

3.3.1. Fluorescent antibody test on tissue smears prepared from Bluetongue virus infected L.longipalpis

3.3.1.1. Materials and methods

Virus

The virus used in these experiments was BT type 4, strain A/SOT 1 (see section 2.2.2.).

Insects

L.longipalpis females obtained from the colony at Imperial College Field Station were inoculated with virus as described in section 2.2.3.2. Sandflies were incubated at 25°C for 8 days and were then frozen at -70°C prior to processing.

Antiserum

The production of BT antiserum was described in section 3.2.1.3. The R20 BT antiserum was used in these experiments. The conjugate was the commercial FITC conjugated swine anti-rabbit IgG described previously in section 3.2.1.3.

Experimental procedure

Smears of L.longipalpis tissue were prepared from the head, thorax and abdomen of infected insects in exactly the same way as described for C.variipennis in section 3.2.2.1. Smears from uninfected L.longipalpis were made on each slide to provide negative controls. Positive control slides were prepared using BT virus infected and uninfected C.variipennis. All experimental procedures were as described in section 3.2.2. Three dilutions of the antiserum were used, 1:10, 1:20 and 1:100. Three slides of L.longipalpis smears and

two slides of C.variipennis smears were treated with each antiserum dilution. One slide from each group was subsequently treated with the FITC conjugate at a dilution of 1:16. The remaining slides from each group were treated with a 1:40 dilution of the conjugate.

Smears were examined using an incident light source, with a BG38 3mm filter and a 2 x KP 490 built in filter, as described in section 3.2.2.3.

3.3.1.2. Results

Smears prepared from L.longipalpis were thicker than those prepared from C.variipennis. The overall background non-specific fluorescence was therefore brighter and slides were not so easily assessed. Ten slides were prepared, each with 2 to 4 infected, and 2 to 4 uninfected tissue smears per slide. On only one slide was specific fluorescence obvious in the infected smears. The dilution of the antiserum and conjugate on this slide was 1:10 and 1:16 respectively.

All the infected and uninfected smears prepared from C.variipennis on the positive control slides were assessed accurately.

3.3.2. Fluorescent antibody test on tissue smears prepared from Pacui virus infected L.longipalpis

3.3.2.1. Materials and methods

Insects and Virus

The virus, insects and methods of infection are as described in section 2.2. L.longipalpis adults were inoculated with PAC virus and were killed after 6 to 8 days incubation at 25°C. The virus concentration at this time was approximately 5.0 $\log_{10}MLD_{50}$/insect.

Antiserum

The method previously used to prepare specific BT antiserum in rabbits was also used to prepare specific antiserum to PAC virus.

two slides of C.variipennis smears were treated with each antiserum dilution. One slide from each group was subsequently treated with the FITC conjugate at a dilution of 1:16. The remaining slides from each group were treated with a 1:40 dilution of the conjugate.

Smears were examined using an incident light source, with a BG38 3mm filter and a 2 x KP 490 built in filter, as described in section 3.2.2.3.

3.3.1.2. Results

Smears prepared from L.longipalpis were thicker than those prepared from C.variipennis. The overall background non-specific fluorescence was therefore brighter and slides were not so easily assessed. Ten slides were prepared, each with 2 to 4 infected, and 2 to 4 uninfected tissue smears per slide. On only one slide was specific fluorescence obvious in the infected smears. The dilution of the antiserum and conjugate on this slide was 1:10 and 1:16 respectively.

All the infected and uninfected smears prepared from C.variipennis on the positive control slides were assessed accurately.

3.3.2. Fluorescent antibody test on tissue smears prepared from Pacui virus infected L.longipalpis

3.3.2.1. Materials and methods

Insects and Virus

The virus, insects and methods of infection are as described in section 2.2. L.longipalpis adults were inoculated with PAC virus and were killed after 6 to 8 days incubation at 25°C. The virus concentration at this time was approximately 5.0 $\log_{10}$ MLD_{50}/insect.

Antiserum

The method previously used to prepare specific BT antiserum in rabbits was also used to prepare specific antiserum to PAC virus.

An emulsion containing a 1:1 mixture of Freund's complete adjuvant and PAC virus (mouse brain passage stock virus in 0.1% BA, 7.5 $\log_{10}$ MLD_{50}/ml) was used for the first intramuscular inoculation. Subsequent subcutaneous injections were of virus alone. The neutralising end point titre of the antiserum produced (R25) was 1:400.

Experimental procedure

Smears were prepared from the head, thorax and abdomen of infected sandflies. Untreated insects were used as uninfected controls. Twelve slides of mixed samples were prepared with smears from 2 to 4 infected and 2 to 4 control insects per slide.

An indirect FAT was performed on these 12 slides using the same procedure as described for the detection of BT viral antigen in Culicoides tissue smears (see section 3.2.2.2.).

A series of antiserum dilutions were tested. These dilutions were 1:1, 1:10, 1:20 and 1:50. Three slides were treated at each dilution. One of each three was subsequently treated with FITC conjugate at a dilution of 1:16. The remainder were treated with a 1:40 dilution of the conjugate.

3.3.2.2. Results

The results are shown in Table 3.6. Good specific fluorescence was observed in tissue smears prepared from PAC virus infected sandflies. The best distinctions between positive and negative samples were made on smears from the thorax and abdomen. The fluorescence appeared similar in form to that observed when the indirect FAT was performed on BT virus infected Culicoides. There was an overall difference in brightness between positive and negative samples with a 'granular' appearance of infected tissue.

The best results, i.e. easiest to assess and most consistent, were obtained using antiserum and conjugate dilutions of 1:50 and 1:40 respectively. Antiserum dilutions of 1:10 and 1:20 also gave good

Table 3.6. Indirect fluorescent antibody test on smears from L.longipalpis 6 to 8 days after inoculation of Pacui virus

Body part		Head			Thorax			Abdomen		
Anti-serum dilnt.	Conjugate dilnt.	Contrast of smears		No.accurate / No.tested	Contrast of smears		No.accurate / No.tested	Contrast of smears		No.accurate / No.tested
		Infected	Uninfected		Infected	Uninfected		Infected	Uninfected	
1:1	1:16	+ + +	+ + +	1/4	+ + +	+ + +	0/4	+ + +	+ + +	3/4
	1:40	+ +	+ +	2/3	+ +	+ +	0/3	+ + +	+ +	2/3
	1:40	+ +	+ +	0/3	+ +	+ +	1/3	+ +	+ +	1/3
1:10	1:16	+ +	+ +	0/4	+ + +	+ + +	0/4	+ + +	+ +	3/4
	1:40	<+	<+	0/4	+ + +	+ +	4/4	+ + +	+ +	4/4
	1:40	+	<+	1/3	+ + +	+ +	3/3	+ + +	+ +	3/3
1:20	1:16	<+	<+	0/4	+ + +	+ +	4/4	+ +	+	4/4
	1:40	+	<+	3/3	+ +	+	3/3	+ +	+	3/3
	1:40	<+	<+	1/3	+ +	+	2/3	+	<+	2/3
1:50	1:16	+	<+	0/3	+ +	+	3/3	+ +	+	3/3
	1:40	+	<+	2/3	+ +	+	3/3	+ +	+	3/3
	1:40	+	<+	2/3	+ +	+	3/3	+ +	+	3/3

results. Positive smears were not reliably distinguishable using a 1:1 dilution of antiserum since both the infected and the control smears appeared very bright.

Smears could be stored overnight at 4°C after fixation and before staining. Longer periods of storage were not attempted.

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3.4 Preparation of sections of Culicoides tissue

3.4.1. Paraffin wax sections

3.4.1.1. Materials and methods

C.variipennis females were killed by freezing at -70°C. Insects were then embedded in paraffin wax. Several different schedules were employed for the pre-embedding stages of fixation, dehydration and clearing. These are described in Table 3.7. Between 4 and 6 insects were prepared by each method.

The successive pre-embedding stages were carried out in 5 ml bijoux bottles or in 90 mm glass petri dishes. Insects were embedded in paraffin wax at 56°C in small 30 mm diameter solid glass watchglasses. These were first smeared with glycerol to facilitate removal of the cooled wax. Clean hot wax was poured into a prepared watchglass. An insect was removed from the final wax bath using a warmed small wide-mouthed pipette, and was placed in the molten wax in the watchglass. It was orientated in a flat horizontal plane using warmed watchmakers forceps or fine needles. The wax was then cooled and hardened in a bath of cold water.

Small blocks of wax, each containing an insect, were trimmed and mounted individually on wooden microtome blocks following normal

Table 3.7. Preparation of paraffin wax sections of C.variipennis

Method 1 : Routine histological method with acetone fixation at room temperature

Stage	Treatment	Time (mins)	Comments
Fixation	Acetone	60	No good sections were obtained. Head tissue : torn in all slides. Thorax muscles very torn. Abdomen : Preservation of gut tissue good in 1 insect out of the 4.
Dehydration	Ethanol 95%	2 x 30	
	Ethanol 100%	3 x 30	
Clearing	Xylene	3 x 30	
Embedding	Wax at 56°C	4 x 30	
	Embed in wax		

Method 2

Stage	Treatment	Time (mins)	Comments
Fixation	Acetone* 50%	2 x 30	Sections all badly torn but structure of salivary glands, some parts of the gut, malpighian tubules and ovaries intact in most sections.
Dehydration	Ethanol 50%	2 x 30	
	Ethanol 70%	2 x 30	
	Ethanol 95%	2 x 30	
	Ethanol 100%	2 x 30	
Clearing	Xylene	2 x 30	
Embedding	Wax at 56°C	3 x 30	
	Embed in wax		

* 50% acetone in distilled water plus 0.1% hederol as wetting agent.

Method 3

Stage	Treatment	Time (mins)	Comments
As for method 2 except for the fixative, in this case 100% acetone.			Sections very badly torn. Some gut structure fairly well preserved.

Continued.....

Table 3.7. Continued.....

Method 4

Stage	Treatment	Time (mins)	Comments
As for method 2 except for the fixative, in this case methanol.			Sections fair. Head tissue: fair. Thorax: muscles badly torn. Abdomen: gut tissues, ovaries and malpighian tubules visible.

Method 5 : Sainte-Marie method. All stages at +4°C except for wax

Stage	Treatment	Time (mins)	Comments
Fixation	Ethanol 95%	60	Very poor results. All sections fragmented.
Dehydration	Ethanol 100%	4 x 30	
Clearing	Xylene	3 x 30	
Embedding	Wax at 56°C	4 x 30	
	Embed in wax		

Method 6

Stage	Treatment	Time (mins)	Comments
Fixation	Carnoy	1 x 60 or 2 x 60	
Dehydration	Butyl alcohol	3 x 30 or overnight	All sections good
Embedding	Wax at 56°C	4 x 30	
	Embed in wax		

Continued.....

Table 3.7. Continued.....

Method 4

Stage	Treatment	Time (mins)	Comments
As for method 2 except for the fixative, in this case methanol.			Sections fair. Head tissue: fair. Thorax: muscles badly torn. Abdomen: gut tissues, ovaries and malpighian tubules visible.

Method 5 : Sainte-Marie method. All stages at +4°C except for wax

Stage	Treatment	Time (mins)	Comments
Fixation	Ethanol 95%	60	Very poor results. All sections fragmented.
Dehydration	Ethanol 100%	4 x 30	
Clearing	Xylene	3 x 30	
Embedding	Wax at 56°C	4 x 30	
	Embed in wax		

Method 6

Stage	Treatment	Time (mins)	Comments
Fixation	Carnoy	1 x 60 or 2 x 60	
Dehydration	Butyl alcohol	3 x 30 or overnight	All sections good
Embedding	Wax at 56°C	4 x 30	
	Embed in wax		

Continued.....

Table 3.7. Continued.....

Method 7 : Routine histological procedure with fixation in Carnoys fluid

Stage	Treatment	Time (mins)	Comments
Fixation	Carnoy	60	Sections fair. Preservation of head tissue and abdomen fair. Thoracic muscles torn.
Dehydration	Ethanol 95%	2 x 30	
	Ethanol 100%	3 x 30	
Clearing	Xylene	3 x 30	
Embedding	Wax at 56°C	4 x 30	
	Embed in wax		

histological procedures (Pantin, 1948; Humason, 1967). Serial longitudinal sections of tissue 3 µm in thickness were cut using a rotary microtome. They were divided into suitable lengths and were mounted on cleaned 76 x 26 mm glass microscope slides smeared with egg albumen. The sections were flattened by pipetting a small volume of water onto the slide under the sections and passing the slide 2 or 3 times through a bunsen flame. As the water warmed, the sections flattened. Excess water was carefully drained from the slide while holding the sections in position with a fine needle. The volume of water on the slide was kept to an absolute minimum to prevent leaching of material should infected samples be used.

The slides were dried and were routinely stained,either with Ehrlich's acid haematoxylin and eosin or with Mallory's triple stain, to determine the quality of sections produced after each method of embedding.

3.4.1.2. Results

Only one of the methods described resulted in the production of good sections. This was method 6 using Carnoys fluid as a fixative, followed by dehydration in butyl alochol and embedding in wax at 56°C. Clearing in xylene is not necessary in this method because butyl alcohol is miscible with paraffin wax. Sections from all four insects prepared by this method were as good as can be expected when using the double embedding technique. The detail of the internal structure was sufficiently clear to enable virus localisation studies to be performed if this were possible. The morphology of the head is shown in Figure 3.12 and 3.13. The salivary glands are illustrated in Figure 3.14. The morphology of the gut diverticulum and abdomen can be seen in Figure 3.15 and 3.16 respectively and the ovary in shown in Figure 3.17. The only

Figure 3.12. C.variipennis L.S. head, H. and E., x 440. CG, cephalic ganglia; SO, suboesophageal ganglia; S, salivary glands; MP, mouthparts.

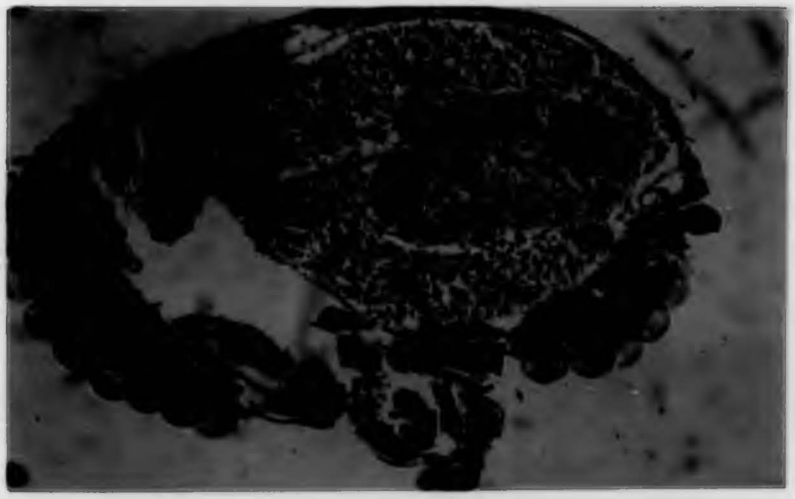

Figure 3.13. C.variipennis T.S. head, H. and E., x 720. CG, cephalic ganglia; MP, mouthparts, L, compound eye lenses; O, ommatidia

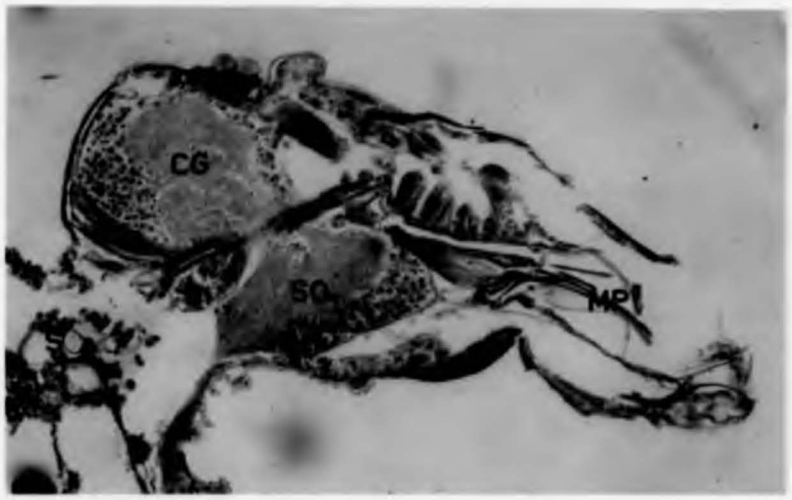

Figure 3.12. C.variipennis L.S. head, H. and E., x 440. CG, cephalic ganglia; SO, suboesophageal ganglia; S, salivary glands; MP, mouthparts.

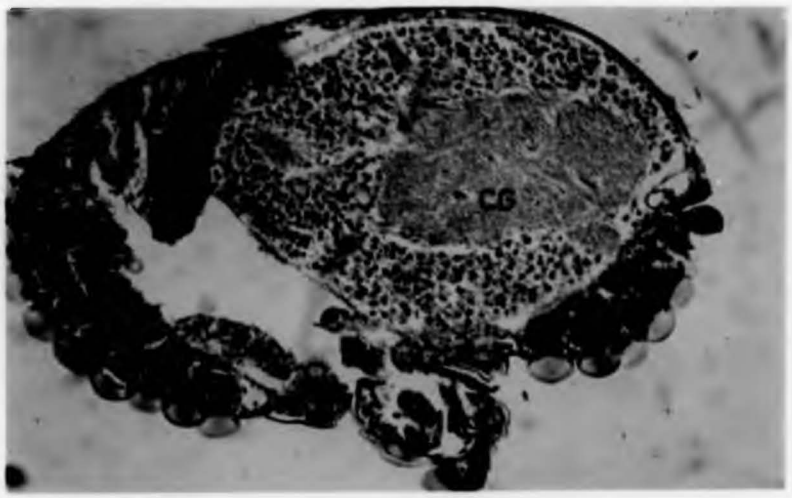

Figure 3.13. C.variipennis T.S. head, H. and E., x 720. CG, cephalic ganglia; MP, mouthparts, L, compound eye lenses; O, ommatidia

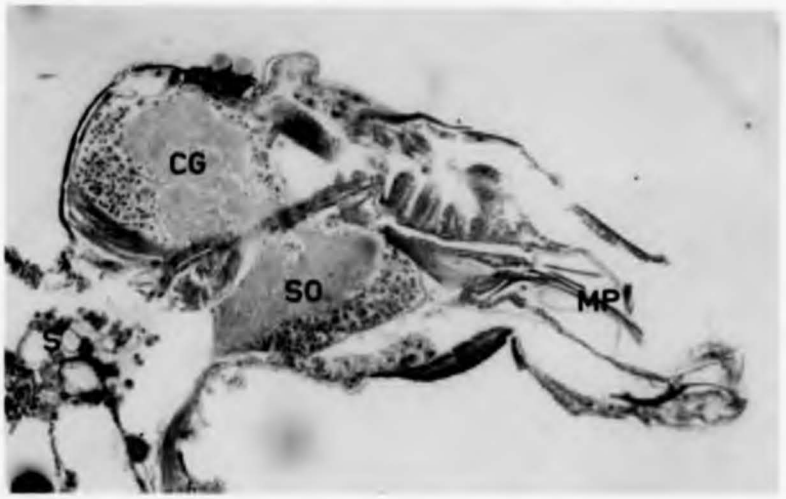

Figure 3.12. C.variipennis L.S. head, H. and E., x 440. CG, cephalic ganglia; SO, suboesophageal ganglia; S, salivary glands; MP, mouthparts.

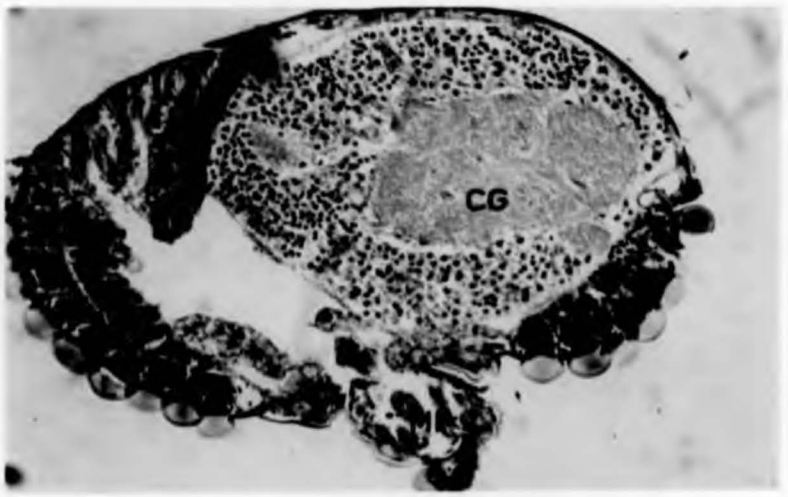

Figure 3.13. C.variipennis T.S. head, H. and E., x 720. CG, cephalic ganglia; MP, mouthparts, L, compound eye lenses; C, ommatidia

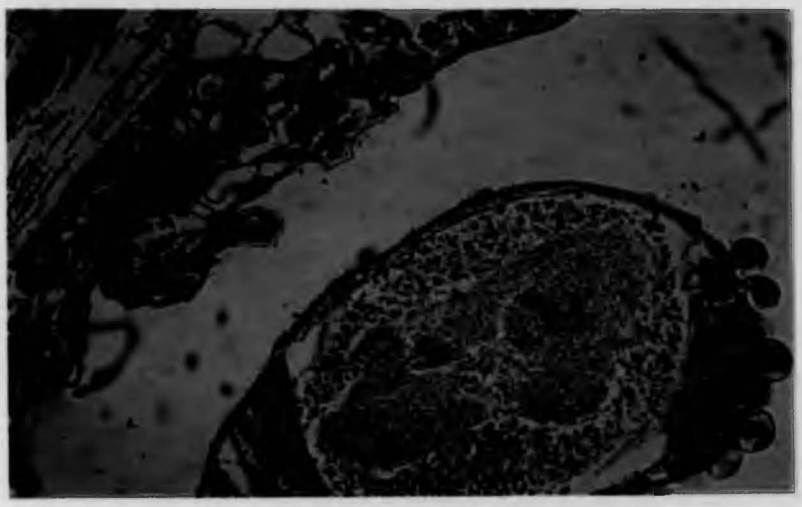

Figure 3.14. C.variipennis T.S. head and salivary glands, H. and E., x 720, CG, cephalic ganglia; SG, salivary glands.

Figure 3.15. C.variipennis L.S. thorax showing gut diverticulum, H. and E., x 720, D, gut diverticulum; TG, thoracic ganglia

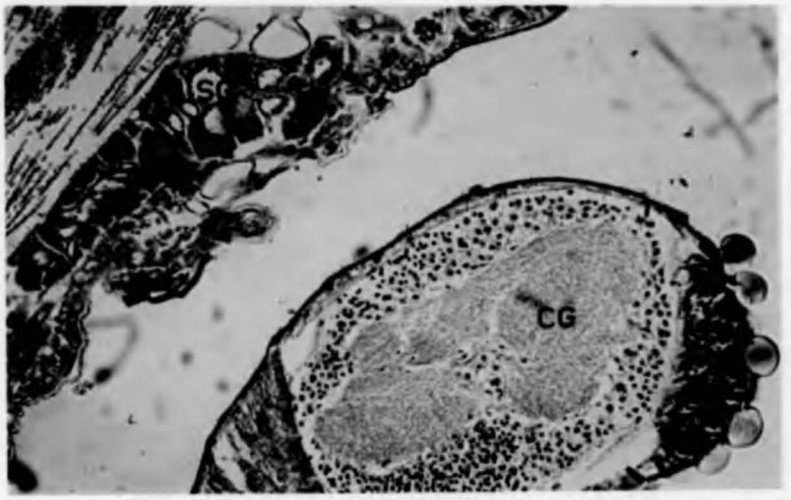

Figure 3.14. C.variipennis T.S. head and salivary glands, H. and E., x 720, CG, cephalic ganglia; SG, salivary glands.

Figure 3.15. C.variipennis L.S. thorax showing gut diverticulum, H. and E., x 720,
D, gut diverticulum; TG, thoracic ganglia

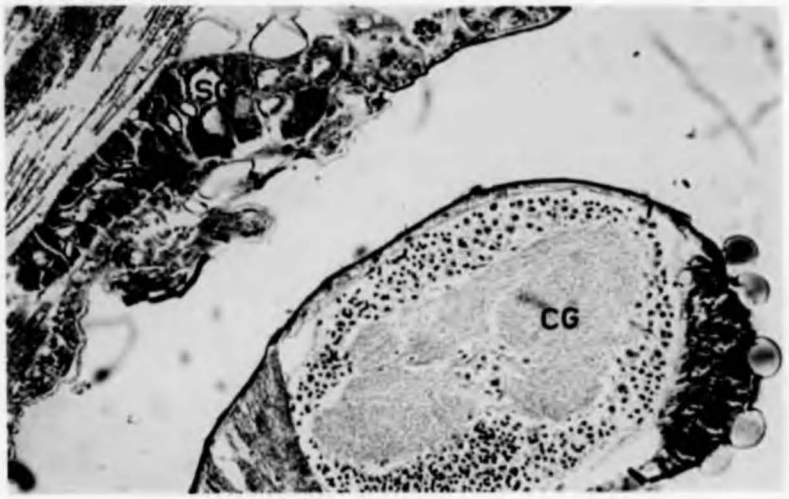

Figure 3.14. C.variipennis T.S. head and salivary glands, H. and E., x 720, CG, cephalic ganglia; SG, salivary glands.

Figure 3.15. C.variipennis L.S. thorax showing gut diverticulum, H. and E., x 720, D, gut diverticulum; TG, thoracic ganglia

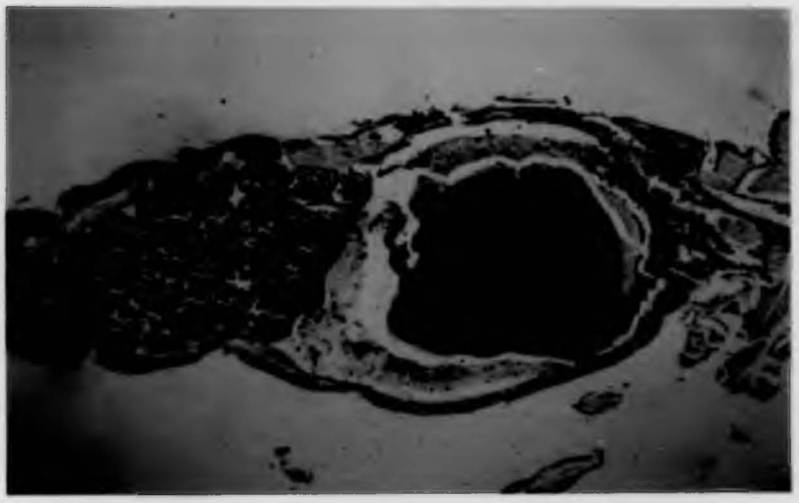

Figure 3.16. C.variipennis L.S. Abdomen, Mallory's triple stain, x 180. BM, bloodmeal; DO, developing ovary.

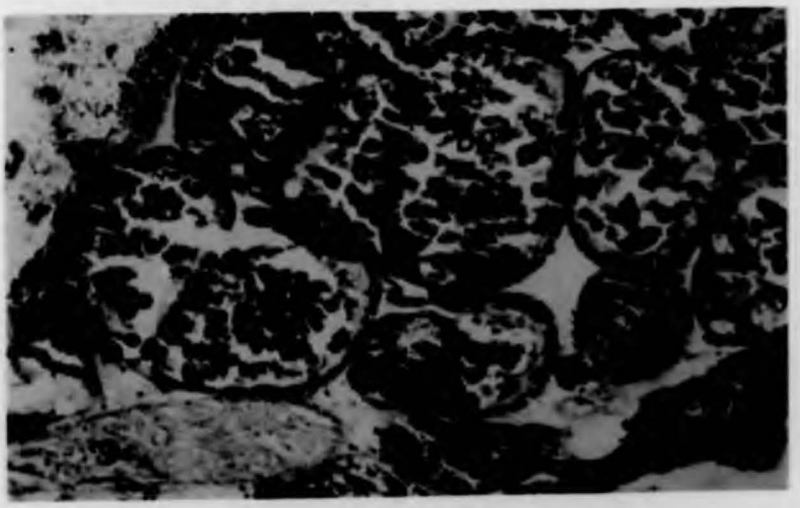

Figure 3.17. C.variipennis L.S. ovary with developing oocytes, Mallory's triple stain, x 720. DO, developing oocyte

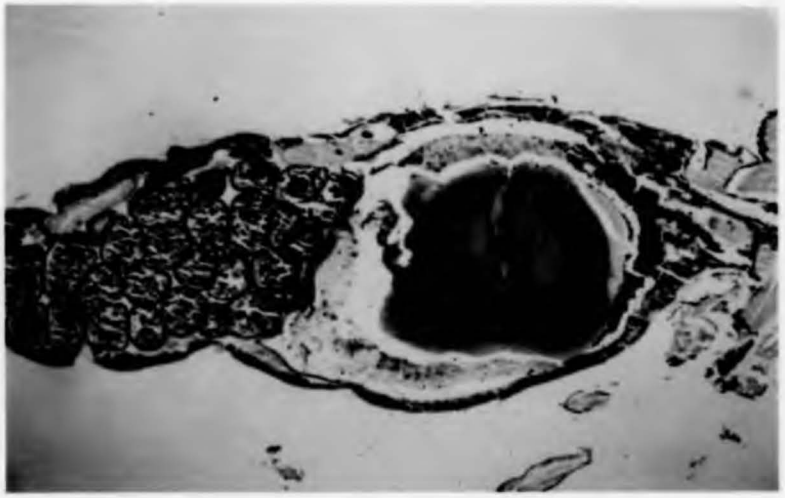

Figure 3.16. C.variipennis L.S. Abdomen, Mallory's triple stain, x 180. BM, bloodmeal; DO, developing ovary.

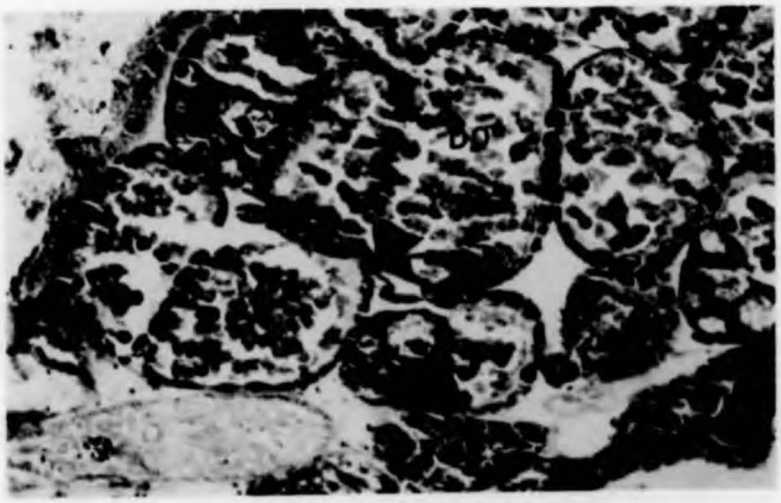

Figure 3.17. C.variipennis L.S. ovary with developing oocytes, Mallory's triple stain, x 720. DO, developing oocyte

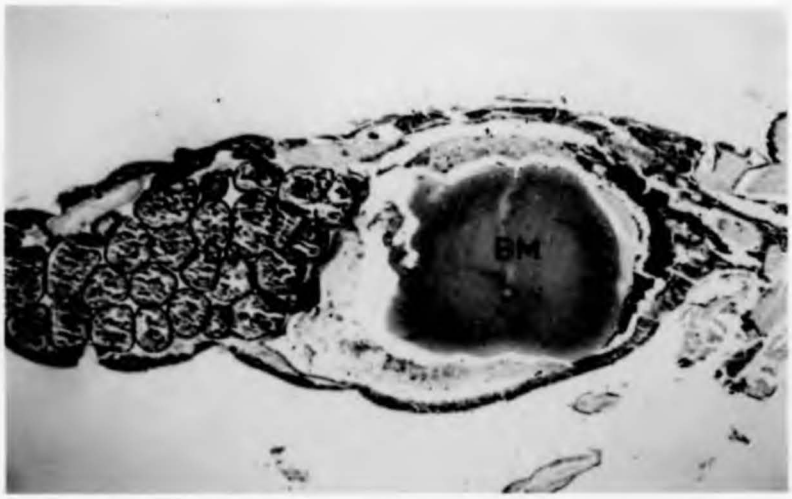

Figure 3.16. C.variipennis L.S. Abdomen, Mallory's triple stain, x 180. BM, bloodmeal; DO, developing ovary.

Figure 3.17. C.variipennis L.S. ovary with developing oocytes, Mallory's triple stain, x 720. DO, developing oocyte

major fault detected in these sections was a number of vacuoles present in the thoracic muscles of one insect. This may have been due to improper wax penetration, or to the use of an insect that was already dead before the original freeze-killing and storage.

The remaining six pre-embedding routines resulted in the production of poor sections, all of which were badly fragmented. However some parts of the internal structure were preserved intact in sections produced after methods 2,3,4 and 7 of the pre-embedding routines. Gut, salivary gland, malpighian tubules and ovarial tissue could be seen on some of the slides.

3.4.2. Frozen sections

3.4.2.1. Materials and methods

Sections of frozen C.variipennis females were prepared using a Cambridge rocker microtome mounted in a -20°C freezing cabinet.

Insects were killed by freezing at -70°C before processing following the procedures of Tobie et al. (1961). They were embedded in a 5% mixture of gelatine in PBS using the following method. A small cylinder, height 10 mm diameter 6 mm, was prepared from a double thickness of aluminimum foil wrapped around the end of a pencil. The cylinder was mounted with one open end on the face of a pre-cooled metal microtome block. A few drops of water were frozen around the base of the cylinder to hold it in position, the whole of the operation being carried out in the freezing compartment containing the microtome. A drop of 5% gelatine was added to the cylinder and an insect was placed on top of this. The insect was held in position until the gelatine had begun to set and another drop of gelatine was then added. The cylinder and block were then frozen in liquid nitrogen for between 45 and 60 seconds. The metal block was fitted to the microtome and sections of 3 to 5 µm were cut at the operating

temperature of -15 to -20°C.

The sections were mounted on 9 x 22 mm coverslips by touching the coverslips against the sections while the latter were still on the microtome knife. Approximately ten sections could be mounted on each coverslip. Serial sections could be produced with care but were more likely to crumple on the knife. The sections were thawed and dried at 37°C. They were stained with Erhlich's acid haematoxylin and eosin stains, employing small 30 x 35 mm Coplin jars to hold the coverslips during staining procedures. After staining, the coverslips were mounted in Canada balsam on microscope slides.

3.4.2.2. Results

Frozen sections were prepared from 20 uninfected C.variipennis females. All the sections were of a poor quality being very torn. They were of a similar standard to the paraffin wax sections obtained after the Sainte-Marie embedding technique described as method 5 in Table 3.7. In some of the sections much of the insect was visible on the slide but the definition of the internal structure was not clear. The major problems with the technique are listed below :

1. There was a tendency for air bubbles to form in the gelatine around the insect during the embedding process.
2. It was essential to arrange insects in a flat horizontal plane in the gelatine since the blocks could not be orientated during sectioning. This was difficult when using insects as small as Culicoides.
3. The whole procedure was very time-consuming since the best results could only be obtained by mounting one section at a time.
4. The quality of the apparatus, i.e. the microtome and knife available, was a severely limiting factor. A finer knife than the one used might give better results.

A few sections were also prepared after freezing insects directly onto the microtome blocks by immersion in dry ice or liquid nitrogen. Insects were first partially frozen onto the surface of a drop of water on the face of the block before the final immersion in liquid nitrogen. This was essential in order to lift them sufficiently far off the surface of the block to enable the knife to make contact with them. Both the ice surround and the frozen insects fractured during section cutting and only fragments of tissue were picked up on the coverslips.

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3.5 Immunofluorescence tests on smears of Bluetongue virus infected insect tissue treated with various histological reagents

3.5.1. Materials and methods

3.5.1.1. Insects and virus

All tests were performed on C.variipennis females which had been inoculated with BT virus (strain A/SOT 1 type 4), and killed after 7 or 8 days incubation at 25°C. Control insects were killed without any prior treatment. Smears were prepared from the heads of insects as described in section 3.2.2.1. Between 5 and 15 mixed infected and uninfected smears were prepared on each slide.

3.5.1.2. Immunofluorescence technique

The indirect FAT was employed throughout these experiments. The test was conducted as described in section 3.2.2.2. The R20 BT antiserum was used at a dilution of 1:20 in PBS and the commercial FITC conjugated IgG was used at a dilution of 1:40.

Slides were examined by transmitted and by incident illumination using the filter combinations described in section 3.2.2.3.

3.5.1.3. Experimental procedure

The prepared tissue smears were subjected to treatment with various reagents used during standard embedding procedures. These included fixatives, dehydrating and clearing agents, and paraffin wax. Treatment with wax was followed by immersion in xylene to remove the wax, and in alcohol to remove the xylene. Two slides were assessed by immunofluorescence after treatment with each successive reagent. Each experiment was repeated at least twice to check the results obtained. Two control slides consisting of smears treated with acetone alone were included in each test.

Slides were either immersed in the individual reagents in Coplin jars, or, when Carnoys and Bouins fixative and butyl alcohol were used, drops of the reagent were placed on the individual smears on the slides held in covered glass petri dishes.

All stages were timed for 5 minutes at $4^{o}C$ with the exception of the wax solvent. Up to 20 minutes treatment with xylene at room temperature was required to remove all wax.

3.5.2. Results

3.5.2.1. Effect of different fixatives on the fluorescent antibody test

The results are shown in Table 3.8. The fixative used and the duration of fixation are listed in the two left-hand columns. The brightness of smears and the contrast between infected and uninfected samples are indicated by the scale of symbols <+ to + + + as seen in Tables 3.2 to 3.6. The number of infected tissue smears in which viral antigen was detected by the FAT is compared to the total number of infected smears tested.

The results obtained after acetone fixation were very good. All infected tissue smears were easily detected with excellent contrast between the brightness of these and that of uninfected smears (i.e. 3+

Table 3.8. Contrast in brightness of fluorescence in infected and uninfected tissue smears after treatment with various fixatives

Fixative (at 4°C)	Time (mins)	Contrast of smears		No.detected infected No.infected	Comment
		Infected	Uninfected		
Acetone	5	+ + +	+	10/10	All showed good contrast
	10	+ + +	+	5/5	
	20	+ + +	+	3/3	
Bouins	5	<+	<+	4/9	
Carnoy	5	<+	<+	9/11	
	10	<+	<+	4/7	
	20	<+	<+	0/3	
Ethanol 95%	5	<+	<+	2/6	Some good contrasts but results very variable.
	10	<+	<+	3/4	
	20	<+	<+	8/13	
Formalin 2%	5	<+	<+	8/9	Very poor contrast.
Formalin 1%	10	<+	<+	0/4	
Formalin 10%	20	<+	<+	0/4	
Glutaraldehyde 1%	5	<+	<+	2/9	
Methanol	5	+ +	+	4/7	Good contrast, but results variable.

infected, 1+ uninfected). None of the alternative fixatives could be reliably used. A proportion of infected smears were almost always identified, but there was very little contrast between the fluorescence of these and that of uninfected smears. These slides all had to be studied closely under both low and high magnifications whereas acetone fixed samples could be rapidly assessed under low magnification. One exception was the specific fluorescence observed in infected smears fixed in methanol. The fluorescence was bright, but the results were inconsistent and not all the smears were scored correctly. Where both infected and uninfected smears in the table are represented by <+ all the samples appeared a pale green colour. Those accurately recorded as containing virus appeared only minimally brighter than the control uninfected samples and there was little evidence of the granular appearance usually observed in infected tissue smears.

3.5.2.2. Effect of post-fixation treatment with alcohol, xylene, and wax on the fluorescent antibody test

The results are shown in Table 3.9. This table illustrates the treatment of smears from fixation through successive histological stages to the FAT. The contrast in brightness of infected and uninfected material is again represented by the <+ to +/+ + system. The total number of smears scored correctly as infected or uninfected is recorded this time, together with the total number of smears examined. Statement only of the number of infected smears detected correctly would be misleading since in this series of experiments many uninfected samples were scored as being infected.

The only slides to be easily and accurately assessed were those fixed in acetone and then treated with either 95% ethanol, or butyl alcohol, or xylene. A high proportion of the remaining acetone fixed

Table 3.9. Contrast in brightness of fluorescence in infected and uninfected tissue smears after treatment with various histological reagents

Slide No.	Pre-embedding treatment				Post-embedding treatment	FAT results			Comment	Slide No.
	Fixative	Alcohol	Clearing agent	Wax m.p. °C		Contrast of smears		No.correct / No.tested		
						Infected	Uninfected			
1		Ethyl 95%	-	-	-	+ + +	+	33/37[a]	Good contrast	1
2		Ethyl 100%	-	-	-	+	<+	7/12	Poor contrast	2
3		Butyl	-	-	-	+ + +	+	12/12	Good contrast	3
4		-	Xylene	-	-	+ + +	+	6/6	Good contrast	4
5	Acetone	Ethyl 95%	Xylene	-	-	+ +	<+	21/26		5
6		Ethyl 100%	Xylene	-	-	+	<+	8/12	Poor contrast	6
7		-	-	56	Xylene	+	<+	9/12		7
8		-	-	56	Xylene	+	<+	12/13		8
9		Ethyl 95%	Xylene	56	Xylene and Ethanol 95%	+	<+	25/28	Uninfected samples very pale	9
10		Ethyl 100%	Xylene	45	Xylene	+	<+	10/12		10
11		Ethyl 100%	Xylene	45	Xylene, PBS and DW	+	<+	6/12	Poor contrast, inconsistent results	11
12	Carnoys	Ethyl 95%	-	-	-	<+	<+	9/15	Inconsistent results	12
13	fluid	Butyl	-	-	-	<+	<+	0/8	No specific fluorescence	13
14		Butyl	-	56	Xylene	<+	<+	7/12	Inconsistent results	14
15	Ethanol 95%	Ethyl 95%	-	-	-	+	<+	21/31	Inconsistent results	15
16		-	Xylene	56	Xylene	+	<+	9/12	Inconsistent results	16
17	Methanol	Ethyl 95%	Xylene	56	Xylene	+	<+	13/19		17
18	Formalin 1%	Butyl	-	56	Xylene	+	+	0/12	All smears brown	18

a. Number of smears accurately scored as infected or uninfected/ Number of smears examined

DW. Distilled water

Figure 3.18 FAT: C.variipennis, Bluetongue virus infected tissue. Head smear treated with alcohol and xylene after routine acetone fixation, 4 min. exposure, x 180

Figure 3.19 FAT: C.variipennis, uninfected control. Head smear treated with alcohol and xylene after routine acetone fixation, 4 min. exposure, x 180

Figure 3.18 FAT: C.variipennis, Bluetongue virus infected tissue. Head smear treated with alcohol and xylene after routine acetone fixation, 4 min. exposure, x 180

Figure 3.19 FAT: C.variipennis, uninfected control. Head smear treated with alcohol and xylene after routine acetone fixation, 4 min. exposure, x 180

Figure 3.18 FAT: C.variipennis, Bluetongue virus infected tissue. Head smear treated with alcohol and xylene after routine acetone fixation, 4 min. exposure, x 180

Figure 3.19 FAT: C.variipennis, uninfected control. Head smear treated with alcohol and xylene after routine acetone fixation, 4 min. exposure, x 180

smears which had been subsequently treated with additional reagents were assessed accurately. However in all of these the contrast between the brightness of infected and uninfected samples was considerably reduced.

Although in the first series of experiments (Table 3.8) acetone was the only satisfactory fixative, several other fixatives were used in combination with other histological reagents in the later tests. All the results obtained from FATs on smears treated in this way were inconsistent. Some infected smears were detected accurately but there was little contrast in appearance between infected and uninfected smears.

Figures 3.18 and 3.19 respectively are positive and negative smears which had been treated with acetone, alcohol and xylene. Smears treated with acetone and xylene; or with acetone, alcohol, xylene, wax and xylene, appeared very similar to these.

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3.6. Discussion

The FAT was first applied to the study of viruses in insect tissue by Nagaraj *et al.* (1961) who used it to detect Wound tumour virus in infected tissue smears from the leaf-hopper *Agallia constricta* Van Duzee. Viruses of vertebrates which have been demonstrated in infected insect tissues using immunofluorescence techniques include Japanese encephalitis (JE) in *C.t.summorosus* (Doi *et al* ., 1967; Doi, 1970), Sindbis (SIN) and Venezuelan equine encephalomyelitis (VEE) both in *Ae.aegypti* (Gaidomovitch *et al*., 1973), La Crosse (LAC) in *Ae.triseriatus* (Beaty and Thompson, 1975, 1976, 1978), Dengue (DEN) in *Ae.albopictus* (Kuberski and Rosen, 1977; Kuberski, 1979), California encephalitis (CE) and Northway

(NOR) each in Ae.aegypti, Ae.communis De Geer, Ae.hexadontus Dyar and Culiseta inornata (McLean et al., 1977, 1979), and Yellow fever (YF) in Ae.aegypti (Aitken et al., 1979). Turlock (TUR), Western equine encephalomyelitis (WEE), and St.Louis encephalitis (SLE) viruses have been demonstrated in infected mosquito cell lines by immunofluorescence (Main et al., 1977), as have Kemerevo (KEM) (Libíková and Buckley, 1971), and CE viruses (Whitney and Deibel, 1971). The technique has not previously been used to study virus infections in Culicoides or sandfly tissues.

The only application of the FAT to BT virus has been for the demonstration of virus in mammalian cells and tissues (Pini et al., 1966, 1968; Bowne, 1967; Ruckerbauer et al., 1967; Stair, 1968; Jochim et al., 1974; Campbell et al., 1978; Lawman, 1979). Pacui virus does not appear to have been investigated by this technique.

The results presented in this thesis demonstrate the suitability of an indirect FAT for detecting BT viral antigen in tissue smears prepared from the vector C.variipennis. The results given in Table 3.1. suggest that for reliable detection of BT infected insects by this technique a virus concentration of at least 3.5 to 4.0 $\log_{10}TCID_{50}$/insect should be present. This level is normally attained in this insect-virus combination between days 4 and 5 post inoculation. The low proportion of infected midges detected in fluorescence tests prior to these days was taken to be indicative of a virus level at or below the threshold of the present test.

When the FAT was applied to smears prepared from BT virus infected L.longipalpis the results were inconclusive, only 4 out of a total of 24 positive smears being detected as such. The virus only multiplies to a

low level in these insects, a mean virus concentration of 3.5 $\log_{10}TCID_{50}$/ fly is normally obtained by day 8. It is therefore present in too low a concentration to be detected reliably by the immunofluorescence technique used in these experiments.

Sufficiently high concentrations of virus were present in the head of BT infected C.variipennis from day 5 post inoculation for the FAT to be carried out on this alone, leaving the remainder of the insect for investigation by other procedures if required. A similar technique, i.e. inoculation of mosquitoes with virus followed by immunofluorescence tests on head smears or squashes prepared after suitable extrinsic incubation periods, is used by Kuberski and Rosen (1977) as a routine method for the isolation and diagnosis of DEN viruses.

The FAT was also successfully used to demonstrate PAC viral antigen in tissue smears from inoculated L.longipalpis sandflies. The results obtained from the thorax and abdomen smears were more reliable than those obtained from the head. The virus content of these insects 6 to 8 days after inoculation of PAC was 3.9 to 5.4 $\log_{10}MLD_{50}$/insect. However, although the comparative concentrations of PAC virus recovered from the head, thorax and abdomen of sandflies was similar to the comparative concentrations of BT virus recovered from the same parts of C.variipennis, the actual concentration of PAC virus in the head, thorax and abdomen of L.longipalpis was lower than that of BT virus in C.variipennis (see Tables 2.26 and 2.28). The threshold level of the FAT as applied to the PAC-sandfly-antiserum combination was not investigated, and the virus content of the head region may possibly have been below the threshold of the test.

The FAT as used in these experiments is neither readily quantifiable nor sufficiently sensitive to be used as a titration system. The main application would appear to be in experiments in which large numbers of

insects have to be screened for possible BT virus infection since as many as 100 insects can be tested by one observer in a day. For example, large numbers of insects are used in both infection rate and transmission experiments. Demonstration of viral antigen in squashes containing the salivary glands, or in the glands themselves, would indicate whether insects were potentially able to transmit virus.

Some background fluorescence was observed in all the smears examined. Non-specific fluorescence such as this may be due to green autofluorescence of the insect tissue, or it may be caused by : the presence of unbound fluorescein dye in the conjugated serum, the "over-labelling" of immunoglobulins with FITC, or the presence of non-specific conjugated antibodies (Schenk and Churukian, 1974). It is unlikely that any of the latter three causes are involved in the production of the non-specific fluorescence observed in these experiments since the FITC conjugate was obtained in a purified form. All unbound FITC and overlabelled antibody had been removed by DEAE chromotography.

Non-specific fluorescence may also result from reactions between the antiserum (and or the conjugate) with heterologous antigen or tissue components such as proteins and amino acids. These reactions are usually due to electrostatic forces between the microscopical preparation and the serum proteins (Nairn, 1969), It was at this form of non-specific fluorescence that the methods used to try to improve the contrast between infected and uninfected smears were directed. None of these methods, i.e. dilution of reagents, shortening of reaction times, and absorption of serum with tissue powders, presented any improvement on the basic test. The use of counterstaining techniques has not yet been investigated. Counterstains have been used by a number of workers as a means of masking non-specific fluorescence, particularly that due to the primary or

autofluorescence of tissue substrates. Severalazo dyes have been particularly useful for this purpose. These include : flazo orange (Hall and Hansen, 1962; Hokensen and Hansen, 1966) and eriochrome black (Fey, 1972; Schenk and Churukian, 1974; Main et al., 1977). Other counterstains which have proved particularly useful for masking background fluorescence include Evans blue (Nichols and McComb, 1962; Boonpucknavig and Nairn, 1967), methyl green, and lissamine rhodamine conjugated serum albumin (Smith et al., 1959; Schenk and Churukian, 1974). The latter has been used by Gaidomovich et al. (1973) for counterstaining mosquito salivary glands infected with alphaviruses.

The FAT has previously been shown to be group antigen specific. Jochim et al. (1974) used the indirect FAT to identify and differentiate BT and Epizootic haemorrhagic disease (EHD) viruses, and Campbell et al. (1978) were able to differentiate BT and Ibaraki (IB) viruses using a similar test. As such it can be used in place of the complement fixation or agar gel precipitin tests to identify or confirm the presence of, for example, different Orbivirus group viruses in suspected vectors. The test does not appear to be type specific. Ruckerbauer et al. (1967) were able to demonstrate three BT virus serotypes with a single conjugated BT antiserum. Similar results were obtained by Pini et al. (1966) with six different BT serotypes.

The specificity of the FAT as used in this project has not yet been evaluated since the requisite heterologous antisera and virus infected insects were not available. Some preliminary experiments were performed with antiserum raised in rabbits against African horse sickness (AHS), an orbivirus, and Maindrain (MD), a bunyavirus. Neither the AHS nor the MD antiserum cross-reacted with the BT viral antigen present in Culicoides tissue preparations. In positive controls, using MD antiserum and MD virus infected Culicoides tissue smears, some specific fluorescence was observed. BT antiserum did not cross-react with these tissue preparations.

No positive controls were carried out with the AHS antiserum.

One of the limitations of the FAT is the requirement for a specific antibody against each separate antigen under investigation. A satisfactory antibody is not always easy to produce in the laboratory animals available. Antisera to three orbiviruses, AHS, EU and IB, are at present being prepared to enable the group specificity of the test to be investigated. Similarly, antisera to several BT virus serotypes, e.g. types 16 and 20, are in preparation in order that the type specificity of the test may be assessed.

The detection of specific fluorescence in smears from isolated organs of infected insects was unsuccessful owing to the extremely small size of the insect and the consequent difficulty of dissecting out individual organs without contaminating each one with virus contained in the haemolymph. Because of their small size organs were difficult to manipulate after dissection which precluded the washing procedures necessary to remove any contaminating haemolymph. With practice however, the following procedure was possible. The salivary glands were dissected out and were placed in a drop of fluid on a slide. Excess fluid was absorbed with the edge of a paper tissue, and the moist salivary glands could then be transferred on the end of a dissecting needle to another drop of fluid further along the slide. By this means it may be possible to inactivate surface contaminating virus by rinsing the salivary glands in successive drops of BT antiserum. If this is prepared from a different animal species to that used to prepare the BT antiserum employed in the routine FAT, then the specific fluorescence observed in subsequent FATs on smears or squashes made from salivary glands treated in this way should be due to virus within the glands rather than from surface contaminating material. These experiments will be attempted as soon as a suitable antiserum from an animal other than rabbit, e.g. from sheep, has been produced.

The routine fixative used in all these experiments, both for BT and PAC virus, was acetone. This is the fixative most commonly used in fluorescence studies with viral antigens. Jochim et al. (1974) and Campbell et al. (1978) used acetone for the fixation of BT and two other orbiviruses, EHD and IB, examined in Vero cell culture by immunofluorescence. Other viruses which have been fixed in acetone without destruction of their antigenic properties include : AHS (Davies and Lund, 1974), JE (Doi et al., 1967), and Coronavirus (Monto and Rhodes, 1977). Acetone has also been used as a fixative for organisms such as Leishmania (Abdalla, 1977) and Schistosoma (Wilson et al., 1977) prior to immunofluorescence studies.

Alcohols such as 95% ethanol or methanol have also been used as fixatives for viruses in immunofluorescence work. For example, ethanol was used by Shi-gie and Pogodina (1964) for the fixation of Far-East tick-borne encephalitis virus (Russian Spring-Summer encephalitis) in frozen sections of infected organs taken from mice and piglets; and methanol has been used in fluorescence studies on the virus of Swine vesicular disease (Chapman and Burrows, 1973). Neither of these reagents proved satisfactory fixatives for BT virus in these experiments even though methanol has been used previously as a fixative for this virus in BHK 21 cells (Dr.M.Lawman personal communication, 1977). This further emphasises the need to evaluate all the experimental conditions for every tissue-antigen-antibody system.

Three other fixatives, glutaraldehyde, Bouins and Carnoys fluid, also proved unsatisfactory for use on BT viral antigen prior to immunofluorescence studies. Glutaraldehyde is used as a fixative in electron microscope studies. Bouins and Carnoys are reagents commonly used as fixatives for invertebrate tissue. Bouins fluid was used successfully as a fixative in immunofluorescence studies on Hepatitis B antigen (Ray and Dasmet, 1975).

Treatment of acetone fixed smears with reagents such as are used in routine paraffin wax embedding procedures, e.g. alcohol, xylene and paraffin wax, prior to performing the FAT, decreased the brightness of specific fluorescence and reduced the amount of "granular" fluorescence apparent in infected smears. The results were always less consistent after such treatment. A lower melting point wax ($45^{o}C$) was used in some experiments to investigate whether the standard embedding temperature of $56^{o}C$ had an adverse effect on the viral antigenicity. There was no improvement in the results. Bluetongue virus is generally considered to have a marked degree of thermostability. Svehag (1963) reports that inactivation of the viral RNA occurs within the range 37^{o} to $46^{o}C$, and inactivation of viral protein occurs between 46^{o} and $56^{o}C$. However Mellor (personal communication, 1979) found that incubation of BT virus for 4 hours at $45^{o}C$ did not affect the viral infectivity, and a 2 hour incubation period at $55^{o}C$ only reduced the concentration of virus in samples from 6.8 to 6.3 $\log_{10}TCID_{50}/ml$. It seems unlikely therefore that the temperature alone at which embedding is carried out would adversely affect the antigenicity of BT virus.

Unfortunately the reagents used to produce the good sections of C.variipennis insects, i.e. fixation in Carnoys, dehydration in butyl alcohol, and embedding in paraffin wax at $56^{o}C$, rendered the FAT ineffective. Fixation of insects in acetone,methanol, or ethanol, resulted in the subsequent production of very poor tissue sections, although sufficient internal structure was visible in some sections to yield some information on virus distribution if the processing methods did not destroy the viral antigenicity.

The use of polyethylene glycol (PEG) waxes for embedding media was considered. These waxes can be used to embed tissue directly from a fixative such as acetone or alcohol, or even directly from water.

The stronger solvents such as xylene are therefore avoided and the histological treatments are reduced to a minimum of two stages. The PEG waxes are available in two forms :

1. Water soluble waxes (Carbowax) : A number of forms of Carbowax are obtainable with a variety of melting points and associated degrees of hardness. Two types, one with a melting point of 32°C - 38°C, have been used in enzyme histochemistry although not yet for immunofluorescence work. These waxes can be difficult to handle since their sectioning properties are strongly affected by temperature and humidity. It is essential to produce thin sections for fluorescence work since autofluorescence is usually considerably increased in thick tissue sections. Using these water soluble waxes thin sections can only be cut in a cool dry environment.

2. Acetone soluble waxes (Ester-wax). Thin sections from 1 to 3 µm can be produced using this as an embedding medium. The melting point is 45° - 48°C. Some of this wax has recently been obtained and its potential use for the production of sections from insects for immunofluorescence studies will shortly be evaluated.

The preparation of frozen sections, which is the commonest histological method for fluorescent antibody studies, proved unsatisfactory when applied to Culicoides. The quality of equipment available was a limiting factor, although these insects are so small that the production of serial sections on the freezing microtome may not be possible. Frozen sections have been successfully prepared from ticks embedded in gelatine (Tobie et al., 1961) or in Tissue-Tek compound (Chernesky and McLean, 1969). Frozen sections of mosquitoes have been obtained by using the latter technique (Kuberski, 1979), and also by employing a mixture of fresh albumen and gum arabic powder as an embedding medium (Doi et al., 1967; Doi 1970). The FAT has been used in conjunction with these sectioning procedures to study the distribution of viral antigens in both groups of arthropods. For example, Chernesky and

McLean (1969) were able to study Powassan (POW) virus in Dermacentor andersoni Stiles, and Doi et al. (1967) and Doi (1970) were able to observe the distribution of JE virus in frozen sections prepared from three species of mosquito C.t.summorosus, C.pipiens pallens Weidemann and C.pipiens fatigans Coquillet. The main disadvantage of using frozen sections for viral location studies is that there may be leaching of the antigen during thawing of sections. In addition the structural definition obtained in frozen sections is not usually as good as that in sections produced after paraffin wax embedding procedures.

The immunoperoxidase technique which employs an enzyme (horseradish peroxidase) labelled antiserum for the identification and location of antigen in cells or tissues is now increasingly used in virological studies. This test has been used to identify enteroviruses (Herrmann et al., 1974) and human myxoviruses and paramyxoviruses (Benjamin and Ray, 1974) in cell culture. It has also been used for the titration of DEN (Okuno et al., 1977), and for the demonstration of CE virus in mosquito salivary glands (McLean et al., 1975, 1977). A similar technique in which glucose peroxidase is substituted for the horseradish peroxidase was also successfully applied to the demonstration of bunyaviruses in mosquito salivary glands (McLean et al., 1979).

The IPT has the advantage that the end product is stable and may be observed under the light microscope. Furthermore, samples can subsequently be prepared and examined with an electron microscope. The method has had limited application to formalin fixed paraffin wax embedded material. Burns (1975b) was able to use the IPT to demonstrate Hepatitis B antigen in sections prepared from this type of material, but in most of this work the antigen has been immunoglobulin, hormones, or enzymes (Burns et al., 1974; Burns, 1975a).

Recent studies have shown that trypsin can be used to "unmask" antigens, including viral antigens such as Hepatitis B, in paraffin

wax embedded tissue facilitating their location by immunofluorescence or immunoperoxidase procedures (Huang et al., 1976, Curran and Gregory, 1977; MacIver et al., 1979).

As stated previously the reagents originally used for the IPT are carcinogenic and therefore the development of the FAT for this project was preferred. However the IPT can now be performed without the use of carcinogenic reagents and its application to the study of viruses in smears and sections of insect tissue should be investigated.

Part 4

Conclusions

Part 4

Conclusions

Insects can be completely refractory or highly susceptible to infection with an arbovirus, or they may display varying degrees of susceptibility between these extremes. The various types of reaction may be observed whether the infection is acquired via the natural oral route or by inoculation into the haemocoele. This has been illustrated by the experimental work contained in this thesis. For example C.variipennis and C.nubeculosus are completely refractory to infection with PAC and SFN viruses, and similarly L.longipalpis is completely refractory to infection with SFN virus, whichever method of infection is employed. These viruses are obviously unable to multiply in any of the tissues of these insects. Similar results were obtained when both Culicoides species and L.longipalpis were infected with SFS, although after inoculation the virus persisted for several days in the insect, i.e. it was not rapidly denatured by the internal environment of the insect.

A limited susceptibility to a virus infection was demonstrated by the development of BT virus in the two sandfly species. These insects are insusceptible to oral infection with BT but the virus will multiply to a limited extent after inoculation into the haemocoele. The distribution of virus in infected insects is restricted, only small amounts are found in the head, and transmission of virus does not occur indicating that the salivary glands either contain very little virus or are not infected. Thus in the sandfly - BT virus combinations virus only multiplies if the infection bypasses the gut and multiplication is to a lower level, presumably because of limited susceptibility of the usual range of tissues or because a more limited range of tissues support virus growth.

Pacui virus also multiplied in both sandfly species after infection by intrathoracic inoculation only, but the pattern of virus development was different. The virus multiplied to a high concentration and was distributed throughout the insect including the head. Transmission of

virus by L.longipalpis was demonstrated proving that the salivary glands were infected. Similar results were obtained when C.nubeculosus was infected with BT virus. In both these insect-virus combinations the virus multiplied readily in the insects' tissues once the gut had been bypassed, i.e. tissues other than the gut were highly susceptible to the PAC/BT infection. This suggests that some form of "gut barrier" exists which limits the susceptibility of these insects to infection with these viruses via the oral route.

C.variipennis like C.nubeculosus, is highly susceptible to intrathoracic infection with BT but the former species is also susceptible to oral infection with this virus. However whereas BT virus multiplies to a high concentration in 100% of inoculated C.variipennis, a much smaller number develop an equivalent infection when infected orally. Sixty-five per cent of the C.variipennis colony are refractory to infection and the extent of virus development in the remaining 35% varied. Some individuals are highly susceptible, high concentrations of virus develop throughout the insect and virus transmission occurs. However the level of virus multiplication in some insects is much lower; whether these individuals can transmit virus is not known. Since the 65% which are refractory to oral infection can be infected by intrathoracic inoculation it would appear that the factor limiting susceptibility after ingestion of virus must be a "gut barrier" of some form. The remaining 35% obviously do not have such a "barrier", although some other factor must limit the extent of virus multiplication in the proportion of these that support low levels of multiplication only.

P.papatasi is the presumed vector of SFS and would thus be expected to be highly susceptible to infection with this virus either orally or by intrathoracic inoculation. Insufficient insects were available to confirm this during my project. The development of SFS virus in inoculated sandflies

was similar to that of BT virus in inoculated sandflies where a low level of virus multiplication was associated with limited virus distribution and inability to transmit. The concentration of SFS (and other Phlebotomus fever group viruses) in infected sandflies which is necessary to indicate that transmission of virus will occur is not known, nor is the quantity of transmitted virus required to produce infection of the vertebrate host.

The results obtained in this thesis from the infection of sandflies with the Phlebotomus fever group viruses are particularly interesting since research of this kind has not previously been carried out with sandflies. The results may not be completely analogous to the situation which occurs in nature because of the number of variables (for example variation in individual insect populations and virus stocks) which can affect laboratory studies of this sort. Particular care should always be taken when interpreting negative results such as obtained with L.longipalpis and SFS and SFN viruses. The research presented in this thesis however provides further evidence towards the view that the Phlebotomus fever group viruses have a more restricted host range than many of the mosquito-borne arboviruses, particularly on comparison with mosquito-borne alpha and flaviviruses. The number of sandfly species in the wild which have been implicated as vectors of the Phlebotomus fever group viruses is small, i.e. P.papatasi in the Old World, L.flaviscutellata, L.trapidoi and L.ylephilator in the New World. However this may merely be a reflection of the number and distribution of the laboratories conducting field studies on these insects. The experimental host range of the Phlebotomus fever group viruses in different sandfly species has not been tested for the reasons described in this thesis. The number of laboratory colonies available for study is increasing, and the development of the two artificial infection techniques described here will greatly facilitate further detailed investigations into the development of these and other

arboviruses in sandflies.

The epidemiology of the Phlebotomus fever group as a whole is interesting. Members of the group have been isolated from the Palearctic, Ethiopian, Oriental and Neotropical zoogeographical zones. According to Tesh et al. (1975) there are distinct differences between the geographically separated viruses with, for example, viruses isolated in Panama being antigenically distinct from those isolated in Brazil or Iran. While there is some geographical overlap particularly of the viruses of SFN and SFS, each region appears to have a different virus or combination of viruses. Most of the viruses in the group are presumed to be transmitted by sandflies. The vectors in the New and Old World areas belong to separate genera i.e. Lutzomyia in the New World and Phlebotomus in the Old World. Occasional virus isolations have also been made from Sergentomyia in the Old World (Barnett and Suyemoto, 1961). The New and Old Worlds separated during the lower Cretaceous period and their respective sandfly fauna has therefore evolved separately since that time (Lewis, 1978). Perhaps the ancestors of the two separate genera transmitted the forerunners of the present Phlebotomus fever group viruses. Both genera include sandfly species with similar habitats and host preferences and it is therefore likely that the viruses which they transmit would develop along similar lines during the course of evolution. Although all the viruses currently included in the group are antigenically distinct at least three of the New World agents cause a disease in man identical to that of the classical sandfly fever of the Old World (in Tesh et al., 1975). It would be interesting to investigate the degree of specificity of these viruses with respect to both New and Old World vector species.

The specificity which exists between arboviruses and their invertebrate hosts is one of the most intriguing aspects of the arbovirus vector relationship. A closer study of the factors which govern host

specificity are required before the fundamental aspects of this relationship can be fully understood. The small size of most insect vectors has hindered investigations into the nature of the barriers which may operate to block an arbovirus infection and thus limit susceptibility. Immunological-histochemical techniques could prove invaluable as a tool for such studies particularly if these methods could be used to study virus in sections of insects. This would enable the progress of a virus infection to be observed at a cellular level and the point at which infections were blocked could be visually demonstrated.

Such techniques have other useful applications as shown by the FAT smear technique developed during the course of the experiments in this thesis. This test provided a rapid method for screening large numbers of insects for virus infection - as many as 100 insects could be conveniently assessed in one day. Certain aspects of the FAT smear test could benefit from further study. It is less sensitive for the detection of virus than titration systems such as suckling mice or vertebrate cell cultures, at least for the BT virus - Culicoides tissue - antiserum combination used in this research, and therefore means of improving the sensitivity could be investigated. The test is probably group specific but the extent of this specificity should be confirmed. Further investigation could also be made of the possibility of using the test for virus distribution studies by using smears prepared from organs dissected out and rinsed in specific antiserum to remove surface - contaminating virus. The final aim however should be the successful application of the FAT to the location of viral antigen in sections of whole insects. Viruses have been studied in frozen sections of mosquitoes using immunofluorescence techniques, however paraffin wax sections would provide a more ideal medium for virus location studies since better definition of internal structure can be observed in such sections.

This is particularly important when working with insects as small as Culicoides and sandflies which fragment easily when frozen and sectioned. The standard methods of producing paraffin wax sections of insects involve time-consuming double embedding techniques. Quicker methods of producing good sections of insects were used successfully in this project, however these have so far proved unsuitable for immunofluorescence studies. Further work is required to investigate the combination of histological techniques which will permit the production of good anatomical sections of insects without rendering subsequent fluorescence tests ineffective.

The field of immuno-histological research is at present in a fairly early stage of development. Techniques such as the immunoperoxidase technique may prove to be more convenient than immunofluorescence for the study of viral antigen in wax-processed sections, but this has yet to be investigated. I feel that one or both of these techniques will finally be applicable to the type of studies suggested above and elsewhere in this thesis, and that they will help in determining the factors which govern insect susceptibility, thus enabling the complex relationships between arboviruses and their vectors to be more fully understood.

Appendices

Appendix 1

Laboratory maintenance of a colony of the sandfly L.longipalpis

The methods used for the maintenance of this colony of L.longipalpis were similar to those described by Killick-Kendrick et al. (1977). The colony was maintained at 25°C ± 1°C, in the dark. The generation interval from egg to adult varied from 30 to 55 days. Diapause did not occur at any time.

Adults

Adult sandflies were held in waxed card pillbox cages as described in section 2.2.1. Approximately 16 sandflies of similar age were held in each cage, in the ratio of 1:1 males to females.

Females were blood-fed between 3 and 7 days post emergence, either by membrane feeding them on heparin-treated mouse blood as described in section 2.2.3.1., or by placing an anaesthetised and shaved adult white mouse on top of the cage.

Mating took place in the cages either before, during or after ingestion of the blood meal. Engorged females were left in their cages for 2 to 3 days after which time they were anaesthetised lightly with CO_2 and were transferred to Hilton pots (Killick-Kendrick et al., 1973) for oviposition. Hilton pots are contructed from 6cm x 4.5cm open-ended plastic pots lined with plaster of paris and filled to a depth of 2cm with the same material (Figure 2.1). The lid consists of a screw cap covered with fine gauze. This can be replaced with a piece of gauze secured with a rubber band to permit easier addition or removal of adult insects. Before use the pots were soaked in distilled water to dampen the surface of the plaster of paris. They were then stored in snap-top plastic boxes lined with moist filter paper. This maintained the humidity

at approximately 100% and ensured that the plaster surface did not dry out.

Eggs

The eggs were laid on the surface of the plaster of paris 5 to 6 days after females had ingested blood. Approximately 50 eggs were laid by each female, 6 females being present in each pot. The insects usually died during or after oviposition, and were removed immediately to discourage the growth of fungi. The eggs hatched between 4 and 9 days after oviposition.

Larvae and Pupae

Larvae were fed daily on desiccated liver powder (Armour Pharmaceutical Co.Ltd., Eastbourne, Sussex) (Gemetchu, 1971). The pots were examined each day and any uneaten food, dead larvae, or faeces, were removed to prevent excess fungal growth on the plaster surface. This was of particular importance during the first and second larval instars since the small larvae were easily trapped by fungal hyphae and died as a result. Care was also necessary at this stage to ensure the correct surface dampness of the plaster lining. If the surface was too dry the larvae desiccated, if too wet they adhered to the plaster and died. Third and fourth instar larvae were easier to maintain. These preferred a slightly drier substrate and were able to eat through any fungal growth on the surface of the pots. The developmental periods for each instar were as follows :

First instar	:	3 to 6 days
Second instar	:	2 to 4 days
Third instar	:	2 to 5 days
Fourth instar	:	4 to 9 days

The larvae pupated after the fourth instar and adults emerged approximately 10 days later.

Appendix 2

Infection of cell lines from Ae.aegypti (Linn.) and Ae.albopictus (Skuse) with Pacui virus

A.2.1. Materials and Methods

Cells

The Ae.aegypti cell line was established from first instar larvae (Jennings unpublished results) following the procedures of Varma nd Pudney (1969). The cells had been subcultured 200 times prior to their use in these experiments.

The Ae.albopictus cells were of Singh's cell line and were obtained from Dr. J. Newman. They had been passaged 190 times in this laboratory.

Cells were grown in 1oz glass bottles containing 3.0ml of culture medium. The growth medium was L15 GM which consisted of : Leibowitz L15 medium (Flow) 93%, tryptose phosphate broth (2% powder in distilled water) 2%, foetal bovine serum (heat-inactivated for 30 mins at 56°C) 5%, penicillin (Glaxo) 500 units/ml and streptomycin (Glaxo) 745 units/ml. Cells were passaged at weekly intervals; they were shaken from the surface of the bottles and reseeded into fresh growth medium at a dilution of 1 in 25 (aegypti), or 1 in 10 (albopictus). Monolayers containing approximately 10^7 cells were formed after 6 days incubation at 27°C.

Chromosome preparations showed the Ae.aegypti cell line to be polyploid (2n=12) with some diploid (2n=6) cells present. The Ae.albopictus cells were predominantly diploid.

Virus

Pacui virus was obtained from Dr.R.Shope of Yale University, U.S.A. It had been passaged four times in suckling mouse brain prior to its use in these experiments.

Infection of cells

Monolayers of cells from both cell lines were infected when 85 to

90% confluent, when each bottle contained approximately 10^6 cells.

The growth medium was poured off and 0.2ml of a suspension of PAC virus in 1% BA (7.5 $\log_{10}MLD_{50}$/ml) was added to each bottle. Control cultures were inoculated with 1% BA instead of the virus suspension. Monolayers were incubated for 1 hour at 27°C for adsorption of virus. The inoculum was then removed and the cells were washed three times with 3ml of L15 maintenance medium (L15MM). L15MM consisted of L15GM with a reduced serum concentration of 2%. 3ml of L15MM was then added to each bottle and a sample of this medium was taken before the cultures were returned to the 27°C incubator. The virus concentration of this sample was taken to be the amount of residual virus present in the medium on day 0. At intervals of 24 hours all the medium was harvested and the cell sheet was washed with L15MM. Fresh L15MM was added to each bottle. Harvested samples were stored at -70°C prior to titration.

Estimation of virus

No CPE was observed in these cells and therefore all virus titrations were performed by the inoculation of samples intracerebrally into suckling mice using the methods described in section 2.2.6. The results were expressed as $\log_{10}MLD_{50}$virus/ml of medium.

A.2.2. Results

Pacui virus multiplied in the Ae.albopictus but not in the Ae.aegypti cell line. The results are shown in Table 2.A.1. The level of virus multiplication in the Ae.albopictus cell line was not high. The maximum virus concentration recorded in the harvested medium was 5.1 $\log_{10}MLD_{50}$/ml on day 7. No CPE attributable to the virus infection was observed at any time.

Table 2.A.1. Susceptibility of Ae.aegypti and Ae.albopictus cell lines to infection with Pacui virus

Days post-infection	Virus content of harvested media ($log_{10}MLD_{50}$/ml)	
	Ae.aegypti	Ae.albopictus
0	3.2	3.2
1	3.5	3.7
2	3.2	4.2
3	2.7	-*
4	1.5	3.5
5	T	4.4
6	-	-
7	0	5.1
8	0	4.7

T : Trace of virus detected but in an amount too low to be calculated accurately (less than 0.5 $log_{10}MLD_{50}$/ml.)

-* : Not tested

The experiment was not continued for longer than 8 days because by then the Ae.albopictus cells in both infected and control bottles had begun to round up and detach from the surface of the glass.

A second passage of virus was made in the Ae.albopictus cells using infected medium harvested on day 5 of the first experiment. The virus concentration at this time was 4.4 $\log_{10}MLD_{50}$/ml. 0.2 ml was used to infect each of four new bottles of Ae.albopictus cells as described previously. The inoculum thus contained 3.7 $\log_{10}MLD_{50}$ of virus. Virus was present in the medium on day 0 at a concentration of 1.9 $\log_{10}MLD_{50}$/ml. By day 4 this concentration had increased to 3.1 $\log_{10}MLD_{50}$/ml. The medium was removed at this time and was stored at $-70^{o}C$.

Both the first and second insect cell passages of virus were used in membrane feeding experiments to infect L.longipalpis (section 2.3.4.4.).

Appendix 3

Experimental procedure for purification of Bluetongue virus

A.3.1. Preparation of virus

Bluetongue virus was grown in BHK 21 cell monolayers in Roux bottles. Cells were grown in EGM and were infected when the monolayers were 90 to 100% confluent. The growth medium was removed and 0.5ml of BT virus (6.5 $\log_{10}TCID_{50}$/ml) was added to each bottle. The virus and cells were incubated together for 1 hour at 37°C. Twenty-five ml of EMM was then added to each Roux and the bottles were incubated at 37°C on a rocker. A marked CPE was evident after 24 hours. The cell sheet usually detached after 48 hours and the whole contents of each Roux were harvested at this time.

A.3.2. Purification procedure

The schedule followed during attempts to purify BT virus is described below. Virus is first concentrated by precipitation of proteinacious material with a saturated solution of ammonium sulphate. Various treatments are then employed to separate the virus from other protein material. These include treatment with detergents, pelleting and fractionation of samples on sucrose gradients. Before commencing the full purification procedure the effect of individual reagents such as detergents and ammonium sulphate solution on viral infectivity was tested. Bluetongue virus could be treated with DOC (sodium deoxycholate) and Tween 80 at concentrations of 0.1 to 1.0% without affecting viral infectivity. However SDS (Sodium dodecyl sulphate) was deleterious to the virus at concentrations above 0.001%. (see Table 3.A.1.) Treatment of BT virus with saturated ammonium sulphate solution to give final concentrations of 40%, 50% and 60% ammonium sulphate did not affect the infectivity. Similar concentrations of virus were precipitated in each

Table 3.A.1. Treatment of Bluetongue virus with detergent

Sample treatment		Virus content of sample ($log_{10}TCID_{50}$/ml)
Original harvest from Roux		8.1
% DOC :	1.0	8.1
	0.1	8.3
% Tween 80 :	1.0	8.1
% SDS :	1.0	4.3
	0.1	4.1
	0.01	7.5
	0.001	7.7
	0.0001	8.2
	0.00001	7.9

case. The 50% final concentration was adopted for routine use. Preliminary experiments to compare the virus concentration in the BHK 21 cells (spun down resuspended in M/25 phosphate buffer pH 7.6, then sonicated for 20 seconds) and in the supernatant medium, showed that there was little difference in the virus concentration of the two samples. All further experiments were performed on the supernatant medium since this provided "cleaner" starting material.

Virus purification schedule

1. Ten Roux cultures of BHK 21 cells were infected with BT virus as described above. The contents of each Roux were removed and pooled 48 hours after infection.

2. The total harvest was centrifuged for 10 minutes at 2,000 g. to remove cellular debris. The clarified supernatant was used for all further stages.

3. A volume of saturated ammonium sulphate solution sufficient to give a final concentration of 50% was added to the supernatant. The mixture was left for 1 hour at $4^{o}C$ for protein/virus precipitation to occur.

4. The precipitate was collected by centrifugation for 30 minutes at 2,000 g. The supernatant was discarded and the precipitate was resuspended in 30 ml of M/25 phosphate buffer. The resultant suspension was centrifuged for 1 hour at 100,000g.

5. The supernatant was discarded, and the precipitate was resuspended in 1ml of 2mM tris buffer, pH 8.0.

6. The suspension was clarified by centrifugation at 2,000 g. for 10 minutes and Tween 80 was added to give a final detergent concentration of 1%. In some experiments SDS (final concentration 0.001%) was used instead of Tween 80.

7. The final suspension was layered on a sucrose density gradient (15-45% sucrose in 0.1M tris buffer pH 7.8 containing 0.1M NaCL, with a

saturated solution of CsCL as a cushion). The gradient was centrifuged at 70,000g for 4 hours.

8. One ml fractions were collected from the bottom of the gradient. Individual samples were examined for the presence of virus particles by electron microscopy. Samples were then pooled in groups of three, and the virus content of each group was determined by titration in BHK 21 cells.

9. Several batches of virus were labelled with 35S methionine prior to purification experiments. The BHK 21 cells were infected as described above. After 24 hours the EMM was replaced with methionine free EMM and 0.1ml of 35S methionine was added to each Roux. The purification schedule was carried out as in numbers 1 to 8 above. 50 µl samples from each gradient fraction were removed for determination of their radioactivity. Each sample was dried onto glass fibre discs and was counted in a liquid scintillation mixture containing a 4 : 0.1gm mixture of POP (2-5 diphenyl oxazole) : POPOP (1,4 - bis (5-phenyl oxazol - 2 - yl) benzene) in a litre of toluene. A Packard-Tricarb liquid scintillation counter was used for counting samples.

A.3.3. Results

Using the methods described here some cellular material was removed from the virus but complete purification was not obtained.

The infectivity assay of gradient samples indicated that the virus was spread throughout the gradient. Similar results were obtained from radioactive counting of labelled fractions.

Examination of samples under the electron microscope showed preparations to contain virus particles and cellular material.

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The infectivity assay of gradient samples indicated that the virus was spread throughout the gradient. Similar results were obtained from radioactive counting of labelled fractions.

Examination of samples under the electron microscope showed preparations to contain virus particles and cellular material.

The experiments were repeated several times and Table 3.A.2. illustrates the distribution of virus throughout the gradient as determined by infectivity assay in each experiment. An example of the radioactivity profile is shown in Table 3.A.3.

Fraction 10-12 from experiment 4 was used to immunise rabbits 20 and 23 in section 3.2.1.3.

Table 3.A.2. The infectivity of samples from sucrose gradient fractionation of Bluetongue virus

Gradient fraction	Virus content of sample ($log_{10}TCID_{50}$/ml)				
	Exp.1	Exp.2	Exp.3	Exp.4	Exp.5
1-3	9.1	8.3	8.9	7.7	8.3
4-6	7.5	7.1	9.3	8.3	8.5
7-9	7.3	6.9	8.3	7.3	8.3
10-12	6.5	7.7	7.5	7.9	8.3
13-15	6.7	6.9	8.3	7.7	8.3
16-18	7.3	5.9	6.7	7.1	7.7
19-21		5.7	6.5	7.5	

Table 3.A.3. Example of the radioactive profile of sucrose gradient fractions obtained during virus purification experiments

Gradient fraction	Counts per minute
1	7182
2	7356
3	7216
4	7512
5	7764
6	7274
7	6992
8	7476
9	7536
10	7704
11	7750
12	7684
13	7524
14	11108
15	7650
16	6360

Appendix 4

Preparation of IgG from whole antiserum by the caprylic acid separation method

Materials

Antiserum

R20, BT antiserum.

Acetate buffer, pH 4.0

0.06 M acetic acid + 0.06 M sodium acetate in the ratio 8:2.

Glycine saline buffer, pH 8.2

10 gm sodium chloride, 75 gm glycine and 1 gm sodium azide were added to 500 ml of distilled water. The pH was adjusted to 8.2, the volume was made up to 1 litre, and the pH was checked again.

Method

1. 5ml acetate buffer was added to 2.5ml rabbit antiserum in a 5ml capacity bijoux bottle
2. The bottle was placed on a magnetic stirrer and 0.18 ml of caprylic acid was added dropwise whilst stirring vigorously.
3. Stirring was continued for 30 minutes at room temperature.
4. The suspension was centrifuged at 18,000 r.p.m. for 30 minutes.
5. The supernatant containing the separated IgG was removed and dialysed overnight against glycine-saline buffer.
6. The IgG was stored in 0.5ml aliquots at -20°C.

Appendix 5

Preparation of Culicoides tissue powders and absorption of antiserum

A.5.1. Preparation of tissue powder

Tissue powders were prepared from C.variipennis adults reared in the Entomology department at A.V.R.I. Pirbright. Adults were killed by freezing at -70°C and were processed as followed :

(i) 5gm of midges were ground in PBS in a small pestle and mortar.

(ii) The ground material was centrifuged at 10,000 r.p.m. for 10 minutes. The supernatant was discarded, and the residue washed in PBS.

(iii)After a further centrifugation the residue was ground in acetone. The product was filtered through filter paper. The deposit on the filter paper was washed twice with acetone to ensure complete dehydration and was then dried at 37°C or at room temperature overnight.

(iv) The dried tissue powder was ground to a fine powder in a mortar and was sieved to remove any coarse pieces.

(v) The powder was stored at 4°C.

A.5.2. Absorption of serum

Samples of both the R20 and R23 antisera were treated. For absorption purposes 100 mg of tissue powder was added to 1ml of the original antiserum. The mixture was shaken for 2 hours at room temperature at a speed which avoided frothing. The supernatant was subsequently removed and stored at -20°C.

The absorbed antiserum was used in place of the whole antiserum in the first stage of the routine FAT in section 3.2.5.3.

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