BUNYAMWERA VIRUS REPLICATION IN ARTHROPOD AND VERTEBRATE TISSUE

by

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Abstract

Bunyamwera (BUN) virus multiplied readily in mosquitoes following intrathoracic injection and also after imbibing an infective blood meal. This agent also multiplied following inoculation of human and avian cells in tissue cultures, with production of cytopathic effects. Both in arthropod and vertebrate tissues, enveloped virions 84 nm diameter were visualized by electron microscopic observation of tissues collected after maximum viral proliferation was attained.

Following intrathroacic injection of $10^{2.2}$ mouse LD_{50} of BUN virus into groups of wild-caught mosquitoes comprising both Aedes canadensis and A. vexans, increments of infectivity were first detected in salivary glands and gut at 3 days, and maximum titres of $10^{5.2}$ mouse LD_{50} per organ were attained in salivary glands at 10 days. However, the virus content of legs, which provided a convenient means of sampling hemocelic fluid, increased at 2 days. Virus transmission by biting mice was demonstrated with mosquitoes injected 10 days previously, but not after shorter intervals. No virus replication was demonstrated following ingestion of $10^{4.0}$ mouse LD_{50} of BUN virus in a blood meal.

Aedes aegypti mosquitoes readily supported the replication of BUN virus following injection with $10^{3.3}$ mouse LD_{50} or imbibing of $10^{4.6}$ mouse LD_{50}. After injection, virus titres of whole mosquitoes declined to $10^{1.7}$ mouse LD_{50} at 12 hours, followed by an increase to a peak amount of $10^{5.0}$ mouse LD_{50} at 2 days. After feeding, virus was first detected in legs and salivary glands at 4 days, and attained

maximum titres of $10^{5.0}$ mouse LD_{50} in salivary glands at 10 days. Transmission of virus to mice was effected by A. aegypti following feeding and injection 10 days previously, but not at earlier intervals.

Following exposure of whole gut cultures of adult A. aegypti mosquitoes to $10^{3.7}$ mouse LD_{50} maximum yields of $10^{6.0}$ mouse LD_{50} per ml. were observed after 4 days incubation at 29°C, after an initial decline of infectivity to $10^{1.8}$ mouse LD_{50} at 12 hours.

Enveloped virions with cores 45 nm diameter and total diameters 80 to 100 nm were observed within vacuoles and lining vacuolar membranes of salivary glands and gut cells of A. aegypti mosquitoes 10 days or more after infection with BUN virus. No particles were observed earlier, despite high virus titres 4 days or more after injection.

After inoculation of continuous live tissue cultures of human epidermoid corcinoma cells (H.Ep. 2) with $10^{6.5}$ mouse LD_{50}, the highest amount of virus produced was $10^{7.0}$ mouse LD_{50} per ml. cell suspension after 24 hours incubation at 37°C. Maximum yields of BUN virus ($10^{6.2}$ mouse LD_{50} per ml. cell suspension) were attained 24 hours after inoculation of primary chick embryo fibroblast monolayers with $10^{5.2}$ mouse LD_{50} following incubation at 37°C. However, a peak titre of $10^{5.0}$ mouse LD_{50} was attained 3 days after inoculation with $10^{3.7}$ mouse LD_{50} in cultures incubated at 29°C. Before an increment of virus titre was observed infectivity declined to zero during the initial 4 hours after inoculation of cultures incubated at 37°C, and a tenfold decline of infectivity was noted in cultures incubated at 29°C.

Enveloped virions with total diameter 84 nm which contained electron-dense nucleoids 44 nm diameter were observed extracellularly in thin sections of chick embryo fibroblasts infected 12 hours previously with BUN virus. These particles were released by budding. Precursor particles 41 nm diameter were associated with intracellular membranes in occasional cells sectioned at 4 hours. Extracellular virions released one day after inoculation of H.Ep. 2 cultures were tagged by ferritin-labelled anti-BUN antibody.

Enveloped virions with mean diameters 100 nm were observed in suspensions of suckling mouse brain infected with BUN virus and stained negatively with phosphotungstic acid.

These results show clearly that BUN virus exhibits the essential biological and morphological characteristics of a mosquito-borne arbovirus.

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Abbreviations

BUN	Bunyamwera virus
CEF	chick embryo fibroblasts
DDSA	Dodecenyl succinic anhydride
ELY	Earle's balanced salt solution with lactalbumin hydrolysate and yeast extract
GLY	Gey's balanced salt solution with lactalbumin hydrolysate and yeast extract
HBSS	Hanks' balanced salt solution
H.Ep.2	Human epithelial cell line. Derived from carcinoma of the larynx
HI	hemagglutination inhibition
LD_{50}	lethal dose killing 50% of individuals tested
mg	milligram
ml	milliliter
NI	neutralization index
nm	nanometer
pH	logarithm of the reciprocal of the hydrogen ion concentration
RNA	ribonucleic acid
rpm	revolutions per minute

Introduction and Literature Review

Arboviruses

a) General Characteristics

Arboviruses are unique among viral agents in possessing the ability to multiply in both vertebrate hosts and hematophagous invertebrate vectors. They are small (20 nm to 100 nm), contain RNA, and their infectivity is inactivated by sodium deoxycholate and by diethyl ether, due to disruption of their lipoprotein envelope. (4,74) All arboviruses are pathogenic for newborn mice. Following intracerebral injection, they induce an acute nonsuppurative encephalitis with perivascular lymphocytic cuffing, degeneration of neurons and focal areas of inflammation. (42,46) Some agents induce systemic manifestations, e.g. hepatitis due to yellow fever, hemorrhagic fever due to dengue, and mild febrile illness due to Ilesha. It should be noted that arbovirus infections are most often subclinical and a substantial portion of the world's population has at one time or another been so affected. (5)

Natural transmission of arboviruses is mediated by Culicidae (mosquitoes, especially culicine), Chironomidae (Culicoides flies), Psychodidae (phlebotomus flies), and Ixodidae (ticks). Hosts may be mammalian, reptilian, or avian.

b) Classification

Currently there are 271 catalogued arthropod-borne viruses. (3) These are divided into 38 antigenic groups by the hemagglutination and complement fixation reactions of Clarke and Casals. (10,25) Members within a group cross react by hemagglutination or complement fixation tests. Minor factors which affect classification are circumstances of

isolation, effects on tissue culture and laboratory animals. In addition to those that fall into the above catagories, there are an additional 30 or more with no proven relation to each other or the major groups.

Group A contains over 20 viruses all of which have mosquito vectors. Group B, 42, eight of which are transmitted by Ixodid ticks. Group C contains over 10 mosquito-borne agents. (12) The other groups of note are Phlebotomus fever and Changuinola which are phlebotomus-borne, California and Bunyamwera, mosquito-borne, and ungrouped agents with tick or mosquito vectors. Recently discovered relations between Bunyamwera group viruses and other small groups has led to the creation of the Bunyamwera Supergroup. (78)

In general, individual serotypes are localized geographically, except dengue which is prevalent throughout the Tropical Zone. However, with modern transport and migratory birds, they may be transferred to a new host-vector system a great distance away. Localization may be partially explained by the complex epidemiology of the host-vector system, e.g. relative avian insusceptibility to Tensaw virus not only eliminates a natural reservoir, but also prevents long-range migratory spread. (71)

c) Infection Cycles

Arboviruses are maintained in nature by infection cycles. (47) In the case of mosquito vectors these are of two types.

1. Transmission from man to man as with epidemic urban yellow fever. (70)

2. Transmission to man from wild or domestic vertebrates, including

birds, which act as natural reservoirs for the disease.

The links in these cycles may all be considered variables which are affected differently by ecological stresses. The effect of each variable on the propagation of the virus is therefore of the utmost importance. The host and reservoir vertebrates must be susceptible to the virus and capable of maintaining a sufficiently high viremia to infect arthropod vectors. The virus also effects a specific range of vectors, which have a low enough infection threshold to allow viremic animals to infect them. They must also be capable of supporting virus multiplication so infective virus may be transmitted to hosts. (16,62)

Host ranges may be tested in the field by serology and attempts at virus isolation, and in the laboratory susceptibility may be tested by inoculation or arthropod-mediated infection. (6)

d) Replication of Arboviruses in Mosquitoes

The dynamics of virus-mosquito interrelations have been of interest since Reed implicated the mosquito as the vector for the "parasite of Yellow Fever" in 1901. (58) He noted that after a meal from a clinical case of yellow fever there was a twelve day latent period before a mosquito could transmit the disease. Stokes, 27 years later, noted that the agent involved was filtrable, transmitted to monkeys by mosquitoes, infected mosquitoes for life, but was not transmitted transovarially from adult to larvae. (70) Whitman showed yellow fever titres in mosquitoes declined for several days after a meal, only to rise after a week to a level much higher than that of

the inoculum. (77) Evidence of similar invertebrate multiplication with other arboviruses showed direct correlation between insect virus titres, the period of eclipse, and the infectious period which follows a dynamic increase in virus levels. (14,43) During this period similar dynamics were discovered in the analogous plant virus-insect vector systems. These plant viruses also show cases of transovarial virus passage, a factor which has been a subject of controversy but never proven in the case of arboviruses. (8,48) While arboviruses multiply well within mammalian temperature ranges their growth in arthropods is quite slow and dependent on the insect's environmental temperature. The optimum is generally considered to be 80°F. With higher temperatures, the ingested virus rapidly goes into the eclipse phase and the time prior to transmission is shortened, but mosquito viability is decreased and peak virus titres are similar to 80°F incubation. The net result is a decreased infectivity span. Low temperatures result in prolonged virus incubation and potentially lower titres. (15) Increasing inoculum also decreases extrinsic incubation time as well as insuring a higher infection rate.

Within mosquitoes, initial multiplication of virus occurs in the abdominal part of the midgut. The gut itself shows a degree of specificity in the range of viruses it can support, and therefore can act as a barrier to infection. If the gut is by-passed by intra-thoracic infection or by gut puncture the insect can support growth of agents which are outside of its natural range. (44) From the gut, dissemina-

tion takes place via the hemolymph to all parts of the body. The area of major importance is the salivary glands as the mosquito injects saliva containing digestive enzymes and anticoagulents subcutaneously prior to taking a blood meal. (56) Virus multiplying in the salivary glands is shed into the saliva; and with infection transmission to the new host is accomplished. The greatest infectivity per unit weight is in the salivary glands with virus levels of over 6 logs chick LD_{50} being reported. (75) It should be noted that while most arboviruses show no pathological effect on their vectors, Semliki Forest produced salivary membrane degeneration and decreased secretion. (49)

e) Electron Microscopy of Replication

The electron microscope has proven to be a valuable tool in arbovirology within recent years. Early work was confined to general morphology and correlation of virus sizes with information gained from filtration and centrifugation experiments.(64) With the advent of thin sectioning, electron microscopy made it possible to delve further into the histo and cytopathology of arbovirus infection. Morgan studied WEE (Western equine encephalomyelitis) development, observing 22nm spheres, which he considered precursors, differentiating at membranes, passing through them, and in the process becoming mature enveloped particles of 45 - 48 nm. (50)

Group A viruses develop by the enveloping of nucleoids which bud through the plasma membrane. Vacuoles are formed in the cytoplasm but, while relatively few particles are found within them, viral RNA and protein synthesis occurs on their membranes. As cells degenerate

nucleoids are seen around these vacuoles and buds may be seen within those which are not encircled by nucleoids. Group A particles average 55 nm in size. (2,22,29)

Group B particles are smaller at 38 nm. They tend to bud at intra-cellular membranes and collect within distended vacuoles. These vacuoles then pass to the cell's surface to discharge the virions. (1,57)

Arbovirus multiplication in insect tissue has only recently been confirmed by electron microscopy. Blue tongue virus was tentatively identified in the salivary glands of Culicoides flies in 1966. Both enveloped and non-enveloped virions were seen. (9) Filshie and Rehacek discovered that group B viruses behaved similarly in mosquito cell cultures and mammalian cell lines when they observed JBE (Japanese B encephalitis) and MVE (Murray Valley Encephalitis) maturing at intracellular membranes surrounding vacuoles. (27) Although Bergold (7) felt he had discovered yellow fever particles in Aedes aegypti salivary glands in 1962, the first truly positive findings on tissue from infected mosquitoes were made by Janzen in 1970 which Chikungunya virus was visualized in Aedes aegypti salivary glands. Precursor particles (size 25 - 31 nm) were seen not only in the cytoplasm and on vesicles, but also within the nucleus. Particles with diameters 50 - 58 nm were seen within vacuoles and in the extracellular spaces. The only variation from normal function is an increase in the density of salivary secretions. (36) Eastern Equine Encephalomyelitis in mosquito salivary

Table I: The Bunyamwera Group of Arboviruses (47,12)

Virus	Distribution	
Batai	Palearctic, Oriental	W.H.O. 1961. W.H.O. Techn. Rep. Ser. No. 219
Bunyamwera	Ethiopian	Smithburn et al. 1946. Amer. J. Trop. Med., 26: 189
Cache Valley	Nearctice, Neotropical	Holden and Hess. 1959. Science 130: 1187
Germiston	Ethiopian	Kokernot et al. 1960. Amer. J. Trop. Med. 9: 62
Guaroa	Neotropical	Groot et al. 1959. Amer. J. Trop. Med. 8: 604
Ilesha	Ethiopian	Okuno. 1961. Amer. J. Trop. Med. 10: 223
Kairi	Neotropical	Anderson et al. 1960. Amer. J. Trop. Med. 9: 70
Lokern	Nearctic	Catalogue of Arthropod-borne viruses, No. 220
Maguari	Neotropical	Causey et al. 1961. Amer. J. Trop. Med. 10: 227
Main Drain	Nearctic	Catalogue of Arthropod-borne viruses, No. 219
Shokwe	Ethiopian	
Sororoca	Neotropical	W.H.O. 1967. W.H.O. Techn. Rep. No. 369
Tensaw	Nearctic	W.H.O. 1967. W.H.O. Techn. Rep. No. 369
Wyeomyia	Neotropical	Roca-Garcia. 1944. J. Infect. Dis. 75: 160

Table II: Members of the Bunyamwera Supergroup of Viruses

Group	No. in Group
Bunyamwera	14
Group C	11
Guama	8
Capim	7
Simbu	16
Bwamba	2
California	10
Patois	4
Tete	3
Koongol	2
Olifantvlei	2
Unassigned	4

glands shows particles identical to those seen in mammalian cells. No nuclear virus is seen, and random budding from cell membrane systems seems to be the method of release. Virus and salivary secretions accumulate at the apical region of the cell, passing from here into the salivary ducts. Large numbers of virions are not seen in the cytoplasm. (76) The low rate of production and relative lack of overt pathology explains how arboviruses manage to confer life long infectivity on a mosquito without affecting its life span.

General Characteristics of Bunyamwera and the Bunyamwera Group

a) Classification

Bunyamwera is the prototype strain of a group containing 14 agents (see Table I) which show serologic cross reaction by complement fixation and hemagglutination inhibition tests. Initially these cross reactions were felt to be strictly within the group, but more recent study has shown interrelation with some other pre-established groups. (11,24) The resulting aggregation is the Bunyamwera supergroup composed of 11 groups plus unassigned viruses.(78) (See Table II.) The antigenic links within the supergroup are not necessarily very strong, and not all members cross react.

b) History

Bunyamwera was first isolated by Smithburn in 1943 from a large pool of Aedes mosquitoes collected at Bunyamwera, an uninhabited area of the Semliki Forest of Uganda. A monkey which had received a

subcutaneous injection of pooled mosquitoes developed severe symptoms at 44 hrs. A post mortem liver sample was injected intracerebrally into mice which developed encephalitis and died. (66) Further work showed it to be an arbovirus sensitive to ether and sodium desoxycholate of size 70 - 105 nm by filtration. (67,73) It was re-isolated from mosquitoes in Tongaland in 1955 in suckling and weaned mice. The infection rate of Bunyamwera and Rift Valley in mosquitoes in this area was found to be 0.2 - 0.5%. (37) The clinical picture of Bunyamwera infection was brought to light by clinical investigations of the agent's oncolytic properties. (40) No beneficial therapeutic effect was discovered, but one subject developed a near-fatal encephalitis with severe bifrontal headache, marked fever, hyperexcitability and laboratory findings indicative of viral encephalitis. This progressed to coma. On subsequent recovery the patient showed decreased mental function and marked amnesia. (68) A young mosquito collector in Tongaland became the first recognized case of naturally acquired Bunyamwera. His serum provided the first virus isolated from a natural human infection. This case presented with mild fever, headache, stiff neck, but little incapacitation. The symptoms lasted two days, after which a rise in anti-Bunyamwera antibody could be detected by neutralization and hemagglutination inhibition tests. Two mosquito virus isolations were made on the same day, in the same area where the boy was working. (38)

Ukauwa, Ilesha and Germiston were also isolated in Africa during this period and were promptly classified as members of the

Bunyamwera group. (54) Accidental laboratory infections with Germiston gave pictures similar to, but less severethan those reported for Bunyamwera. (39)

Calovo, Ilesha, Guaroa and Ukauwa have also been associated with clinical disease. (47)

Two members of this group are endemic in North America. First isolated was Cache Valley from mosquito pools collected in Utah in 1956. (30) Subsequent studies showed a distribution that extended as far as Brazil. (13) In 1961 Tensaw was isolated in mosquito samples taken from Arkansas. The virus is 70 - 122 nm in size by filtration, shows widespread distribution in Alabama, Florida and Georgia, and further isolations have been made from mosquitoes and a dog. (18,19, 20,21) Suckling mice appear to be the only animal in which Tensaw gives overt infection. Dogs show a transient viremia and mosquito transmission from dog to mouse has been accomplished. (71)

c) Replication of Bunyamwera

Intracellular replication of Bunyamwera group was first studied with Guaroa in a mammalian tissue culture system. Using flourescent antibody, acridine orange and electron microscope, virus replication was shown to be strictly cytoplasmic with diffuse areas of RNA production and scattered, descrete foci of viral antigen production. (69) The histopathology of fatal Bunyamwera encephalitis in suckling mouse brain shows interstitial and perivascular edema, neuronal necrosis, a lack of inflamatory response (due to the animal's immaturity) and no

inclusion bodies. Cytopathology first shows at 20 hrs. with the occasional appearance of virions. By 34 hrs. virus is present in the extracellular spaces, Golgi and endoplasmic reticulum membranes increase in quantity, and there are signs of mitochondrial degeneration. The nucleus appears undisturbed. Virus titres within the cell are very high prior to the outset of marked degenerative change. Virus buds into cisternae, attached to the membrane by a stalk which pinches off. Accumulations of nucleoids are not seen. Elongated, striated particles of the same diameter (98 nm) as round virions are also present. In late stages large vacuoles distended with particles, and small vacuoles with individual viruses, are seen. It is felt that virus release is by cell disruption and/or fusion of the small vacuoles' membranes with the plasma membrane followed by virus egestion. This mechanism and morphology seems to be common to all of the Bunyamwera and California group viruses. (51, 52)

Ukauwa virus multiplies in A. aegypti after feeding and injection and in A. canadensis and A. triseriatus following intrathoracic inoculation. In A. aegypti an extrinsic incubation period of 8 days was noted following an infectious meal. Peak titres of 4.3 log mouse LD_{50} in the thorax, 5.3 log mouse LD_{50} in the salivary glands, 4.3 log mouse LD_{50} in the legs and 4.6 log mouse LD_{50} in the gut were reached on day 14 after the infectious meal. With parenteral inoculation infectivity was detected within 12 hrs. and had peaked by 4 days. Virus titres were slightly higher by this method, the highest levels

being found in the thorax. Virus transmission to mice was possible 48 hrs. after infectivity showed in the salivary glands, provided that a threshold titre of at least 4.3 log mouse LD_{50} was present. (53)

A comparative study of Bunyamwera virus reproduction in vertebrate and insect tissues is the objective of this thesis.

Materials and Methods

Materials and Methods

Virus

The prototype strain of Bunyamwera virus from the American Type Culture Collection was obtained in its forty-sixth suckling mouse brain passage.(66) A stock virus preparation was prepared by inoculation of a family of 10 suckling mice aged 2 days with 10^5 mouse LD_{50}. Brains were removed aseptically from mice two days later when signs of encephalitis were observed. The brains were homogenized using a mortar and pestle and suspended in ELY plus 20% calf serum, centrifuged at 2000 rpm for 3 minutes to remove gross tissue debris, and stored at -70°C in 0.1 ml. aliquots in sealed glass ampuoles.

Virus Assay

Virus assays were carried out by intracerebral inoculation with 0.03 ml. of serial tenfold dilutions of test material into groups comprising 3 Swiss mice each aged 3 weeks. Virus caused fatal encephalitis within 5 days. Virus dilutions were made in ELY or sterile 0.15 M sodium chloride plus 20% calf serum. The mouse LD_{50} titres were calculated by the method of Karber.(47)

Blood samples were diluted 1:2 on collection with Alsever's solution. Mosquito organs and whole mosquitoes were ground in a mortar with 0.9 ml. of sterile 0.15 M sodium chloride containing 20% calf serum. Tissue culture scrapings suspended in ELY + 20% calf serum and

tissue culture supernates were also used as starting suspensions.

Serology

Neutralization tests were carried out by mixing 0.15 ml. of undiluted anti-Bunyamwera rabbit serum with 0.15 ml. of serial tenfold dilutions of virus. (45) These samples, along with a serial tenfold dilution virus control, were held at 25°C for 30 minutes prior to intracerebral injection into groups of weaned mice. The virus control determined the number of LD_{50} of virus actually used in the test. Mice receiving unneutralized virus developed fatal encephalitis within five days. The $\log_{10}$ neutralization index was the difference between the control virus titre and that of the serum-virus mixture.

Hemagglutination inhibition antibodies were measured by the method of Clarke and Casals (25) using disposable Microtitre plates. (63) Serial two fold dilutions of 1:5 serum suspensions which had been acetone extracted were tested against 8 hemagglutinating doses of sucrose-acetone extracted, Bunyamwera infected mouse brain antigen.

Preparation of Anti-Bunyamwera Serum

A $10^{7.5}$ mouse LD_{50} suspension of Bunyamwera in 1 ml. of 20% calf serum saline was injected into the marginal ear vein of two New Zealand white rabbits. Serum samples were taken at selected intervals to assess the production of antibody. A second dose of 10^{6} mouse LD_{50} was injected in the marginal ear vein 4 weeks later. One week after this booster injection 20 ml. of blood was collected from an ear vein.

Following centrifugation at 2000 rpm to remove the clot, the serum was removed and stored at -20°C in screw cap vials.

Viremia Experiments

a) Rabbits

Blood samples were drawn from ear veins at 0, 2, 3, 4, 6, and 8 hours after the injection of $10^{7.5}$ mouse LD_{50} into the marginal ear vein of New Zealand white rabbits. These samples were stored in Alsever's solution at -20°C.(23)

b) Mice

1. Intraperitoneal inoculation. Three 3 week old mice each received 0.1 ml. of $10^{6.33}$ LD_{50}/ml. and another three received 0.1 ml. each of 10^{2} LD_{50}/ml. of Bunyamwera intraperitoneally. Blood was removed from the tail vein and by cardiac puncture when symptoms of encephalitis were seen. This was placed in Alsever's solution and stored at -70°C prior to testing.

2. Intracerebral inoculation. Six three week old mice received $10^{4.8}$ mouse LD_{50} intracerebrally in 0.03 ml. each of a Bunyamwera suspension. They were bled from the tail vein at daily intervals until they died from encephalitis. These blood samples were stored in Alsever's solution at -70°C until used.

3. Artificial viremia. Mice were injected in the tail vein with 0.5 ml. of a $10^{7.5}$ LD_{50}/ml. Bunyamwera suspension in a method analogous to that used by Chernesky for creating artificial viremias

in rabbits with feeding ticks. (23) Blood samples were taken at the time of infection, 30 minutes, 1 hour, 2 hours later, and then tested for virus titres.

Tissue Culture

Infected vertebrate tissue cultures were used as a comparison for insect tissue virus growth. H.Ep. 2, mouse macrophage, and primary chick embryo fibroblast (CEF) cultures were tried. CEF was found to be most satisfactory in terms of virus production and convenience.

CEF cultures were produced by aseptically removing embryos from eggs which had been incubated at 37°C for 10 to 12 days. The limbs, eyes, and beak were removed. The remaining tissue was placed in a sterile petri plate and finely minced with scissors. Hanks' balanced salt solution +0.25% trypsin was added, and the mixture agitated on a magnetic stirrer for five minutes at room temperature. This supernate was discarded and the tissue transferred to a trypsinizing flask. The washing was repeated, this supernate also being discarded.

Eight ml. of 0.25% trypsin-HBSS was added per embryo and stirred for 30 min. at 25°C. The supernate was pipetted into a 40 ml. centrifuge tube containing 1.5 ml. of calf serum. This extraction was repeated three times. The cells were deposited by centrifugation at 1500 rpm for 10 minutes, and the supernate was discarded. The upper part of the packed cells which was free of erythrocytes, was removed and suspended in GLY. The cell concentration of this suspension was

estimated by diluting a sample 10^{-2} in 0.1 M citric acid and 0.1% crystal violet and counting in a hemocytometer. The concentration was adjusted to 2 x 10^6 cells/ml. using GLY plus 5% calf serum. The resulting suspension was dispensed in 5 ml. amounts into 60 mm. plastic petri plates which were incubated in small, sealed battery jars at 37°C until the cells formed monolayers. (41)

Infection of CEF Tissue Cultures

After the tissue culture media had been decanted, the cells were covered with 1 ml. of GLY-5% calf serum virus suspension containing $10^{5.2}$ mouse LD_{50} of Bunyamwera per ml. After 30 minutes at 37°C the inoculum was removed and replaced with 5 ml. of GLY plus 5% calf serum maintenance medium, the plates were then incubated in sealed small battery jars at 29°C or 37°C. These were checked daily for cytopathic effect using an inverted stage light microscope. Virus titres were measured at 0, 4, 8 and 12 hours, and 1, 2, 4, and 10 days. Cell samples were removed for electron microscopy at the same intervals.

Mosquito Colonies

a) Adult Female Aedes aegypti mosquitoes were provided from the colony at the British Columbia Research Council Laboratories at the University of British Columbia. These were kept in cardboard and nylon mesh containers within a bell jar at 80% humidity and 25°C. Maintenance feeding was accomplished by placing split raisins or cotton wool balls

soaked in a sucrose-water solution on the cage tops.

b) Aedes aegypti eggs were also provided by the British Columbia Research Council Laboratory. These were hatched by placing them in a deionized water plus yeast extract solution in a pan within a nylon mesh holding cage. When pupae matured into adults they were removed to small cardboard and nylon cages and kept as in a.

c) Wild Aedes vexans and Aedes canadensis collected by hand at sites around Penticton, British Columbia were air freighted to Vancouver in small cages within a tape-sealed styrofoam cooler. These were then transferred to small holding cages and maintained as in a. These two species were pooled for experiments.

Mosquito Infection

a) Inoculation

Quarter inch Pyrex tubing was heated over a bunsen burner flame and drawn out till it had a diameter of approximately 10 μ. This was attached to a 0.25 ml. syringe by a rubber tube coupling. The plunger was operated by a low pitch screw which minimized volume variation in serial injections. By weighing volumes expressed from the syringe it was found that one quarter turn of the screw delivered 1 mg. (.001 ml.) of the inoculum. The virus stock was suspended in sterile saline plus 10% calf serum and pulled into the syringe. Single mosquitoes were aspirated from the holding cage and placed into a three inch length of one inch glass tubing which had one end covered with

nylon mesh and the other stoppered with cotton wool. Carbon dioxide fumes from a tank or a bottle of dry ice were directed into this chamber to anaesthetize the mosquito, which was then tipped onto the stage of a dissecting microscope. The syringe's capillary tube was inserted through the cuticle of the thorax midway between the base of the wing and base of the hind leg and the screw was given a 3/4 turn. The insects were then placed in holding cages in the insectary at 25°C until they were sampled.

b) A sugar cube was placed on the mesh top of a cage of mosquitoes which had been starved for one day. A measured dilution of virus in defibrinated rabbit blood was pipetted onto the cube. The mosquitoes which fed within three hours and were engorged were removed to a holding cage at 25° to await sampling. The titre of virus on the cube was measured before and after feeding.

c) An adult Swiss white mouse was secured to a table with adhesive tape, lightly anaesthetized with ether, and its abdomen shaved. A 26 gauge needle was inserted into the tail vein and 0.5 ml. of virus in saline-10% calf serum was injected. A mosquito cage was immediately secured over the mouse in a manner which would allow the insects to feed on the mouse through the cage's mesh. Blood samples for viremia tests were taken from the tail vein at the time of injection and at the end of feeding (30 minutes). Engorged mosquitoes were removed and placed in a holding cage at 25°C.

In all cases a sample pool was taken immediately after feeding (0 hours) to titrate the infectivity of the meal. Samples for

virus titration and electron microscopy were taken at 0, 2, and 12 hours, and 1, 2, 3, 4, and 10 days.

Pools of three mosquitoes were anaesthetized by placing them in -4°C for five minutes and dissected in sterile saline with 20% calf serum under a dissecting microscope. The wings were removed using a sterile scalpel and discarded. The legs were cut off using a new sterile blade and pooled in a mortar for titration. Salivary glands were expressed by traction on the head of the mosquito while applying gentle pressure to the thorax. These were divided with a sterile blade -- half for electron microscopy and half pooled in a mortar for titration. The gut was removed by nicking the wall of the abdomen at the last segment and extracting the gut by gentle traction on the separating segment. The midgut was cut away and halved for titration and electron microscope procedures. Material for titration was placed in screw cap vials and stored at -70°C.

Transmission

Suckling mice were secured to a bench top with tape and cages containing individual infected mosquitoes were placed over them. Engorged mosquitoes were harvested for titration and electron microscopy after two hours and mice that had been bitten were returned to their mother. The criterion for transmission of infection was development of signs of encephalitis within five days of being bitten. The harvested brains of these moribound mice were sampled for infectivity.

Mosquito Organ Culture

Primary mosquito gut cultures were produced to provide a convenient and easily controllable arthropod tissue environment for virus replication. Tissues were grown in Leighton tubes at 28°C using Grace's insect tissue culture medium (Grand Island Biological Co.) plus 5% calf serum in place of insect hemolymph. (28)

a) Gut tissue was obtained from sterile first instar larvae of Aedes aegypti. Eggs were laid on damp strips of filter paper. These were allowed to dry and then were washed by dipping the strips into 95% ethanol, drying them in a sterile petri plate and repeating the procedure two more times. The eggs were hatched in a sterile 0.5% yeast extract-water solution. The anterior thirds of the larvae were cut off using a sterile scalpel blade and discarded. The remainder was diced with a sterile scalpel blade, suspended in 1 ml. growth medium per five larvae, dispensed in 1 ml. amounts into Leighton tubes and incubated at 28°C until cell attachment was seen. The cells are fibroblast-like and grow from the ends of tissue fragments. Degeneration of the cells took place within one week after culturing.

b) Adult Aedes aegypti were fed on mice. The distended midguts were removed and placed into Grace's insect tissue culture medium along with antibiotics to supress bacterial and yeast growth. Two guts in 1 ml. of medium were used per tube which was then placed in a 29°C incubator. These preparations were viable for 10 to 12 days.

Electron Microscopy Techniques

Electron microscopy was used to study the cytopathology of Bunyamwera in both mosquito and chick embryo tissues. (55)

a) Thin sectioning

Tissue culture specimens were scraped from the glass using a rubber policeman, pelleted by centrifugation and immediately placed into 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for 30 minutes at 4°C. This was given three 1 hour washes in phosphate buffered sucrose (0.2 M) at 4°C. Tissues were post fixed in 1% OsO_4 pH 7.2 phosphate buffer at 4°C. The fixative was aspirated and the sample was given three 15 minute washes in 4°C distilled water. Dehydration was carried out by passing the specimen through a graded series of ethanol concentrations: 15 minutes in 30%; 5 minutes each in 50%, 75%, and 95%, and three 5 minute changes of absolute ethanol.

Samples were embedded in Epon 812 resin using DDSA and methylnadic anhydride as curing agents. The procedure involved two 15 minute changes of propylene oxide, a one hour change of Epon-propylene oxide 1:1 and one hour of Epon alone. The samples were transferred to Beem capsules which were filled with Epon, incubated at 37°C for 12 hours to facilitate resin penetration, and cured at 60° for 36 hours.

Mosquito samples were treated in the same manner as tissue cultures except all times in the fixation and dehydration procedures were doubled to counteract poorer tissue permeability.

The hardened blocks were cut using a LKB III microtome and a glass knife. The sections were collected on carbon coated formvar films on 400 mesh copper grids. These were positively stained with a saturated alcoholic solution of uranyl acetate for 1 minute, washed with distilled water for five minutes, and then post-stained with Reynold's lead citrate at room temperature for 10 to 15 minutes. (60) Grids were washed with distilled water and air dried prior to examination with a Phillips EM 300 electron microscope.

b) Negative Staining

The cells of an infected mouse brain preparation were disrupted by rapid freeze-thawing. The sample was placed on a metal plancet which was alternatively touched to a block of dry ice and the palm of a hand. The cell clumps were pelleted by low speed centrifugation and the pellets resuspended in distilled water. A drop of this suspension was mixed with a drop of 3% PTA (phosphotungstic acid) pH 6.5 on a 300 mesh carbon and formvar coated copper grid. The excess liquid was removed by touching the edge of the grid with filter paper. The grid was air dried and observed in a Phillips EM 300 electron microscope.

c) Immunochemical Staining for Electron Microscopy

Rabbit anti-Bunyamwera serum was treated with 1.436 M sodium sulfate to isolate the globulin fraction. This fraction was conjugated

with six-time recrystallized cadmium-free ferritin by the method of Rifkind et al utilizing toluene 2, 4- diisocyanate. (61,65)

Tissue samples were fixed in phosphate buffered formalin 5% at pH 7.2 for 10 minutes, then quick frozen in an alcohol-CO_2 bath and thick sectioned on a cryostat. These sections were stained with the conjugate, then washed, fixed with phosphate buffered osmium tetroxide 1% at pH 7.2 and embedded for electron microscopy.

Results

Results

Viremia Studies

a) Rabbits

Two rabbits received $10^{7.5}$ mouse LD_{50} of Bunyamwera (BUN) virus in 1.0 ml. amounts. Average viremia titres were $10^{4.3}$ mouse LD_{50} per ml. at zero hours decreasing to $10^{3.1}$ mouse LD_{50} per ml. at four hours, and by eight hours virus became undetectable (Fig. 1). Virus was cleared at a rate of $10^{0.5}$ mouse LD_{50} per ml. per hour.

Serum samples taken prior to inoculation did not possess any anti-BUN antibodies. Neutralizing antibody first appeared at nine days post injection at a neutralizing index of 2.5 mouse LD_{50} per ml. of serum. By 25 days this had risen to 4.0 mouse LD_{50} per ml. of serum. Booster injections of $10^{6.0}$ mouse LD_{50} given at eight weeks were followed by an antibody titre of 5.4 mouse LD_{50} neutralizing doses per ml. one week later (Fig. 2).

Control virus incubation at 25°C with non-immune serum showed an infectivity loss of $10^{1.0}$ mouse LD_{50} per ml. per hour for the first three hours.

Anti-hemagglutinin was first detected seven days after infection at a titre of 20 and increased to a maximum of 400 seven days after the second injection (Fig. 2).

b) Mice

1. After Intracerebral and Intraperitoneal Infection

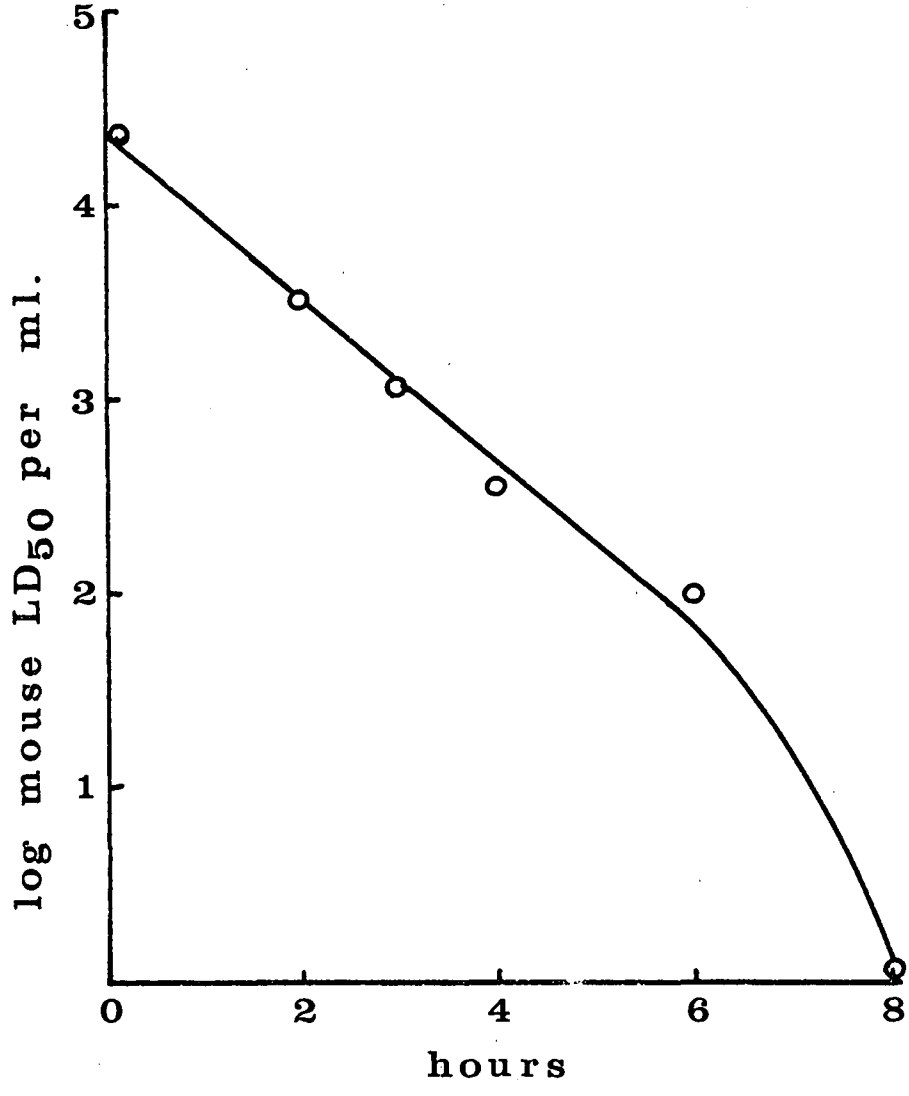

Fig. 1. Viremia in New Zealand white rabbits following injection of $10^{7.5}$ mouse LD_{50} per ml. of Bunyamwera virus intravenously.

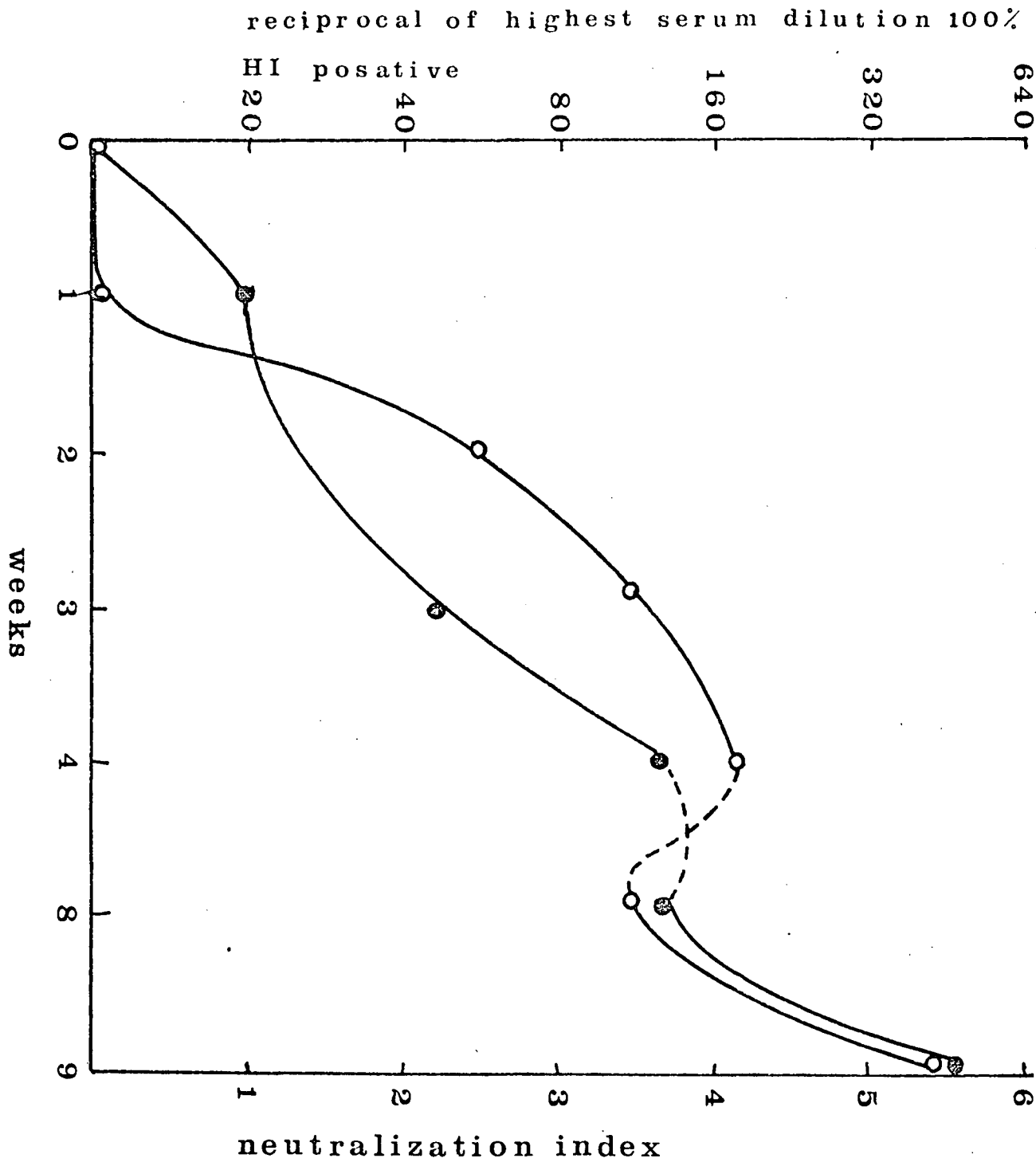

Fig. 2. Serological response of rabbits to Bunyamwera virus following intravenous injection of $10^{7.5}$ mouse LD_{50} of virus as tested by hemagglutination inhibition (●) and virus neutralization (○).

Intraperitoneal injection of $10^{5.3}$ LD_{50} BUN virus resulted in a viremia of $10^{2.0}$ mouse LD_{50} per ml. at three days. After intracerebral injection of $10^{4.8}$ LD_{50} of virus the peak titre of viremia, $10^{4.3}$ mouse LD_{50} per ml., was attained at four days, by which time the mice were moribund (Fig. 3).

2. Artificial viremia

Mice were inoculated intravenously with $10^{7.7}$ mouse LD_{50} of Bunyamwera. Blood samples taken at the time of infection showed $10^{6.3}$ mouse LD_{50} per ml. Mosquitoes were fed on the mice. Another sample taken at 30 minutes post infection, showed a viremia of $10^{5.6}$ mouse LD_{50} per ml. of blood (Fig. 4). During the first two hours post infection virus was cleared at a rate of $10^{2.0}$ mouse LD_{50} per ml. per hour.

Virus Development in Tissue Culture

a) H.Ep. 2 cells

H.Ep. 2 monolayers were infected with $10^{6.5}$ mouse LD_{50} of BUN virus. Following a steady decline of infectivity to undetectable levels at four hours, initial evidence of virus production was observed after 12 hours. The peak virus titre ($10^{6.8}$ mouse LD_{50} per ml. of cell-supernate suspension) was attained at two days following which titres declined due to complete destruction (Fig. 5).

b) Chick Embryo Fibroblasts at 37°C

Within two hours after inoculation of CEF monolayers, virus titres declined to zero from an initial $10^{5.2}$ mouse LD_{50} per ml. Thereafter the virus titre increased to a peak of $10^{6.2}$ mouse LD_{50} at 24 hours (Fig. 6). No virus was recovered at four days, by which time cell destruction was complete.

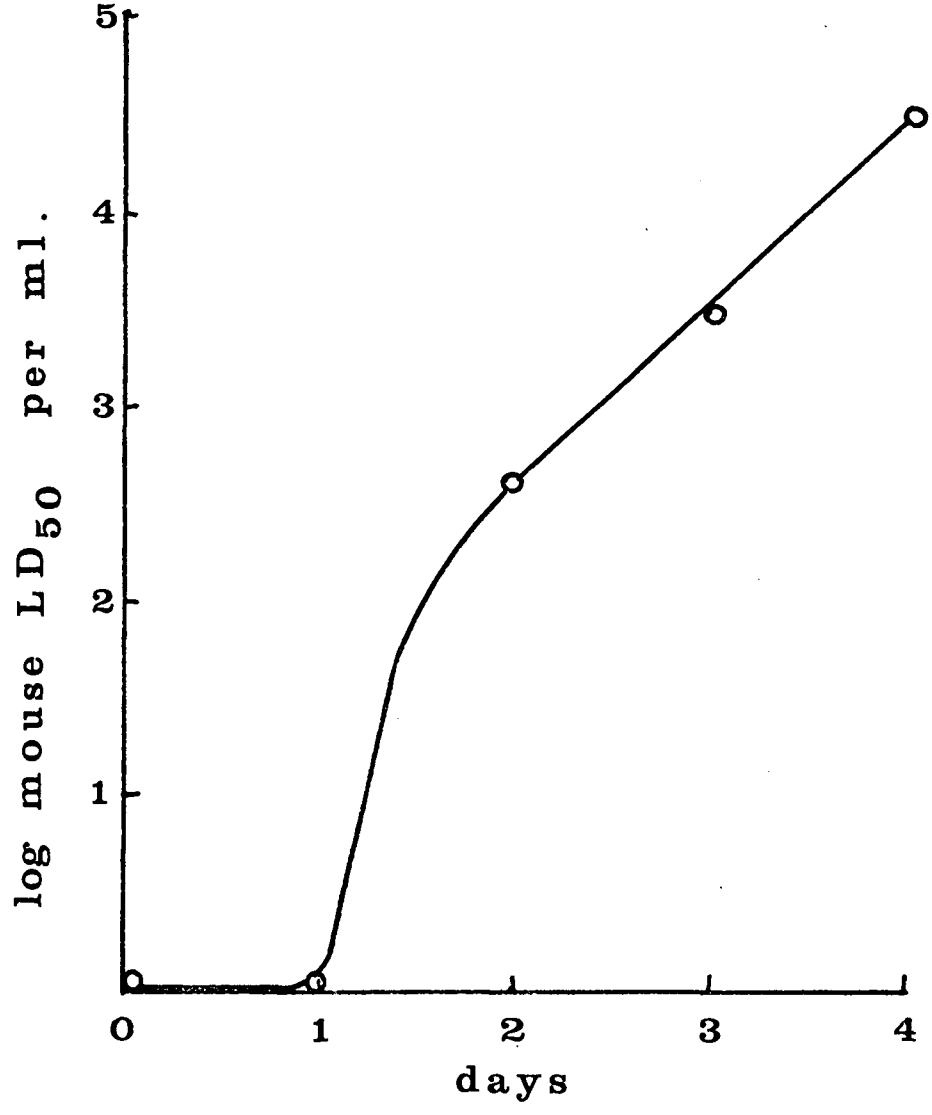

Fig. 3. Viremia in Swiss white mice following intracerebral injection of $10^{4.8}$ mouse LD_{50} of Bunyamwera virus.

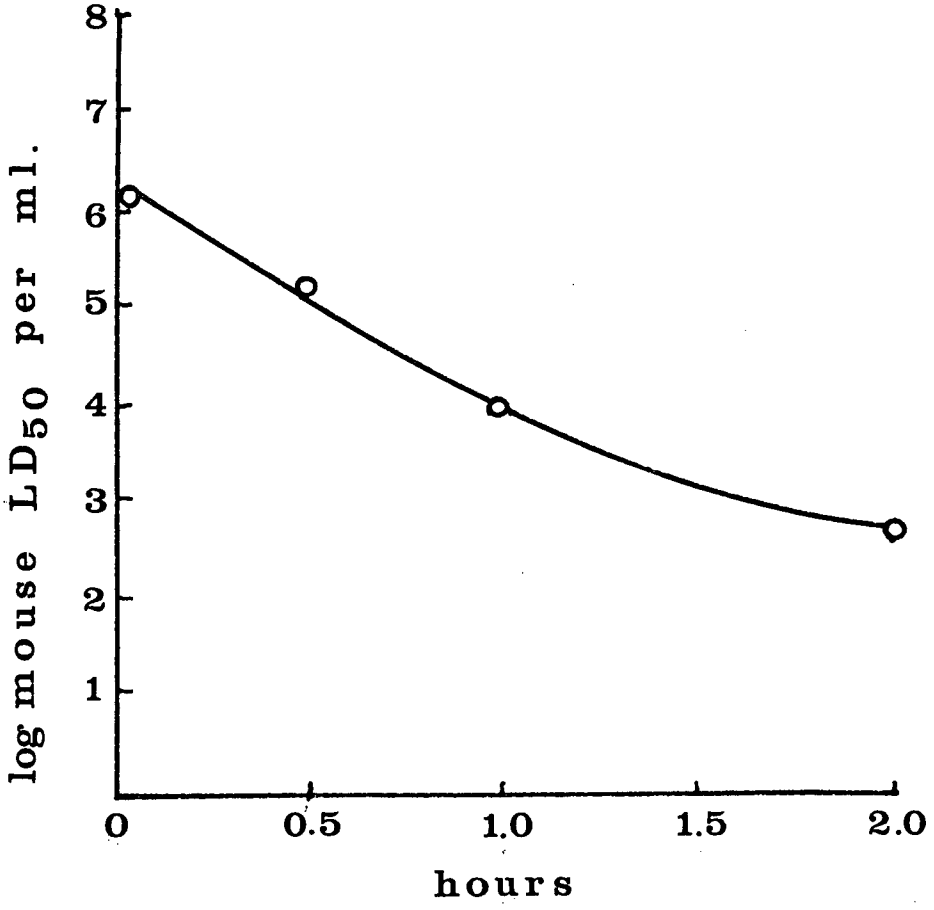

Fig. 4. Artificial viremia in Swiss white mice following intravenous injection of $10^{7.7}$ mouse LD_{50} of Bunyamwera virus.

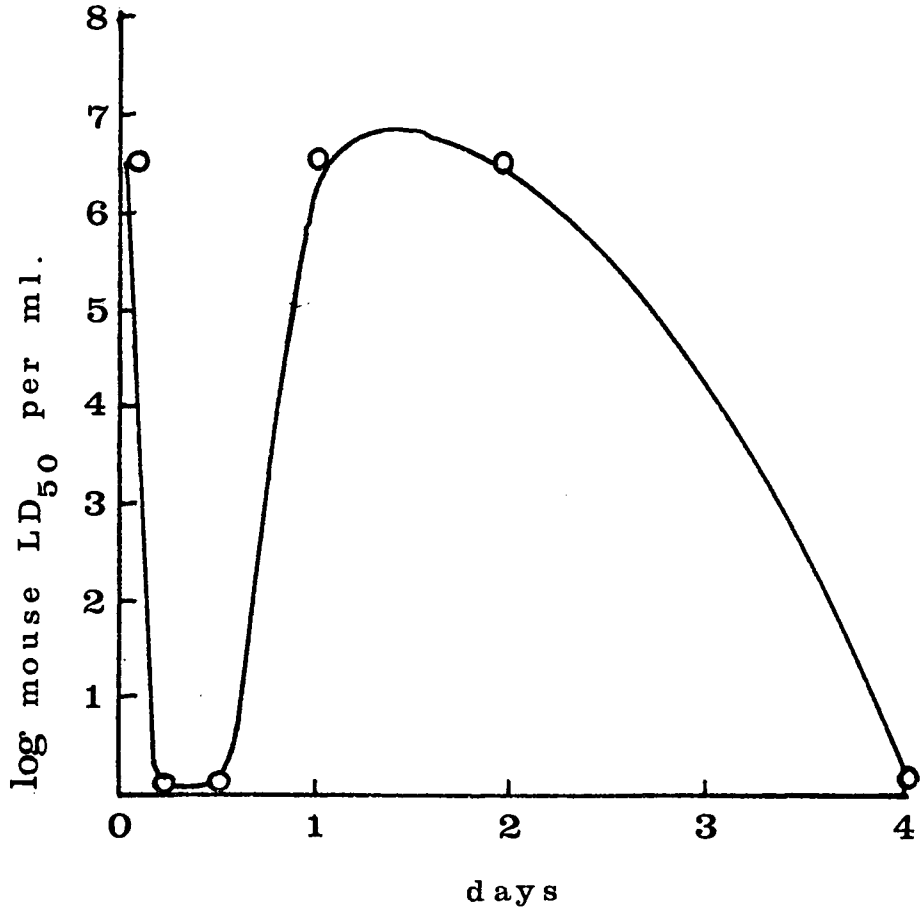

Fig. 5. Development of Bunyamwera virus in H.Ep. 2 cells following infection with $10^{6.5}$ mouse LD_{50} per ml.

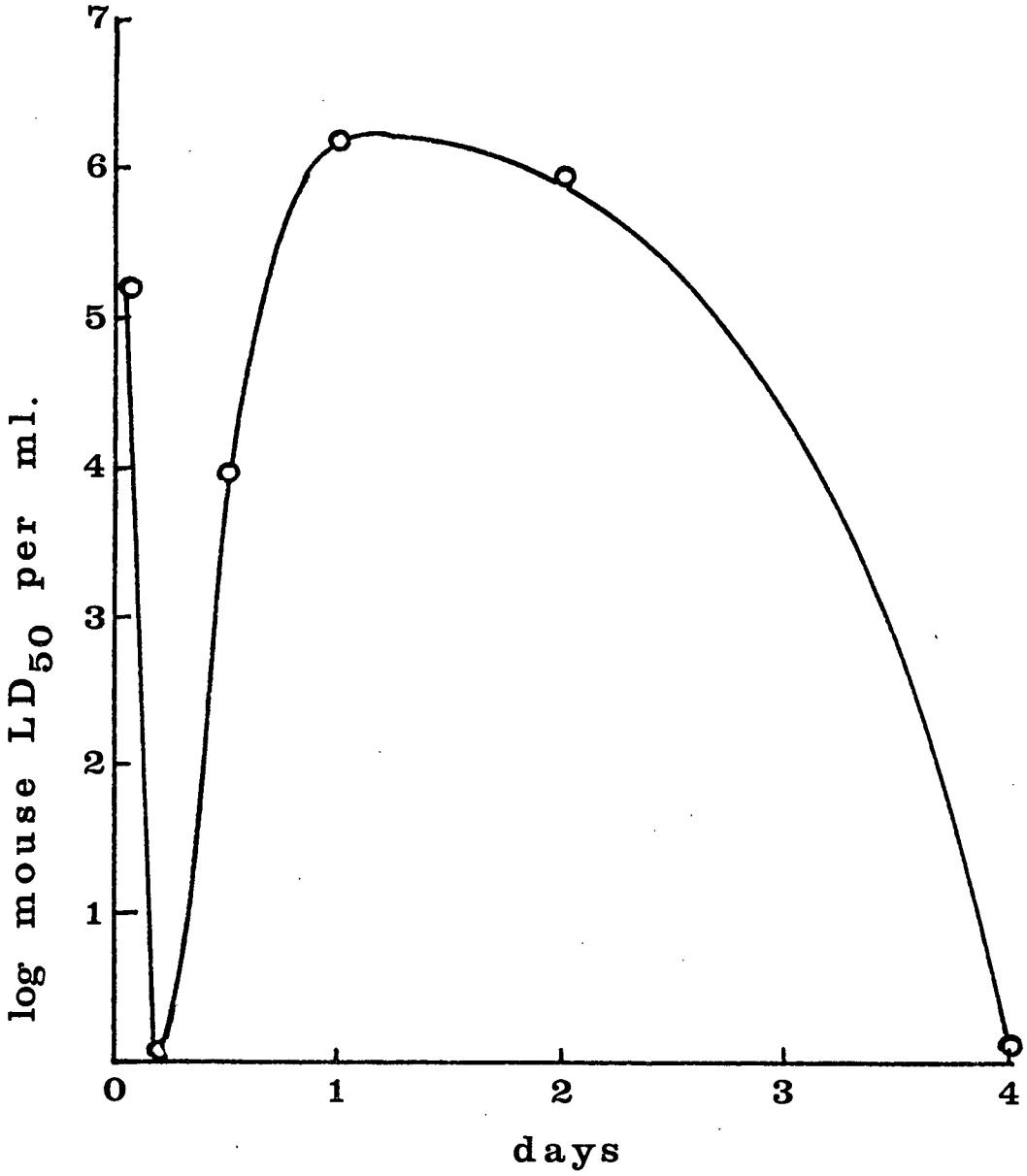

Fig. 6. Development of Bunyamwera virus in chick embryo fibroblasts following inoculation with $10^{5.2}$ mouse LD_{50} per ml. and incubation at 37°C.

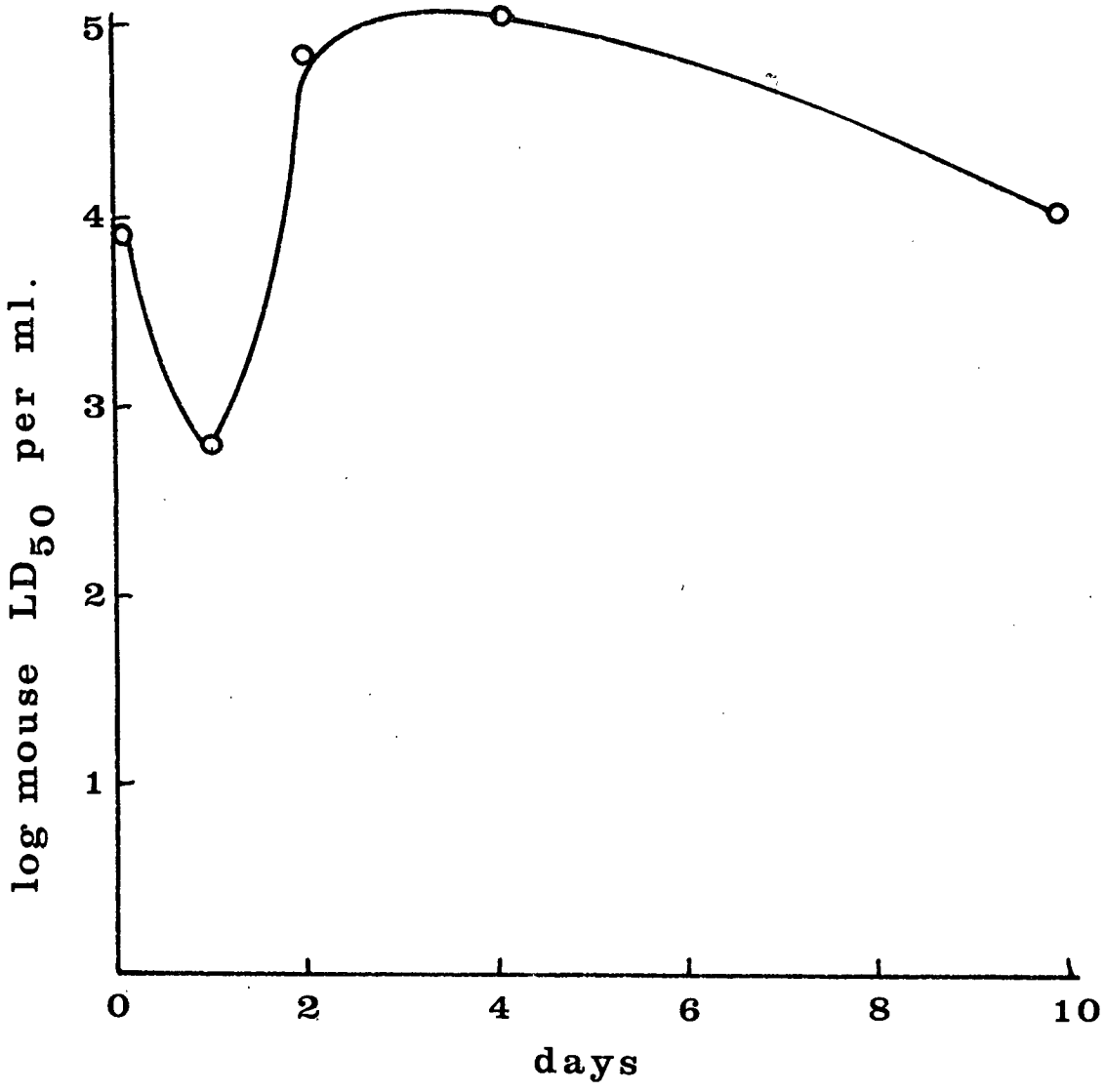

Fig. 7. Virus development in chick embryo fibroblasts infected with $10^{3.7}$ mouse LD_{50} per ml. of Bunyamwera virus and incubated at 29°C.

c) Chick Embryo Fibroblasts at 29°C

The virus titre declined from an initial $10^{3.7}$ mouse LD_{50} to $10^{2.7}$ at 12 hours, followed by an increase to $10^{5.0}$ at four days and subsequent decrease to $10^{4.0}$ at 10 days (Fig. 7). At that time complete destruction was observed in 90% of cells.

Virus Growth in Whole Mosquitoes

Mosquitoes were injected with a blood-virus mixture containing 10^{7} mouse LD_{50} per ml. of virus. Pools titrated immediately after infection contained $10^{5.6}$ mouse LD_{50} per mosquito for the fed insects and $10^{5.5}$ mouse LD_{50} per mosquito for injected insects.

a) Injection of Aedes vexans and Aedes canadensis Pools

The virus content of whole mosquitoes was determined daily following intrathoracic injection of mosquitoes with $10^{3.2}$ mouse LD_{50} per insect. Infectivity declined to $10^{1.4}$ mouse LD_{50} at 1 day, followed by an increase to $10^{2.6}$ at 2 days and a maximum of $10^{5.0}$ at 10 days (Fig. 8).

After injection, virus infectivity was rapidly lost from the hemolymph, but infectivity was maintained in salivary gland and midgut organ samples. Both gut and salivary tissue demonstrated active virus multiplication two days post injection, and virus was found in leg samples. Virus titres at ten days were $10^{5.2}$ mouse LD_{50} in salivary glands, $10^{4.0}$ mouse LD_{50} in gut and $10^{2.7}$ mouse LD_{50} in leg samples (Fig. 9).

Hydrachnellid mites infect many mosquito species and pass

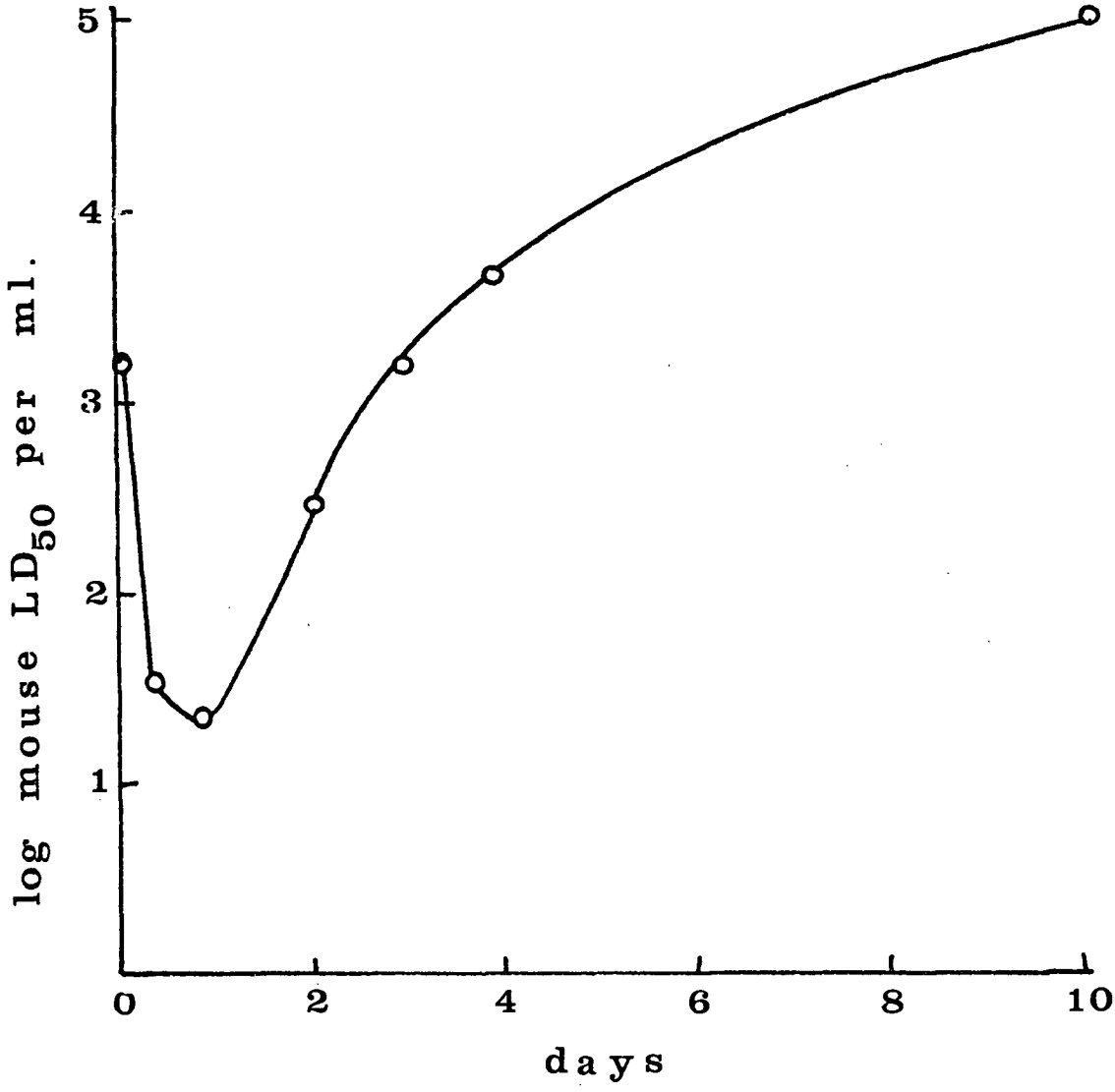

Fig. 8. Virus development in Aedes vexans and Aedes canadensis pools following intrathoracic injection of $10^{3.2}$ mouse LD_{50} of Bunyamwera virus.

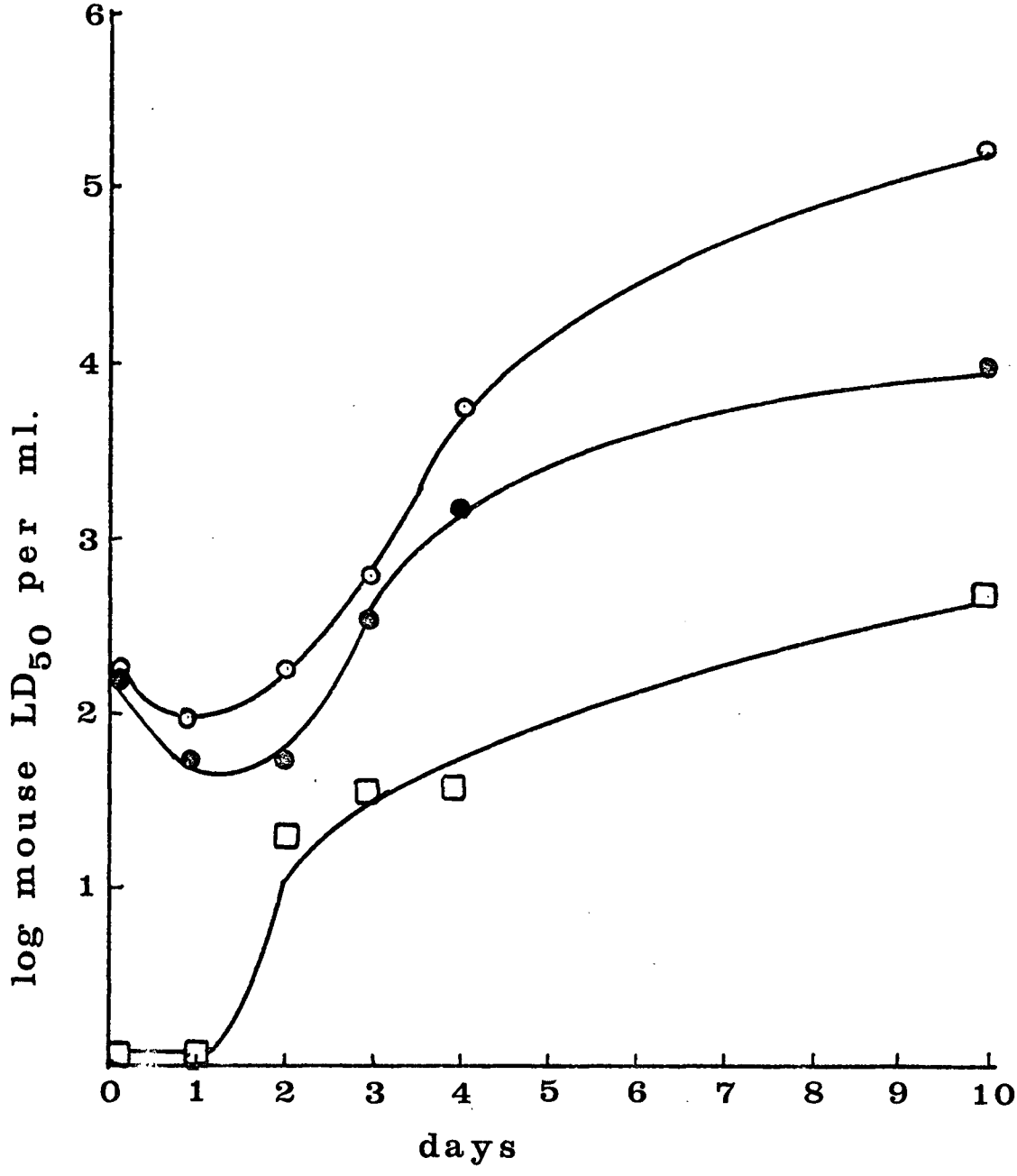

Fig. 9. Virus titres in salivary glands (o), gut (●), and leg (□) samples from Aedes vexans and Aedes canadensis pools following intrathoracic injection with $10^{2.2}$ mouse LD_{50} of Bunyamwera virus.

from insect to insect when mosquitoes are in close contact. (33,34) Mites taken from wild mosquitoes at the end of the ten day virus incubation period contained no detectable virus. Therefore, mosquito to mosquito transmission of virus by mites is not probable.

Transmission of virus by biting suckling mice was accomplished by two of three mosquitoes 10 days after injection, but not earlier. Thus a salivary gland virus titre of $10^{5.2}$ correlated with the ability of a mosquito to transmit BUN virus to mice.

b) Aedes vexans and Aedes canadensis Fed on Virus-Blood Mixtures

Mosquitoes fed on virus-Blood mixtures containing $10^{6.5}$ mouse LD_{50} per ml. ingested $10^{4.0}$ mouse LD_{50}. At eight days the virus levels were $10^{4.0}$ mouse LD_{50} and declined to $10^{2.8}$ mouse LD_{50} by 17 days. Virus transmission was not accomplished with these pools.

c) Aedes aegypti Injected with Virus

Mosquitoes injected with $10^{3.3}$ mouse LD_{50} of BUN virus were sampled over a period of four days. An initial fall in virus titre ended by 12 hours, by two days virus levels had risen to $10^{5.0}$ mouse LD_{50}, and at four days the titre was $10^{5.2}$ mouse LD_{50} per mosquito (Fig. 10).

d) Aedes aegypti Fed on Viremic Mice

Mosquitoes were readily infected with substantial amounts

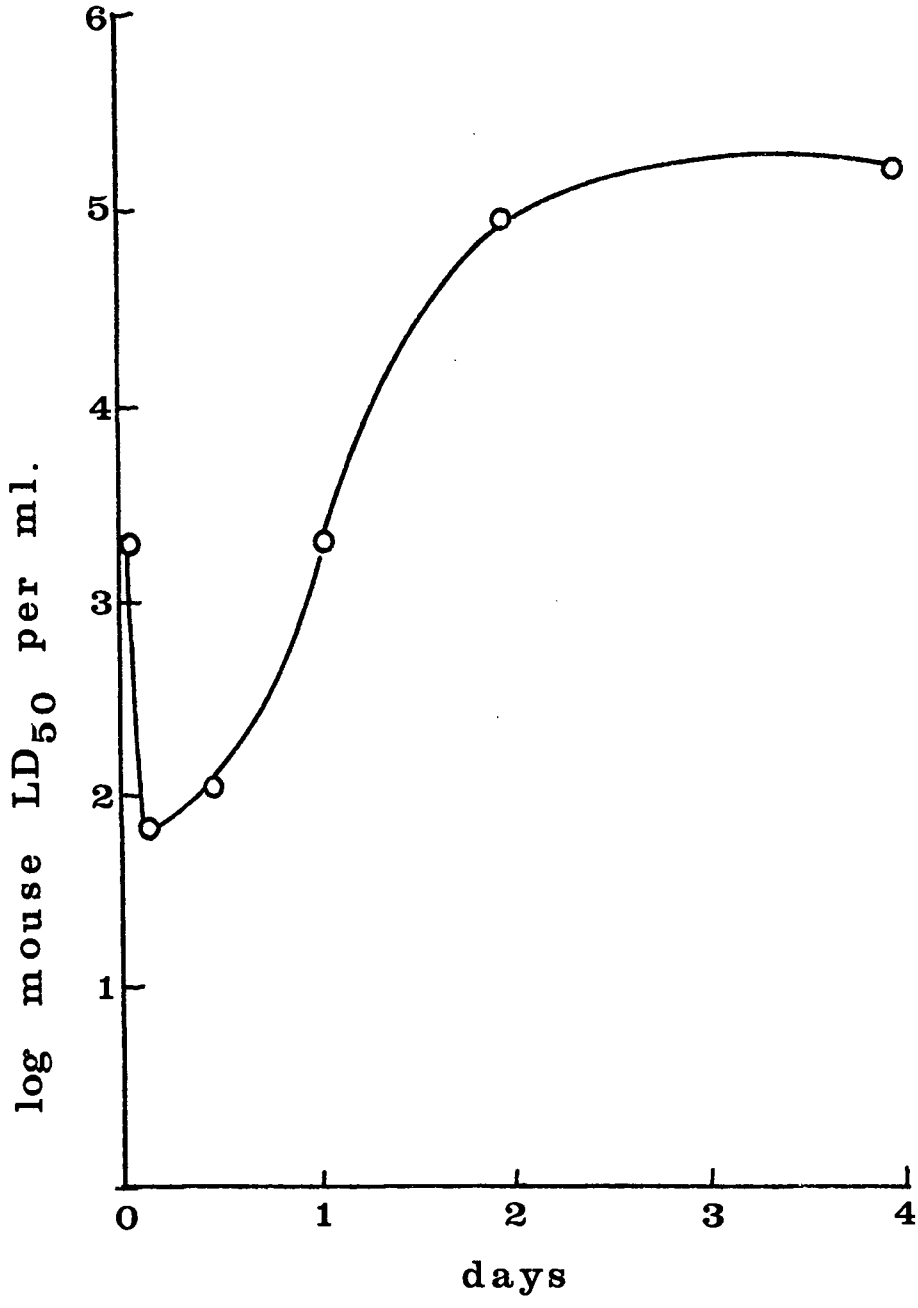

Fig. 10. Virus development in Aedes aegypti following intrathoracic injection with $10^{3.3}$ mouse LD_{50} of Bunyamwera.

of virus by feeding on mice with artificial viremias. Gut levels of $10^{4.6}$ mouse LD_{50} were attained at the time of the meal. This fell slowly to $10^{2.6}$ mouse LD_{50} at one day and rose to a peak level of $10^{3.3}$ mouse LD_{50} by 10 days. The detection of virus in the salivary glands followed the dissemination of virus in the hemolymph as monitored by titration of legs. Salivary gland levels reached $10^{5.0}$ mouse LD_{50} by 10 days (Fig. 11). At this time transmission of virus to mice was demonstrated in both pools tested.

Insect Tissue Culture

a) Gut Cells from First Instar Larvae

BUN multiplication occurred at a low level in larval tissue cultures. Initial titres of $10^{5.7}$ mouse LD_{50} per ml. fell to undetectable levels by one day. The peak titre of $10^{3.0}$ mouse LD_{50} per ml. was reached at four days. Cellular degeneration was evident at this time and by five days virtually all tissue fragments were destroyed and had detached from the glass. Cell death by five days was non-specific because the cells in the control tube showed a similar cycle.

b) Mosquito Gut Organ Culture

Whole gut cultures infected with BUN virus at $10^{3.7}$ mouse LD_{50} per ml. dropped to a titre of $10^{1.8}$ mouse LD_{50} per ml. at 12 hours. Rapid growth in the second 12 hour period brought the titre to $10^{5.0}$ mouse LD_{50} per ml. at one day. Peak titres of $10^{6.0}$ mouse

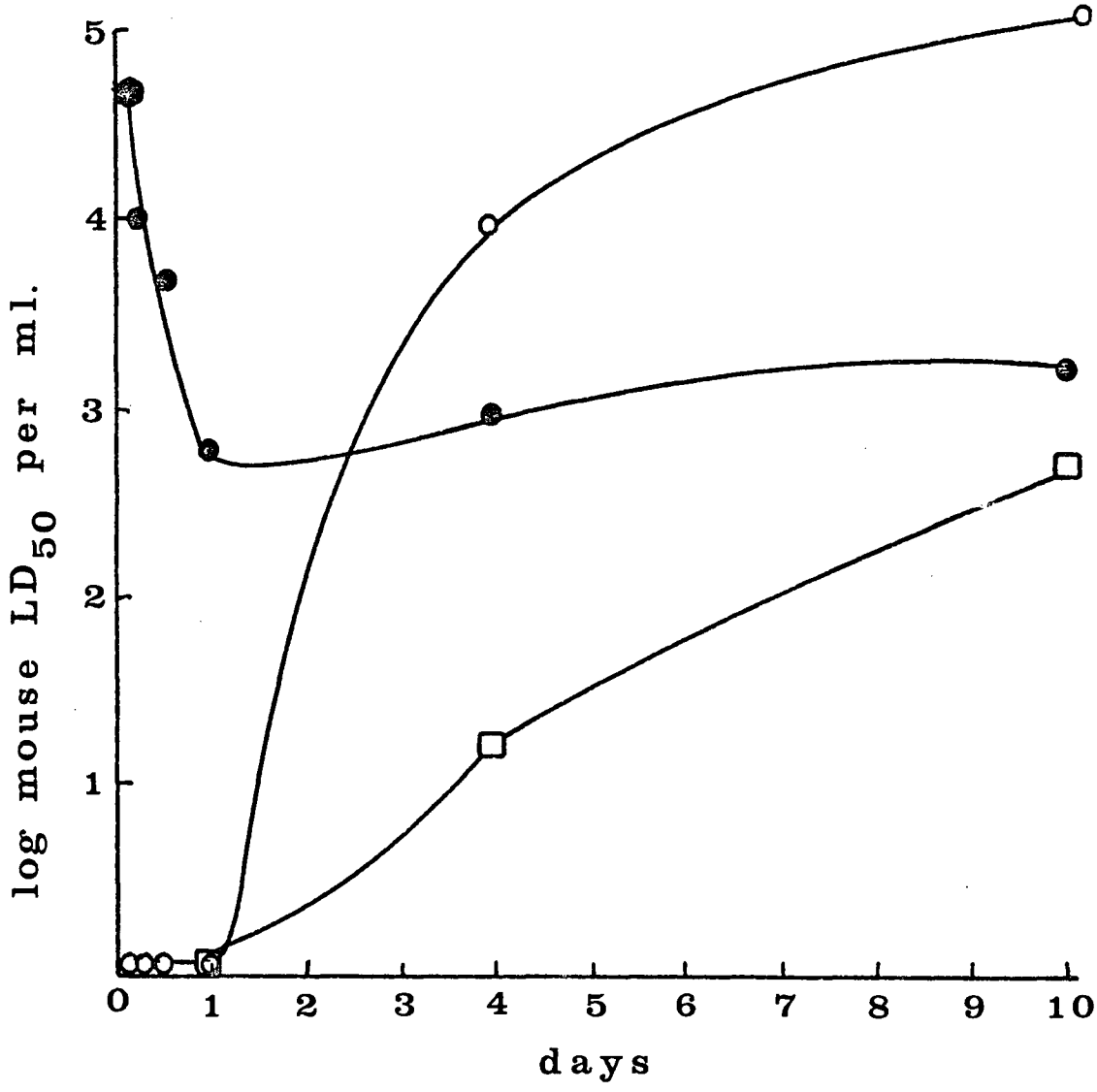

Fig. 11. Bunyamwera virus development in salivary gland (o), gut (●), and leg samples (□) from Aedes aegypti fed on Swiss white mice with a viremia of 10^6 mouse LD_{50} per ml.

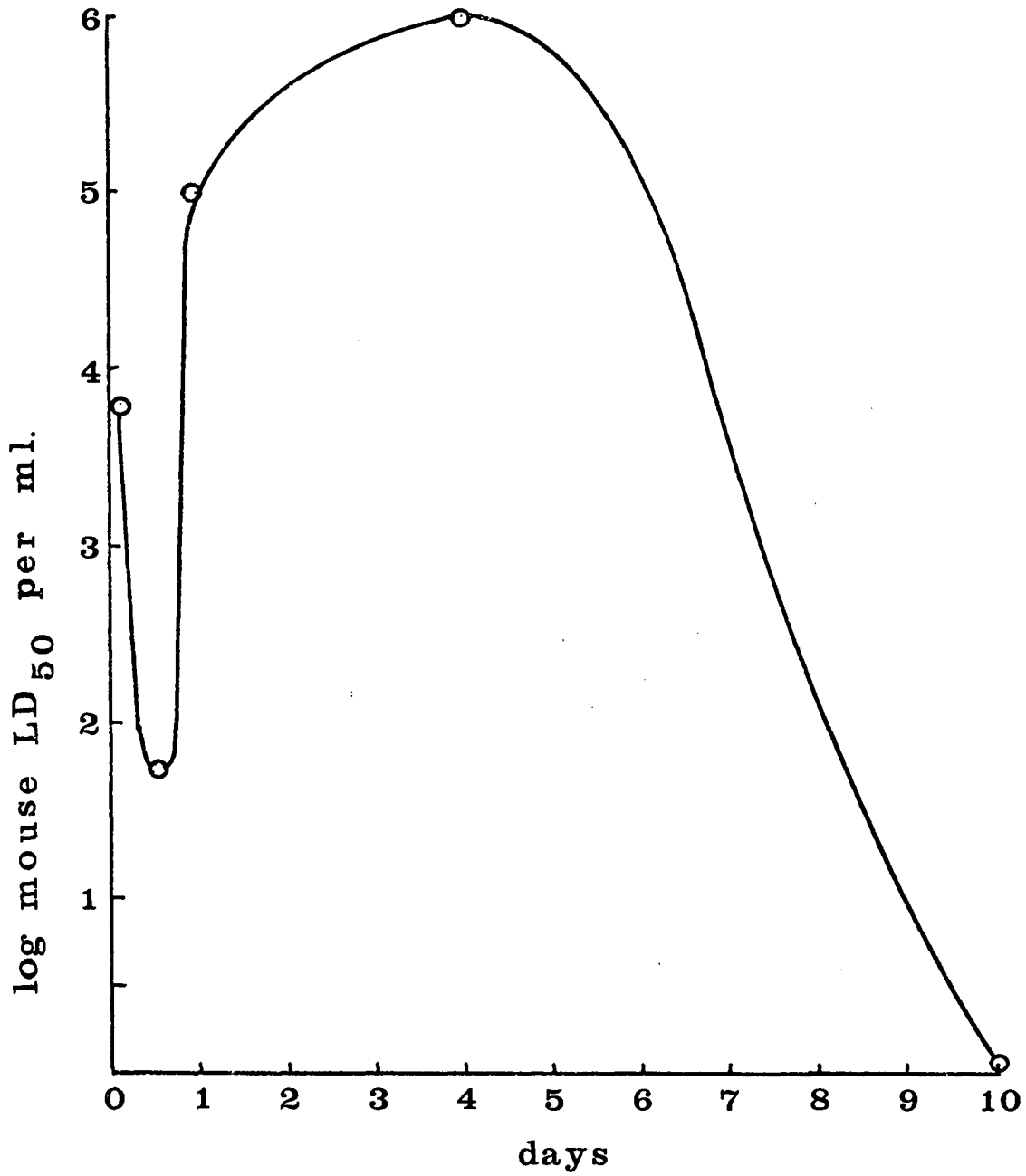

Fig. 12. Virus development in Aedes aegypti gut organ culture following inoculation with $10^{3.7}$ mouse LD_{50} per ml. of Bunyamwera virus.

LD_{50} per ml. were achieved at four days after which they dropped to undetectable levels by ten days. The reason for loss of cell viability in this experiment probably arose from the culturing technique rather than the virus infection, as the control cultures also lost viability by ten days (Fig. 12).

Electron Microscopy

a) Negative Stain Using Phosphotungstic Acid

A suspension of freeze-thawed BUN infected mouse brain was negatively stained and examined in a Phillips EM 300 electron microscope. In addition to cell debris and myelin-like structures numerous particles with a mean diameter of 100 nm were observed. In cases where the external structure of the particles had been disrupted a loosely defined inner component of 48 nm mean diameter was seen. It was not possible to positively identify these as virus as structural definition was poor. Uninfected mouse brain did not show a similar pattern of 100 nm particles.

b) Thin Sectioning: Chick Embryo Fibroblasts

Thin sections of uninfected chick embryo fibroblast cultures showed well preserved micro anatomy with intact cell membranes, mitochondria, and undistorted endoplasmic reticulum. Small secretory vacuoles were occasionally seen, but virus was not detectable intra or extra cellularly (Fig. 13).

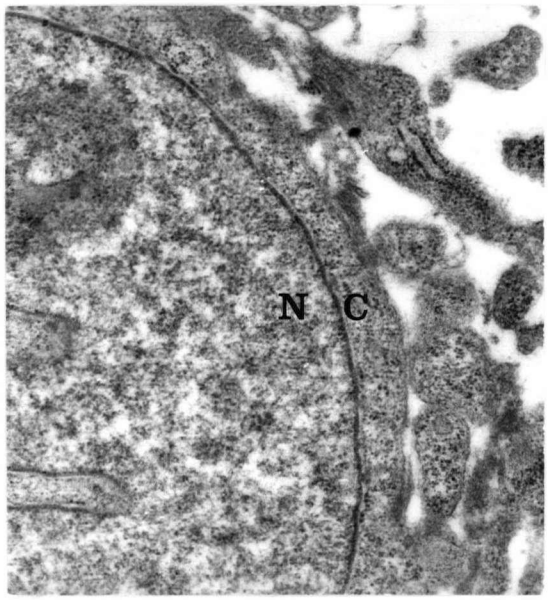

Fig. 13. Uninfected chick embryo fibroblast cell showing normal nuclear and cytoplasmic structure. Magnification x20,000.

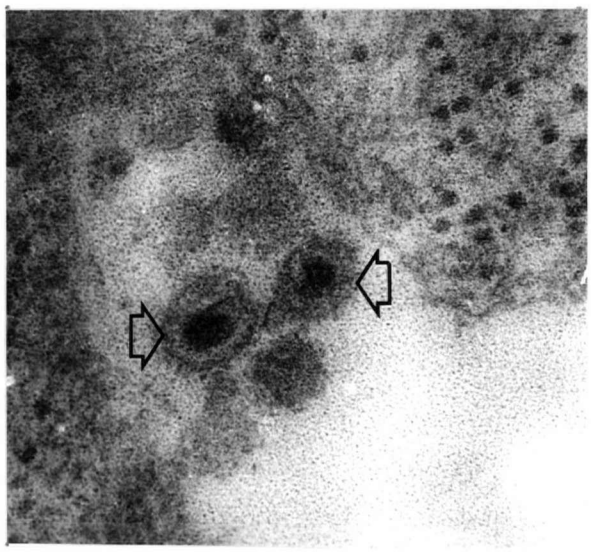

Fig. 14. Virus-like particles at the cell membrane of chick embryo fibroblasts at the time of inoculation. Magnification x105,000.

Samples taken at the time of inoculation had structural details identical to the uninfected control. One section contained a clump of virus-like particles attached to an invagination in the cell membrane -- apparently in the process of being phagocytozed (Fig. 14).

Evidence of virus replication was first observed four hours after inoculation. At this time virus precursor-like particles of 41 nm mean diameter were seen associated with intracellular membranes, vacuoles, and rarely with the cell membrane. These were smaller than BUN virus and larger than ribosomes (Fig. 15).

At 12 hours mature virus-like particles of mean external diameter 84 nm could be found extracellularly. These contained electron dense nucleoids of 44 nm diameter. These mature particles seemed to develop by budding into vacuoles and by budding from the cell surface. Figure 16 shows a bud with a dense core surrounded by a diffuse cytoplasmic coat. Free virus was not observed in the cytoplasm and no evidence was found of nuclear involvement.

Wide-spread cellular destruction with corresponding high levels of extracellular particles was evident on the second day (Fig. 17). Cell boundaries became discontinuous, empty vacuoles were numerous, a large amount of cellular debris was free in the media, and nuclear and mitochondrial degeneration was prominent. A small number of vacuoles contained single or multiple virus-like structures (Fig. 18). Extracellular virus had an external diameter of 100 nm and dense internal core of 48 nm mean diameter.

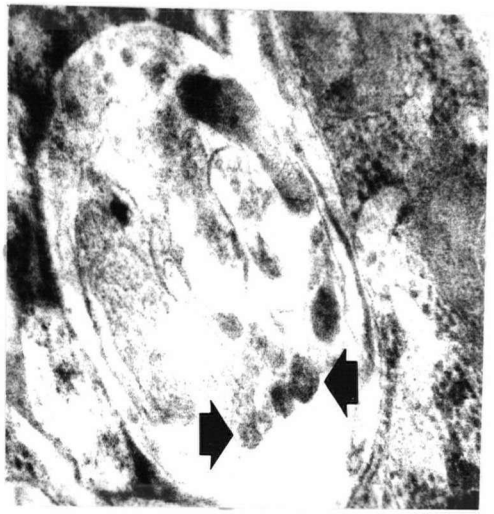

Fig. 15. Precursor-like particles associated with intracellular membranes in chick embryo fibroblasts four hours post infection. Magnification x62,580.

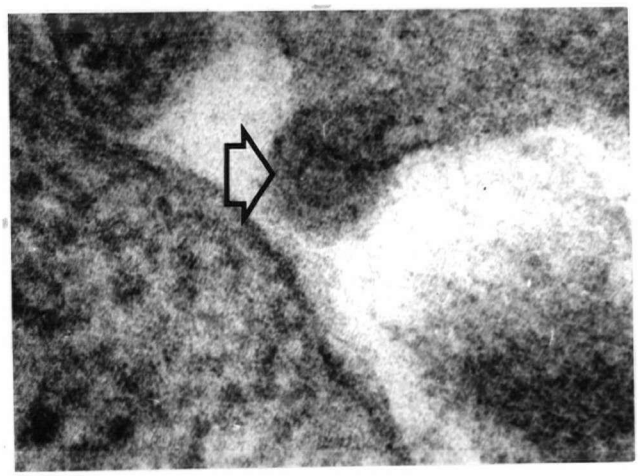

Fig. 16. Budding particle at the surface of a chick embryo fibroblast cell 12 hours after infection. Magnification x115,000.

c) Ferritin Labelled H.Ep. 2 Cells

Uninfected control H.Ep. 2 cells showed no sign of virus or cytopathic effect by electron microscopy. When control cells were labelled with a ferritin-anti BUN serum conjugate there was a minimal random non-specific staining of cellular and extracellular material. The labelling procedure caused distortion and destruction of cells and ultrastructural components, but cell boundaries, nuclei and mitochondria were recognisable.

Infected cells contained virus-like particles in vacuoles and extracellular virus by one day post infection. Ferritin labelling of intracellular virus components was not successful. Cell components such as ribosomes readily took up the heavy metal stain and interfered with visualization of the smaller, less electron-dense ferritin particles. This limited the technique's use to the labelling and identification of extracellular BUN virus.

Section from the one day sample showed isolated virus particles labelled with antibody (Fig. 19). Ferritin tagging of intracellular virus could not be demonstrated.

d) Mosquito Tissue

Despite relatively high virus levels as demonstrated by infectivity titrations, ultrastructural evidence of virus multiplication was very rare. Attempting to accomplish this on the basis of cytopathic effects is not sound as uninfected insect tissue contains

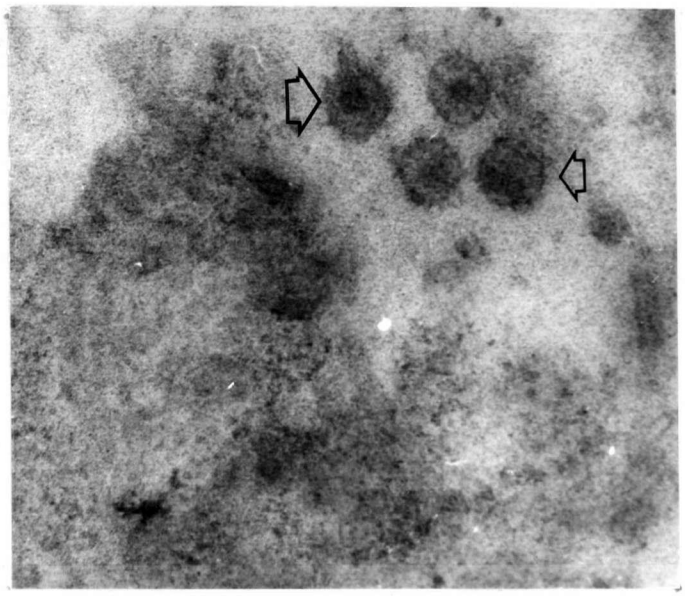

Fig. 17. Extracellular particles in chick embryo fibroblast cultures two days after inoculation with Bunyamwera virus. Magnification x79,200.

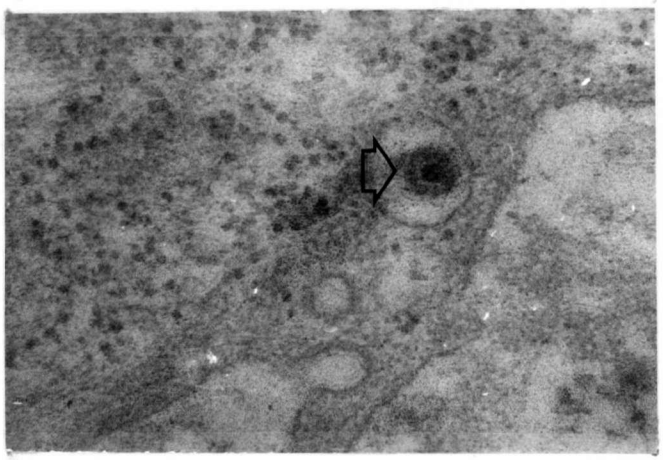

Fig. 18. Virus containing vacuole in a chick embryo fibroblast two days after inoculation. Magnification x57,750.

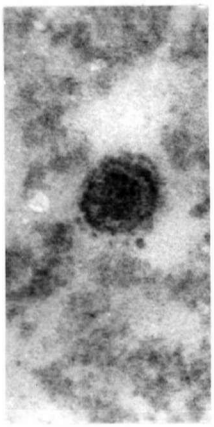

Fig. 19. Ferritin labelled extracellular virus in a H.Ep. 2 culture 24 hours after infection. Magnification x105,800.

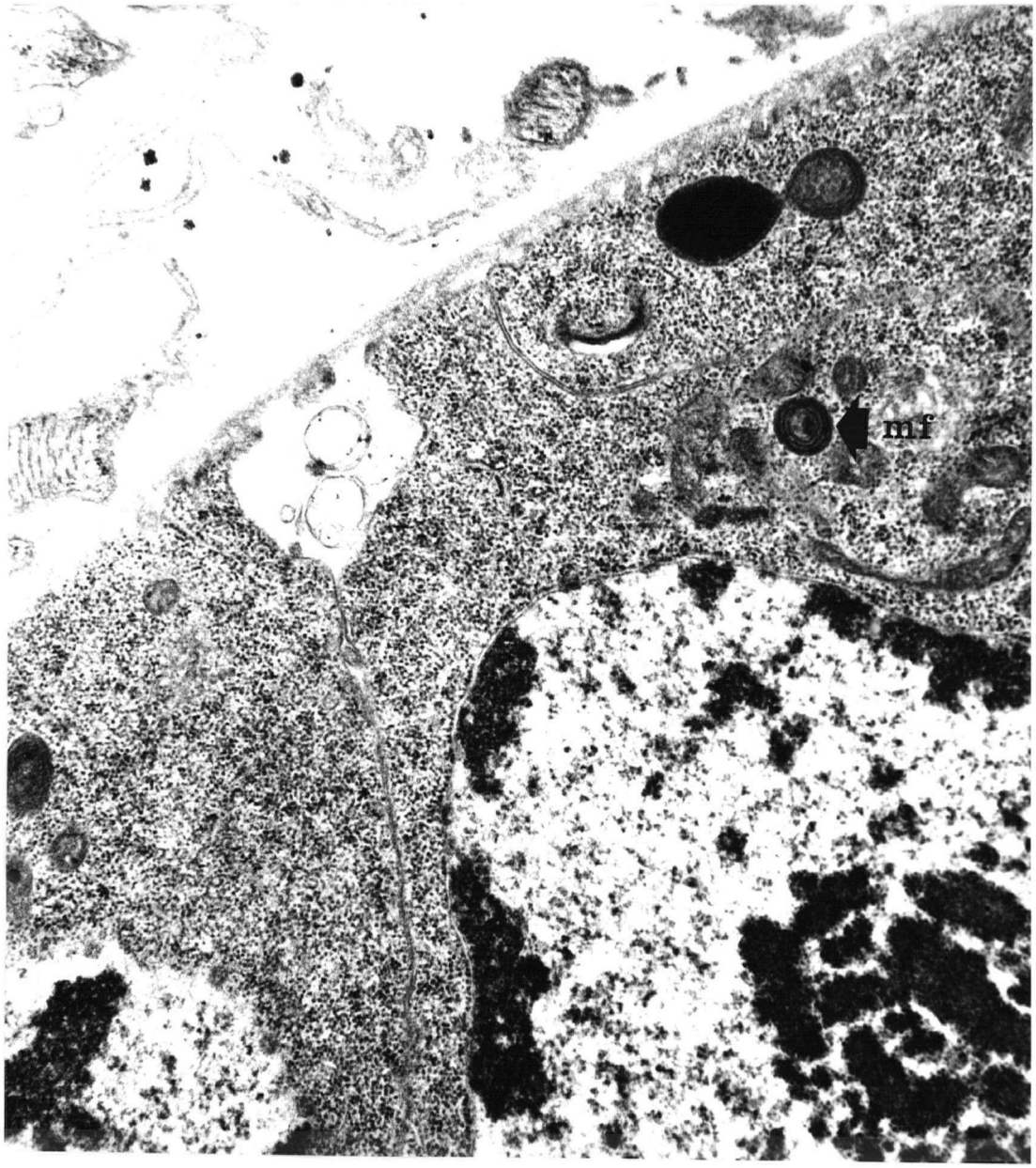

Fig. 20. Midgut cells from uninfected Aedes aegypti. Magnification x20,000.

mf: myelin figure-like structure

structures similar to those found in arbovirus infected mammalian cells, for example, the myelin figure-like material within the gut cells in Figure 20.

Virus-like particles with core diameters of 45 nm were seen extracellularly in fat body tissue surrounding the salivary glands of mosquitoes which had been infected for 12 days. Figure 21 shows a cell from this area with a large vacuole containing membranous structures and virus-like material. Salivary glands and gut tissue from a 12 day mosquito showed 80 to 100 nm particles within the cytoplasm and lining vacuolar membranes, but faulty staining obscured structural detail and made identification difficult. Figure 22 shows a large vacuole in tissue associated with the gut muscle of a mosquito which had been infected for 10 days. In addition to non-specific membrane structures it contains particles with 42 nm cores surrounded by distorted envelopes of 83 nm to 125 nm diameter.

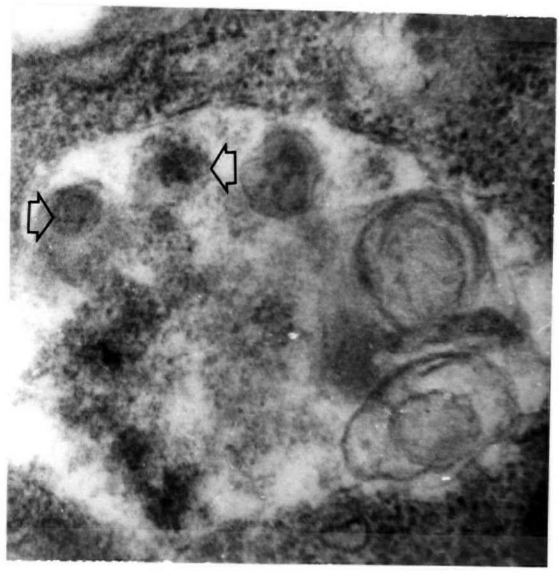

Fig. 21. Virus-like particles in mosquito fat body cells 12 days after virus ingestion. Magnification x47,200.

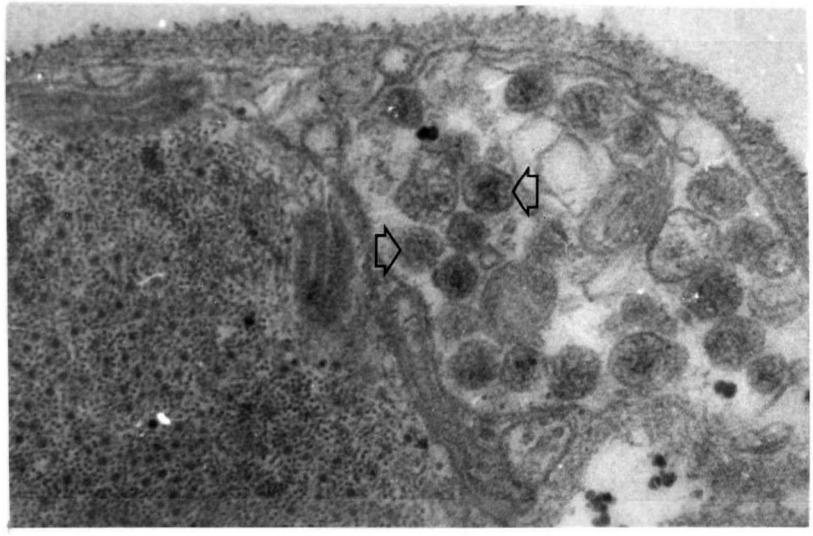

Fig. 22. Virus-like particles in mosquito gut 10 days after virus ingestion. Magnification x50,400.

Discussion

Discussion

These results show that the prototype strain of Bunyamwera virus conforms to the accepted definition of an arbovirus by multiplying in vertebrate and insect tissue and by being transmitted from host to host by a mosquito. Ogunbi has demonstrated similar properties using the Ukawa strain of Bunyamwera. (53)

The insect-mediated mechanical transmission of virus seen with some plant diseases does not play a role in arbovirus infections. Multiplication of virus within mosquitoes resulting in high titres within salivary tissue and secretions is necessary for transmission. (17) The concentration of virus required to infect a mosquito varies greatly from species to species and if this infection threshold is high enough it effectively prevents a mosquito from being an arbovirus vector. (14,16) The threshold phenomenon seems to be dependent on virus susceptibility of gut cells as multiplication must take place here prior to hemolymph dissemination to non-central tissues. (44)

Arboviruses may be transmitted by mosquitoes other than their natural vectors and certain North American mosquitoes have shown the capacity to transmit viruses not endemic in North America. (59,62) All Aedes species used in this research were easily infected by feeding on mice with artificial viremias. Since infection threshold levels were not determined and blood virus levels were one hundred fold higher than those found in mice with overt Bunyamwera encephalitis this should be interpreted cautiously with regard to natural host-vector systems.

The inability of virus fed Aedes canadensis and vexans to transmit Bunyamwera to highly susceptible hosts (suckling mice) tends to rule out their potential involvement in natural Bunyamwera cycles. Transmission of Bunyamwera group viruses by British Columbian mosquitoes could prepare an ecological niche suitable for North American Tensaw and Cache Valley viruses.

Intrathoracic injection of virus by-passes the gut barrier and allows mosquitoes to support the growth of agents which are not infective by ingestion. Even tissues of non-hematophagous insects such as grasshoppers, houseflies and carpet beetles can support arbovirus growth after injection, as can tissue culture cells derived from the moth Antheraea eucalypti. (35,72)

Following the injection of virus into the thoraces of Aedes canadensis and vexans there was a decline in titratable virus. This correlates with previous findings and may be due to a combination of virus inactivation and eclipse phase. (14,43,44) After ten days incubation, pools of whole mosquitoes showed virus levels approximately one hundred fold greater than the titre of the inoculum. Salivary tissue titres increased one thousand fold and gut titres increased one hundred fold over a ten day period. Virus replication rates and peak titres were highest in the salivary glands.

Aedes vexans and canadensis were capable of maintaining the 10^4 mouse LD_{50} level of virus ingested for eight days. This is indicative of a low level of multiplication without which all infectivity would be lost due to virus inactivation. (14) Bunyamwera levels in

these wild mosquito pools dropped over tenfold by day 17. Danielova noted a similar decrease with Tahyna in Aedes vexans, which was probably due to mosquito aging or a form of immunity rather than virus induced pathology. (26)

After two days Aedes aegypti injected with Bunyamwera showed virus titres three hundred fold higher than Aedes vexans and canadensis which received a similar virus dose. Peak titres were comparable, but were attained six days earlier in Aedes aegypti. This correlates well with the differences in Ukauwa virus production between North American wild mosquitoes and Aedes aegypti. (53)

Virus was constantly present in the gut of Aedes aegypti following an infective meal. After a one day lag corresponding to the time required for gut multiplication and subsequent virus release into the hemolymph, infectivity was detected in other regions. The lag in virus dissemination was very short compared to that found in other mosquito-virus systems. (26,53) This may be due to an exceptional susceptibility of Aedes aegypti gut to the prototype Bunyamwera strain, (Fig. 11 & Fig. 12) and/or the high virus titres ingested from artificially viremic mice. (44,75) The detection of virus in hemolymph samples and the initiation of rapid replication in the salivary glands occurred at the same time. Transmission was not accomplished at four days post infection but a tenfold higher salivary titre at ten days resulted in transmission to suckling mice. Chamberlain and Sudia showed that prevention of virus dissemination to salivary tissues greatly reduced the

transmission rate and high virus levels in salivary secretions are necessary for transmission. (17)

In Uganda, Bunyamwera was transmitted by Aedes circumluteolus but evidence presented here suggests that Aedes aegypti would make an excellent vector in urban areas. (31,37,38,66)

The adaption to mice of Chernesky's intravenous virus injection artificial viremia technique produced an instantaneous viremia one hundred fold greater than that attained by mice with Bunyamwera encephalitis. Within one half hour over 75% of mosquitoes exposed to these mice had taken blood meals versus 40% for a three-hour period when blood-virus soaked sugar cubes were used. Although the blood virus clearance rate of Bunyamwera in mice is approximately twice the rate of thermal inactivation at room temperature, the six fold longer time used for sugar cube feedings results in a less controllable variation in meal infectivity from mosquito to mosquito. With lengthy methods the potential for ingestion of virus below the infection threshold is also increased giving rise to potential error in virus development studies. Because the circulatory volume of mice can be calculated it is possible to produce a controlled level of viremia using a virus suspension of known infectivity. Such a system could be as useful in the accurate determination of mosquito infection thresholds as it has been for Powassan virus in Dermacentor andersoni ticks attached to rabbits. (23)

Infected vertebrate tissue cultures incubated at 29°C gave

comparatively rapid growth cycles with cell destruction and subsequent loss of detectable virus after four days. An initial drop in virus titre corresponding to that seen in mosquitoes was followed by a phase of rapid proliferation concurrent with the appearance of cytopathic effect. Cytopathic effect was not found in mosquito tissue even when comparable levels of virus were being produced.

Chamberlain and Sudia have shown that the rate of virus production in mosquitoes is subject to thermal regulation and that a temperature of 29°C is optimal for virus growth with mosquito longevity. (15) Bunyamwera produced similar growth curves in 29°C chick embryo fibroblasts (Fig. 7) and mosquito tissue (Fig. 8,9,10,11,12) as opposed to the findings in 37°C H.Ep. 2 and chick embryo fibroblasts. Cytopathic effect in the 29°C cells was first seen four days after the onset of virus production. This suggests that to a degree, temperature effects are responsible for the slow production of virus and lack of cytopathic effect seen in most insect tissues.

Bunyamwera replicated faster and produced higher titres in mosquito organ cultures than in whole mosquitoes. This suggests the presence of a growth limiting factor in whole mosquitoes.

Electron microscopy of infected chick embryo fibroblasts demonstrated a reproduction cycle similar to Murphy's findings in Bunyamwera infected mouse brain. (51,52) Virus entry appears to be by phagocytosis. Cytopathology seen at four hours involves virus precursor-like particles in conjunction with intracellular membranes

not described by Murphy. The observation of complete virus at 12 hours correlates well with the onset of infective virus production (Fig. 6). Maturation occurs by budding into cisternae or from the cell surface in a manner similar to group A arboviruses. (2,50) Virus replication leads to cell vacuolation and cisternae containing virus may be seen near the cell's surface prior to releasing their contents into the extracellular space. Nuclear involvement and free nucleoids within the cytoplasm were not seen.

The diameter of the virus envelope varied from 80 to over 100 nm in diameter depending on variations in electron microscopy preparation and the type of host tissue used. The dense nucleoids, however, remained relatively constant at 45 ± 3 nm diameter. Immunotracing with ferritin labelled anti-Bunyamwera serum identified similar particles in infected H.Ep. 2 cells as Bunyamwera viruses.

The presence of arboviruses within insect tissues is rarely confirmed by electron microscopy, and only Semliki Forest virus infected mosquito salivary glands demonstrate cytopathic effect by light microscopy. (49) Whitfield and Murphy have shown that complete Eastern Equine Encephalomyelitis virus is not observed in mosquito salivary glands until four days after the end of the extrinsic incubation period. A further eight day period is required before these particles are numerous. (76)

Cytoplasmic particles the size of Bunyamwera virus were seen in salivary tissue containing $10^{5.0}$ mouse LD_{50} of Bunyamwera. Tissue from the fat body and gut associated cells of infected mosquitoes

demonstrated that virus accumulates in vacuoles as it does in vertebrate tissue. No evidence was found of the nuclear involvement reported by Janzen. (36)

The prevalence of virus in the fat body near the salivary glands suggests that care should be taken to avoid contamination when salivary glands are being dissected out for titration.

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