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MAX ALEXANDER CHERNESKY

B.S.A. University of Guelph 1965
M.Sc. University of Toronto 1967

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POWASSAN VIRUS MULTIPLICATION IN DERMACENTOR ANDERSONI TICKS

ABSTRACT

All stages of Dermacentor andersoni (Stiles) ticks were infected by feeding on rabbits injected intravenously with large doses of Powassan virus 48 to 72 hours after the ticks became attached. Viremia titers at least $10^{2.5}$ mouse LD_{50} per ml were required to establish infection in 1 to 5% of ticks ingesting viremic blood. The "infection-threshold" of D. andersoni adults for Powassan virus was elevated when viremia was induced towards the end of engorgement. When each stage in the tick's life cycle was infected a viral-eclipse phase was demonstrated in gut cells. Other organs such as salivary glands, Gene's organ glands and accessory glands supported virus multiplication. This was demonstrated by infectivity titrations and observation of fluorescent foci when the tissues were stained with Powassan virus antiserum conjugated with fluorescein isothiocyanate (FITC). Transstadial transfer of Powassan virus was observed through ecdysis of larvae to nymphs and from nymphs to adults. This occurred both after feeding on hamsters (which produced a substantial viremia) and guinea-pigs (which did not become viremic). Virus was detected only in larval gut and invaded nymphal salivary glands during the molt. Infection localized in gut and salivary glands of engorged nymphs and flat adults, and was not detected in adult female accessory and Gene's glands until repletion. Adult males maintained high virus titers in salivary glands only. Transovarial transfer was not detected although infectivity was present on the surface of eggs laid by the mediation of an infected Gene's organ gland. Powassan virus was detected in salivary gland secretions but not in rectal excretions in adults or nymphs. Transmission of Powassan virus to hamsters, guineapigs, and rabbits was demonstrated by the bite of D. andersoni nymphs and adults which were infected by ingestion of blood by their antecedent larvae.

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Field of study: Virology

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ABSTRACT

All stages of Dermacentor andersoni (Stiles) ticks were infected by feeding on rabbits injected intravenously with large doses of Powassan virus 48 to 72 hours after the ticks became attached. Viremia titers at least $10^{2.5}$ mouse LD_{50} per ml were required to establish infection in 1 to 5% of ticks ingesting viremic blood. The "infection-threshold" of D. andersoni adults for Powassan virus was elevated when viremia was induced towards the end of engorgement. When each stage in the tick's life cycle was infected a viral-eclipse phase was demonstrated in gut cells. Other organs such as salivary glands, Gené's organ glands and accessory glands supported virus multiplication. This was demonstrated by infectivity titrations and observation of fluorescent foci when the tissues were stained with Powassan virus antiserum conjugated with fluorescein isothiocyanate (FITC). Transstadial transfer of Powassan virus was observed through ecdysis of larvae to nymphs and from nymphs to adults. This occurred both after feeding on hamsters (which produced a substantial viremia) and guineapigs (which did not become viremic). Virus was detected only in larval gut and invaded nymphal salivary glands during the molt. Infection localized in gut and salivary glands of engorged nymphs and flat adults, and was not detected in adult female accessory and Gené's glands until repletion.

Adult males maintained high virus titers in salivary glands only. Transovarial transfer was not detected although infectivity was present on the surface of eggs laid by the mediation of an infected Gené's organ gland. Powassan virus was detected in salivary gland secretions but not in rectal excretions in adults or nymphs. Transmission of Powassan virus to hamsters, guinea-pigs, and rabbits was demonstrated by the bite of D. andersoni nymphs and adults which were infected by ingestion of blood by their antecedent larvae.

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ABBREVIATIONS

CE - California encephalitis

CTF - Colorado tick fever

DNA - deoxyribonucleic acid

EEE - eastern equine encephalomyelitis

FITC - fluorescein isothiocyanate

H & E - hematoxylin and eosin

HI - hemagglutination inhibition

i.c. - intracerebral

IgG - (7S) gamma globulin

IgM - (19S) gamma macroglobulin

i.p. - intraperitoneal

i.v. - intravenous

IUDR - 5-iodo-2'-deoxyuridine

JBE - Japanese B encephalitis

KFD - Kyasanur Forest disease

LAN - Langat

LD_{50} - lethal dose killing 50% of individuals tested

LI - louping ill

mg - milligram

ml - milliliter

MVE - Murray Valley encephalitis

NEG - Negishi

Abbreviations (Continued)

NI	-	neutralization index
nm	-	nanometer
NT	-	neutralization test
OMSK	-	Omsk hemorrhagic fever
PBS	-	phosphate buffered saline
POW	-	Powassan
PTA	-	phosphotungstic acid
RNA	-	ribonucleic acid
RSS	-	Russian spring summer
RSSE	-	Russian spring summer encephalitis
s.c.	-	subcutaneous
SLE	-	St. Louis encephalitis
TBE	-	Tick-borne encephalitis
TMV	-	Tobacco mosaic virus
UFM	-	unconjugated fluorescent material
WEE	-	western equine encephalomyelitis

Introduction and Literature Review

Historical

Powassan virus is the only known North American representative of the Russian spring summer (RSS) complex of Group B tick-borne arboviruses. These agents are also prevalent in the British Isles, Northern, Central and Eastern Europe and extend through Asia, USSR, India, Malaysia and Japan (41), (Table I).

The first member to be isolated was louping ill (LI) virus from the brain of a sheep which had died from encephalitis in Selkirkshire, Scotland in 1929 (57). The incidence of LI virus parallels the distribution of Ixodes ricinus (L) ticks through the Scottish lowlands and Ulster. Voles, mice and sheep are reservoirs. Human infections are manifested as aseptic meningitis.

The Sofjin strain of Russian spring summer encephalitis (RSSE) virus was first isolated in May 1937, in the far eastern taiga region of the USSR (67). Frequent isolations of this serotype have been made from humans with fatal encephalitis and from ixodid ticks, especially Ixodes persulcatus (Schultze) in many localities of the temperate zone of the USSR.

The Kubrin strain of Omsk hemorrhagic fever (OMSK) virus was isolated in 1947 from the blood of a child from the Omsk Oblast of Siberia who had developed hemorrhagic fever with leukopenia (16). This disease is confined to the Omsk and Novosibirsk Oblasts and Dermacentor pictus (Herm.) ticks along with forest rodents maintain the virus in nature.

Table: I: Prototype strains of the Russian Spring Summer Complex of Tick-borne Group B Arboviruses

Prototype Virus		Initial Isolation			Tick Vector	Mammal reservoir
name	strain	year	location	reference		
Louping Ill (LI)	Original	1929	Scotland	Pool, W.A. et al. 1930	Ixodes ricinus	voles, mice, sheep
Russian spring summer encephalitis (RSSE)	Sofjin	1937	Far eastern U.S.S.R.	Silber, L.A. 1939	Ixodes persulcatus	forest rodents
Omsk hemorrhagic fever (OMSK HF)	Kubrin	1947	Omsk Oblast U.S.S.R.	Chumakov, M.P. 1948	Dermacentor pictus	forest rodents
Negishi (NEG)	Original	1948	Japan	Ando, K. et al. 1952		
Tick-borne encephalitis (TBE)	Hanzalova	1948	Czechoslovakia	Gallia, F. et al. 1949		
	Absettarov	1951	Leningrad Oblast	Smorodintsev, A.A. et al. 1953	Ixodes ricinus	forest rodents, goats (milk-borne)
	Hypr	1953	Czechoslovakia	Pospisil, L. et al. 1954		
	Kumlinge	1959	Finland	Oker-Blom, N. et al. 1962		
Langat (LAN)	TP21	1956	Malaysia	Smith, C.E.G. 1956	Ixodes granulatus	forest rodents
Kyasanur Forest Disease (KFD)	W371	1957	Mysore, India	Work, T. et al. 1959	Haemophysalis spinigera	monkeys, forest rodents
Powassan (POW)	LB	1958	Ontario, Canada	McLean and Donohue 1958	Ixodes cookei	forest rodents

Adapted from McLean, Tick-borne Group B Arboviruses: General Features. In Textbook of Virology, A.J. Rhodes and C.E. van Rooyen, 5th edition, 1968.

Negishi (NEG) virus is the Japanese member of the group, being isolated in Tokyo in the summer of 1948 (3). The patient had developed encephalitis and the virus was isolated from the cerebrospinal fluid. An additional strain was isolated from another patient's brain later that same year. The tick vectors and animal reservoirs have not yet been identified.

Currently documented prototype strains of the European members of the RSS complex comprise four isolates from I. ricinus ticks or man in various parts of Europe. These tick-borne encephalitis (TBE) viruses show virtually no antigenic differences among each other (19). These strains are Absettarov (72), Hanzalova (28), Hypr (58) and Kumlinge (53). Previous names such as biphasic meningoencephalitis and Central European encephalitis are included in this group. TBE virus has induced encephalitis in man by the drinking of raw milk from goats infected with this virus (56).

The TP21 strain of Langat (LAN) virus was first isolated from a pool of Ixodes granulatus (Supino) ticks taken from two species of Rattus rats collected in the jungle near Kuala Lumpur Malaysia in 1956 (69). Two additional strains were isolated from I. granulatus ticks in 1959.

Work et al. isolated Kyasanur Forest disease (KFD) virus (W371 strain) from the blood and other organs of a Presbytis entellus monkey, collected in the Kyasanur Forest of the Shimoga

District of Mysore State, India, in March 1957 (86). Other representative isolates were recovered from human blood and from Haemaphysalis spinigera (Neumann) ticks. Patients contracting this disease present with hemorrhagic fever.

Powassan (POW) virus was isolated by McLean and Donohue from the brain of a child who died from encephalitis in September 1958 (43). This index case lived on a farm near the village of Powassan in northern Ontario, Canada. Since that time extensive field studies in that vicinity have revealed a natural cycle involving groundhogs (Marmota monax) and red squirrels (Tamiasciurus hudsonicus) as principal reservoirs, with hard ticks including Ixodes cookei (Packard) and Ixodes marxi (Banks) serving as arthropod vectors (44,45,46,47,48). Man and other vertebrates appear to be infected tangentially, and play no part in the maintenance of the virus within natural foci.

The isolation of a virus closely related to Powassan virus from Dermacentor andersoni (Stiles) ticks collected in Colorado in 1952 was reported in 1960 by Thomas et al. (78), and since that time ecological evidence has strongly suggested a potential involvement of D. andersoni ticks as vectors of Powassan virus (49,50).

General Characteristics of Powassan Virus

a. Biological

Powassan virus (strain LB) multiplies readily in the brains of suckling and weaned laboratory mice. Suckling mice die of encephalitis between 4 and 6 days following intracerebral (i.c.), intraperitoneal (i.p.) or subcutaneous (s.c.) inoculation of virus. The average time for death of weaned mice following i.c. inoculation of 100 mouse LD_{50} is 7 days.

A hemagglutinin has been prepared from infected suckling mouse brains by sucrose-acetone, acetone-ether, or borate-protamine extraction. The virus agglutinates erythrocytes of geese, newly hatched chicks or roosters at pH between 6.4 and 6.8, and at temperatures of 4°C, 25°C, 37°C.

b. Biophysical and Biochemical

Powassan virus is inactivated by sodium deoxycholate (1:1000) at 25°C. After 1 hour the titer is reduced 100,000 fold. The virus contains ribonucleic acid (RNA). Infectivity is not inhibited when virus is inoculated into susceptible tissue cultures, in the presence of 5-iodo-2'-deoxyuridine (IUDR) (unpublished data). This substance inhibits the replication of viruses containing deoxyribonucleic acid (DNA)

The virus particle diameter measures 40 to 45 nanometers (nm) by electron microscopic examination both of thin sections of infected cell pellets, and of phosphotungstic acid (PTA) preparations (1). Individual particles show capsids with cubic symmetry surrounded by a delicate envelope with high lipid content. Virus particles are located exclusively in the cytoplasm of infected cells.

c. Medical

Human disease presents with fever, headache, prostration, stiff neck, spastic paresis and pleocytosis of cerebrospinal fluid. (43). Characteristic histopathological lesions include neuronophagia, glial knots, perivascular cuffing with lymphocytes in brain and spinal cord of a fatal human case.

Development of Animal Viruses in Ticks

a. Natural Infection

Ticks become attached to a host by lacerating the tissue with their chelicerae and inserting the hooked hypostome. A canal is formed by the chelicerae and hypostome which receives salivary secretion from the ticks' salivary glands as well as host blood

from the pool created by the rupture of blood vessels. The blood passes through the pharyngeal cavity and short esophagus into the mid-gut and its diverticula (Figure 1), a great deal of regurgitation takes place (31). The majority of ingested blood is dehydrated in the diverticula and its fluid components pass through the cylindrical epithelial cells, membrane propria, and muscles of the gut wall into the hemocoele where it is mixed with the hemolymph. The erythrocytes of the vertebrate are destroyed in the diverticula and the hemoglobin crystallizes. Epithelial cells digest these crystals (79) and near the end of engorgement virtually no epithelial cells remain attached to the membrane propria.

If the host is in the viremic stage of an arbovirus infection, the tick will imbibe virus, and if the amount of virus circulating in the blood is sufficiently high to establish infection in the cells of the tick diverticula the tick will become infected.

b. Laboratory Infection

Several techniques have been employed in the laboratory to infect ticks with arboviruses. Burgdorfer (10) infected D. andersoni ticks by feeding them on ground squirrels circulating Colorado tick fever (CTF) virus in the blood following subcutaneous injection of virus. Ornithodoros moubata (Murray) became

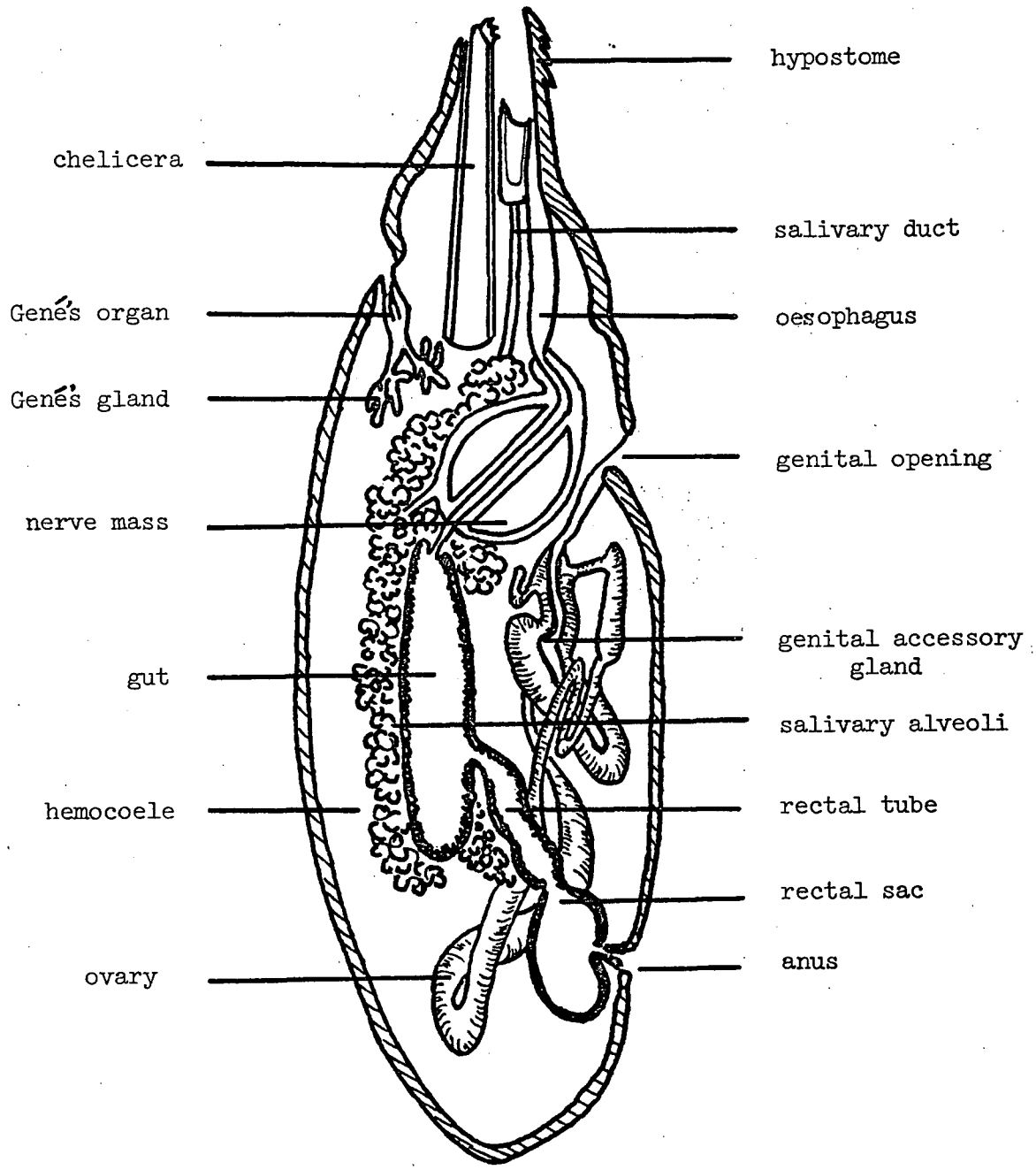


Fig. 1. Lateral view of D. andersoni adult female

infected with western equine encephalomyelitis (WEE) virus by feeding on membranes of embryonated hen's eggs infected with virus (12). Rabies virus has been successfully introduced into the gut of D. andersoni when a virus blood mixture was channelled through a capillary tube placed over the hypostome (8). A more artificial method of parenteral inoculation of TBE virus directly into the hemocoele successfully established infection in ixodid ticks (64), but some mortality ensued due to leakage of hemolymph.

One of the objectives of this study was to select an easy, reproducible, semi-quantitative method of infecting all actively feeding stages of D. andersoni ticks with Powassan virus.

Infection Threshold

An infection threshold or dosage requirement (14) has been reported previously for tick-borne viruses. The infection threshold is defined as the least amount of virus required to infect 1 to 5% of arthropods ingesting the viremic blood. Dumina (23) reported that I. persulcatus became infected with RSSE virus at a dosage of $10^{8.0}$ mouse LD_{50} per ml. and Burgdorfer (10) found that D. andersoni larvae or nymphs did not acquire a permanent infection of CTF virus unless the level of viremia was at least $10^{2.0}$ mouse LD_{50} per ml.

The amount of virus necessary to infect a species of tick

determines not only the potential of the arthropod to be a biological vector but allows a comparison of vertebrate capacity to become a reservoir by comparing the animal's viremic level with the host preference of each tick stage. For example, if D. andersoni possessed an infection threshold less than $10^{4.0}$ LD_{50} per ml. which is the average titer that groundhogs (Marmota monax) circulate during a viremia of four days duration (80), it is conceivable that if the western groundhog (Marmota flaviventrus) circulated a sufficiently high viremia of Powassan virus to infect D. andersoni ticks, this ecological combination could create a tick-mammal-tick cycle in nature because D. andersoni has a host preference for M. flaviventrus (unpublished data).

Virus Elimination in Feces

Throughout the life cycle of D. andersoni a pattern of defecation ensues in which large amounts of guanine crystals are eliminated immediately following a molt and large numbers of gut cells and host blood cells plus hemoglobin are excreted during active feeding. TBE virus has been isolated from I. ricinus tick feces (6), and eastern equine encephalomyelitis (EEE) virus has been isolated from the feces of Dermacentor marginatus (Sulzer) and I. ricinus ticks during engorgement (62).

In this study, it was necessary to determine the ability of D. andersoni ticks to pass infective virus from the anus during engorgement and immediately following ecdysis.

Virus Distribution in the Tick

Although the development of many arboviruses in mosquitoes has been studied repeatedly (85, 4, 14, 40, 77, 35, 15), only two such studies have been carried out using tick-borne viruses in ticks. Pavlovsky and Soloviev in 1939 and 1940 (54, 55), infected I. persulcatus and Haemaphysalis concinna (Koch) adult female ticks by feeding them for 3 days upon mice which were viremic with TBE virus. Infectivity was detected in the gut, salivary glands and ovaries of I. persulcatus 1, 10, 20 and 25 days following infection in a high titer ($10^{6.0}$ mouse LD_{50}). Smaller amounts of virus were consistently found in the Malpighian tubules, brain, and Genés organ. All organs were infective for TBE virus 40 days and 53 days after feeding in another group of experiments reported in the same paper.

H. concinna adults became infected less readily with TBE virus than I. persulcatus. At approximately 6 days after beginning a period of infective feeding which lasted 3 days, virus was demonstrated only in the diverticula and salivary glands, while in other organs the presence of virus was not detected until 2 days later. All of these experiments employed weaned mice inoculated i.c. for virus assay.

By comparison, a great deal more work has been done on the development of Rickettsiae in ticks than for viruses. The first

organ of Haemaphysalis humerosa (Warburton and Nuttall) to become infected with Q fever (Coxiella burnetii) ingested in a blood meal, was the gut (70), and rickettsiae were present not only in the cytoplasm but also in the nuclei of epithelial cells. Subsequently many organs became infected including Malpighian tubules, ovaries, nervous ganglia, salivary glands and testes.

The fluorescent antibody technique has been employed for studying the distribution of rickettsiae in the organs of vectors. Amosenkova et al. (2) demonstrated C. burnetii in gut smears of infected ticks between the 1st and 210th day after infection and found that the gradual increase in rickettsiae concentration was not affected by ecdysis from nymphs to adults.

The presence of Rocky Mountain spotted fever (Rickettsia rickettsii) was demonstrated in smears of diverticula from D. andersoni. Numerous rickettsiae were found in the brain, epithelial gut cells, salivary glands, Malpighian tubules and muscle fibers as well as in the genital system and developing eggs by organ smears and sections (11,13,65).

Due to the lack of information concerning the sequential development of tick-borne viruses in larvae, nymphs and adults as well as in the two molts that take place during the cycle, it was of interest to trace the sequence of virus invasion of tick organs in each tick stage following infection at each specific stage as well as in an antecedent stage. A correlation of infectivity

with immunofluorescence was employed to trace the development of Powassan virus in all stages of D. andersoni and to determine the relative selectivity, if any, for certain organs throughout the tick's life cycle.

Transstadial Transmission

Conflicting results of virus dynamics during metamorphosis from one tick stage to another have been reported. Benda (5) reported viral increases in the early stages of metamorphosis, and a decrease when the molt was finished, for larval or nymphal I. ricinus infected with TBE virus. Varma and Smith (83) studying Langat virus in Haemaphysalis spinigera (Neumann) recorded a substantial drop in virus titer as the ticks went into a molt, followed by an increase as they emerged into the next stage. Rozeboom and Burgdorfer (65) observed an initial drop in virus titers during metamorphosis followed by an increase in D. andersoni that had ingested small amounts of CTF virus as nymphs. Kennedy et al. (32) obtained comparable results in a similar experiment, but in 2 additional studies with larvae and nymphs, titers increased throughout engorgement, quiescence and molting.

In Rozeboom and Burgdorfer's study, maximum titers of $10^{4.5}$ mouse LD_{50} of CTF virus were recovered from engorged larvae and

flat nymphs yielded $10^{4.0}$ mouse LD_{50} after ecdysis. Engorged nymphs contained $10^{5.0}$ mouse LD_{50} of CTF virus and maintained high titers ($10^{5.6}$ mouse LD_{50}) when they emerged as flat adults.

Divergent results such as those cited above precipitated a necessity to examine the pattern of development of Powassan virus both in whole ticks and individual organs through each successive stage in the tick's development.

Transovarial Transmission

Equally fragmentary and conflicting are the results reported concerning transovarial transmission of viruses in ticks. If this term is defined as the transmission of virus from one generation to the second via eggs, with the second generation beginning with hatched larvae, then "true" transovarial transmission must result in infected larvae and not only infected eggs.

Although attempts in 1917 by Stockman (73) to passage LI virus by I. ricinus eggs suggested transovarial transmission did occur, 44 years later MacLeod (51) failed to confirm these original findings. KFD virus has been detected in larvae of H. spinigera collected from the forest floor (60), although repeated attempts to demonstrate transovarial passage by experimentally infected female ticks were unsuccessful. In 1963, Singh et al. (68) showed evidence of transovarial transmission by 1 of 33 females after

sucking blood in contrast to 17 of 29 after inoculation of massive doses of KFD virus into the hemocele of H. spinigera adults (68).

Chumakov (17) has reported transovarial transfer of RSSE virus through 3 generations of I. persulcatus, its natural vector. Smorodintsev (71) observed that although many regions of the eastern USSR yielded uninfected adult female ticks, many isolations were readily obtained from larvae reared from maternally infected females. Transovarial transmission of RSSE virus has been reported in I. ricinus, Dermacentor nuttalli (Olenev), Hyaloma dromedarii (Koch), H. turkmeniense and H. asiaticum (18).

Benda has investigated the development of TBE virus in I. ricinus. He reported about 6 percent of experimentally infected female ticks transmitted virus ovarially (6). This rate of infection was increased as high as 20% by feeding the ticks massive doses of TBE virus. Rehacek (63) found the transovarial infection rate of females fed TBE was 3.3% and it increased to 21% in females infected by inoculation. In contradistinction to these findings, Van Tongeren (82) was unable to find TBE virus in the offspring of experimentally infected I. ricinus.

Although TBE has been isolated from larvae collected in a focus in Austria (59), Loew (38) argues that ovarial transmission does not play an important role in virus dissemination as shown by the fact that only 1 isolate was obtained from 162 pools of larvae.

Among the ungrouped arboviruses, the first tick-borne virus shown to be transmitted transovarially was Nairobi sheep disease virus in Rhipicephalus appendictulatus (Olenev) (21,37). Florio et al. (26) reported that the same phenomenon occurred in D. andersoni infected with CTF virus; however Eklund et al. (24) failed to confirm this, and these workers report that CTF is seldom isolated from D. andersoni larvae collected in nature.

Transovarial transmission of mosquito-borne viruses in ticks has been reported for St. Louis encephalitis (SLE) virus in Dermacentor variabilis (Say) (7) and WEE virus in D. andersoni (74).

To investigate the mechanism of transovarial transmission of Powassan virus in D. andersoni ticks in the present study it was necessary to look at the sexual apparatus (ovary, oviduct, vagina) and ovipository organ of Gené as well as the presence of virus through eggs and larvae.

Transmission to Vertebrate Hosts

Benda (6) showed that the greatest amount of TBE virus in I. ricinus ticks is secreted by the salivary glands of adult females (one female being sufficient to cause encephalitis in a mouse), smaller amounts were secreted by nymphs and the smallest by larvae. Under experimental conditions, TBE virus was transmitted by I. persulcatus nymphs to healthy mice, following feeding on infected mice or humans (5).

The transmission of viruses by tick bite can be measured by production of viremia in the host or by the conversion of a seronegative animal to virus antibody seropositive. Levkovich et al. (36) demonstrated this fact in different species of small rodents bitten by nymphs of I. persulcatus infected with TBE virus, and I. ricinus and Haemaphysalis inermis (Birula) transmitted the same virus to the common shrew (Sorex araneus) (34) and to roe deer (Capreolus capreolus) (52). Intensive viremia of CTF virus in porcupines (Epethizon dorsatum) and ground squirrels (Citellus lateralis) caused by bites of infected adults (9) and nymphs (10) has been reported by Burgdorfer.

As well as demonstrating the presence of Powassan virus in the salivary glands of transmitting D. andersoni and detection of viremia and/or virus antibodies in hosts, isolation of Powassan virus collected from tick saliva would substantiate D. andersoni's ability to transmit.

Materials and Methods

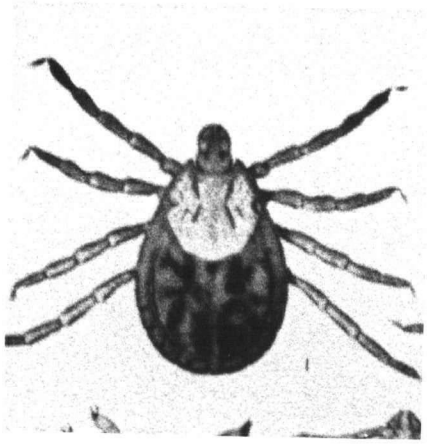
Tick Rearing

A colony of Dermacentor andersoni (Stiles) ticks was established upon receipt of ten male and ten female adults (Figure 2) from Mr. J. D. Gregson, Research Station, Canada Department of Agriculture, Kamloops, British Columbia, Canada. These were obtained from the Kamloops colony after more than twenty generations of laboratory propagation.

Ticks were fed on the shaved back of a young adult New Zealand white rabbit. A mailing tube type capsule (33) was taped on the back to restrain the ticks in one area (Figure 3). All adults began to feed within twentyfour hours. Matings took place during feeding. Individual engorged ticks began to drop off the rabbit after 9 days of feeding and by 14 days all ticks were removed.

All of the females were placed in individual insect vials (7.5 x 2.5 mm) which were closed with loose absorbent cotton plugs. The males were discarded, except in virus infection studies, in which case they were processed for the presence of virus. The vials were placed in a glass dessicator over a saturated solution of sodium chloride at 24 to 27°C. Oviposition commenced between 10 to 20 days after repletion of adult ticks. Eggs hatched to larvae 10 to 15 days later.

Flat larvae were fed upon a rabbit 3 to 4 weeks after hatching. All the progeny from two females were sprinkled into an ear of a



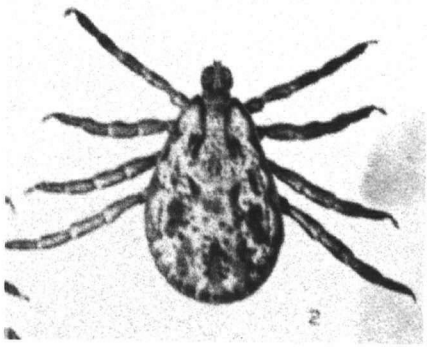


Fig. 2. The external anatomy of female (1) and male (2) Dermacentor andersoni (Stiles) adult ticks.

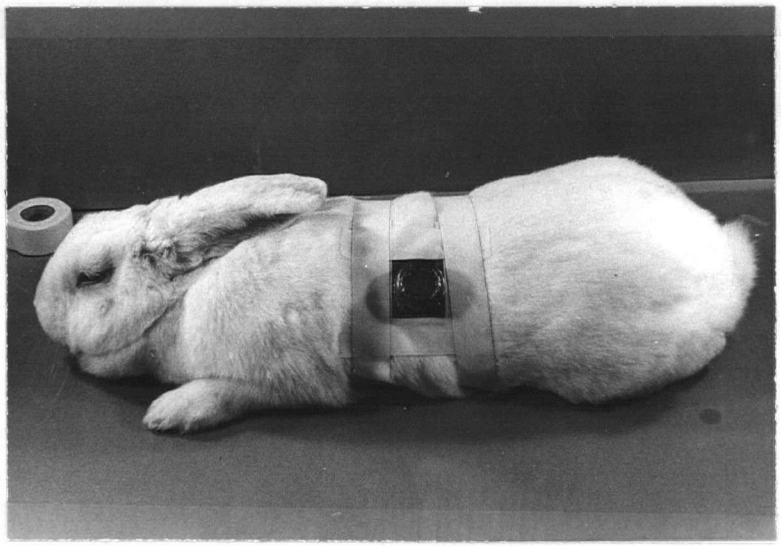


Fig. 3. Ticks feeding under a restraining capsule on a rabbit.

rabbit (30). The ear was covered with a fine mesh bag, 2 inches wide by 5 inches long, and sealed to the shaved base of the ear by porous adhesive tape, one inch wide. This ear was then held down to the rabbit's back by a loose loop of tape around the animal's thorax with a thin piece of absorbent cotton between the tape and the ear bag on the upper part of the loop. Adhesive tape was applied to the rabbit's paws to prevent the animal from removing the bag by scratching.

Virtually all larvae became completely engorged after 4 to 6 days of feeding. The bag was removed from the ear on the sixth day. The contents of the ear bag were funnelled equally into two insect vials, and each vial received a piece of light white cardboard (2 x 5 mm) containing the pertinent rearing data marked in pencil. Each vial was stoppered with a rubber stopper (number 4) that had a single hole (0.5 mm diameter) for ventilation. A 7 mm square of fine mesh nylon was pulled over the narrow end of the stopper as it was inserted into the vial. The vials were placed into a dessicator over saturated sodium chloride at 24 to 27°C.

Flat nymphs which appeared within 14 days of collection of engorged larvae were fed upon a rabbit 2 to 5 weeks later. A piece of nylon mesh was inserted inside the screened cap of the feeding capsule. All nymphs began to feed within 24 hours and they actively engorged for 6 to 8 days. On the eighth day, all engorged nymphs were placed into insect vials (twenty to a vial)

and stored over saturated sodium chloride solution at 24 to 27°C.

Flat adults first appeared 3 to 4 weeks after their antecedent nymphs became engorged. After being held at the same temperature and humidity for an additional 2 weeks, males and females were put into separate vials (50 to each vial) and held at 4°C for 6 to 8 weeks with 6 hours light and 18 hours darkness per day.

Following this chilling period adults were transferred to 24 to 27°C for 3 to 7 days, following which time they were placed in a capsule and allowed to feed on a rabbit.

Tick Infection

Each feeding stage of D. andersoni was placed on a rabbit. An intravenous dose of Powassan virus was administered at least 48 hours subsequently, by which time all ticks had become attached. Rabbits infested by larvae were always inoculated with virus by 48 hours post-infestation, whereas rabbits to which nymphs became attached were inoculated at 48 or 72 hours. Rabbits infested by adult ticks received virus either 2, 3, 5 or 7 days following the commencement of tick engorgement. Individual ticks which fed slowly or not at all were discarded before virus was administered to the rabbit. At the end of engorgement, all tick stages were maintained as cited in the section on tick rearing.

Tick Virus Transmission to Animals

At the prescribed optimal time for feeding nymph and adult stages after ecdysis, individual ticks were used for transmission studies. If guineapigs were used, a regular Kohl's capsule was taped to the shaved side of an animal, seronegative for Powassan virus hemagglutinating inhibiting (HI) antibodies. The tick was then placed inside and allowed to feed as prescribed in tick rearing procedures. The animal was bled by cardiac puncture 30 days later and the serum tested for HI antibodies to Powassan virus.

The use of hamsters for transmission studies necessitated the use of a 19 x 12 mm, strong, flexible chicken fencing wire having a mesh hole of 1.5 mm in diameter. The animal was anesthetized with ether and rolled securely in the mesh leaving its 4 legs protruding through mesh holes. A 1.5 mm plastic capsule with fine nylon on the top was placed over the tick and snugly inserted into a mesh opening over a shaved part of the animal's lower abdomen. Bond-fast glue was edged around the circumference of the union of the base of the capsule and the animal's skin as well as the capsule and the wire mesh (Figure 4). The hamster was bled by cardiac puncture 4 to 6 days subsequently and the whole blood tested for the presence of virus. Blood was again collected at 20 days and the serum tested for the presence of HI antibodies to Powassan virus.

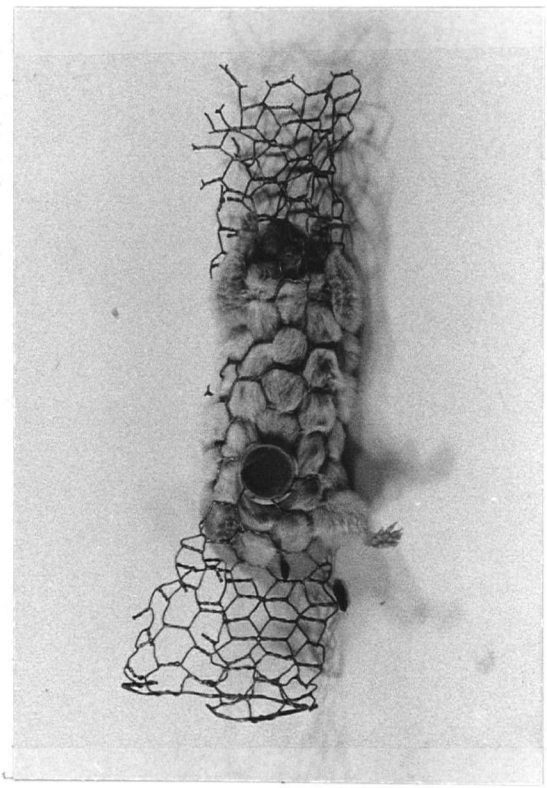


Fig. 4. Dermacentor andersoni (Stiles) ticks feeding upon a hamster.

All ticks employed for transmission were collected following the blood meal. They were either ground in mortar and pestle as a whole, or their organs were dissected. The resulting sample was tested for virus by titration in mice, or by direct fluorescent antibody staining of cut sections.

Tick Dissection

An aluminum foil dish 6 mm in diameter and 1.5 mm deep was filled 3/4 full with a mixture of 50 percent beeswax and 50 percent powdered resin stirred and heated without boiling for 20 minutes. After cooling, this dark brown substance became hard and sticky. Just prior to embedding the tick's legs into this dish, the wax-resin mixture was heated under a 100 watt bulb to soften the surface. The tick's legs and ventral abdomen were placed into the soft surface following which the whole container was immediately placed into a -20°C refrigerator for 2 minutes. The dish was then placed under a dissecting binocular microscope and viewed with a 25 x objective.

Several drops of 20 percent calf serum saline were placed on the tick forming a large bubble covering the entire tick.

A sterile disposable scalpel (Bard-Parker, Inc. number 11, catalogue number 1611) was employed to remove the dorsum of the tick. A slit was made with a scalpel point around the lateral

edge of the tick, beginning at the junction of the capitulum to the scapula on one side and proceeding around the entire tick to the junction at the other side. The entire dorsum was then gently reflected up and forward and pinned inside-out into the wax, in front of the hypostome.

This procedure allowed the exposure of the entire set of organs with the exception of some torn musculature and aorta which were firmly attached to the dorsum.

The entire set of diverticula were removed intact by gently prying them individually with micro-dissection forceps from their place, cutting the stomach at the orifice into the posterodorsal part of the central nerve mass, and across the rectal tube.

The ovary or testes were lifted free from the surrounding trachea and Malpighian tubules and cut at their entrance into the accessory sex gland. The accessory sex gland was removed by cutting underneath at the level of the vagina or ejaculatory duct.

Both lateral masses of salivary gland alveoli were grasped at the anterior proximity of the salivary duct to the sub-cheliceral plate, cut at that point and lifted dorsally and posteriorly, gently pulling the long strings of alveoli from the many trachea.

Large numbers of tracheae were grasped and cut at the entrance into the spiracles and removed. Malpighian tubules were removed similarly to tracheae by cutting at their entrance into the rectum.

The central brain mass was lifted and cut away from the esophagus.

The fat bodies were scooped out of their ventral position by curved fine tipped forceps, and confirmed by Sudan staining.

The majority of the heart was cut from the inside surface of the reflected dorsum.

Gené's organ glands were dissected from their anterior position just under the scapulae.

All individual organs were rinsed in sterile saline for 30 seconds. They were processed for presence of virus by inoculation of mice with homogenates or by direct fluorescent staining of thin sections.

Collection of Tick Saliva

Saliva was collected into capillary tubes from engorging adult ticks by the methods of Gregson (29) and Tatchell (76). Ticks were allowed to feed for 4 days on a rabbit, following which they were removed and taped to a mounted stand, ventral side down. The capillary tube (0.28 mm at the tip) was placed over the hypostome, spreading the palps gently. By a warm lamp and intermittent gentle stroking of the tick's back, saliva readily flowed into the tube.

Tick Sectioning Techniques

Individual organs or whole ticks were embedded in Tissue-Tek O.C.T. compound employing aluminum foil as described by Tobie et al. (81). After quick freezing, the specimen was sectioned in a Harris-International Microtome Cryostat, Model CT1, at 6 to 8 μ thickness. Ribbons of sections were cut employing an anti-roll bar, and picked up from the blade by touching with a clean microscope slide at 24°C. Sections were dried on a slide warmer for 1 hour at 35°C or overnight at 24°C. Following drying the sections were fixed in acetone at 24°C for 10 minutes.

Powassan Virus Antibody Preparation

New Zealand white rabbits were injected into a marginal ear vein with 2.0 ml of Powassan virus grown in suckling mouse brain, containing 10,000 LD_{50} per ml of virus. Subsequent injections of 2.0 ml were administered i.p. every second day for a total of eight injections. All virus inocula had been centrifuged at 3,500 rpm for 20 minutes to remove brain debris before inoculation into rabbits. The animals were rested for 1 week, then given 1.0 ml of virus i.p. on each of 2 consecutive days and bled one week later. After each bleeding of approximately 20 ml from an ear vein, the animals were injected with 1.0 ml of virus i.p. and

rested for 2 weeks before bleeding again. Blood samples were collected into 40 ml centrifuge tubes, allowed to sit at 4°C overnight and rimmed with a sterile Pasteur pipette. The clots were deposited using an International Centrifuge Model CS at 3,000 rpm for 15 minutes. The serum was pipetted into 10 ml. screw cap vials and stored at -20°C until processing for conjugation with fluorescein.

Preparation of Fluorescent Conjugated Antiserum

Twenty ml of saturated ammonium sulfate were slowly added for 20 minutes to 20 ml of hyperimmune antiserum and mixed overnight at 4°C with a magnetic stirrer to precipitate the gamma globulin. The precipitated mixture was centrifuged at 8,000 rpm for 30 minutes at 4°C. The supernatant was discarded and the precipitate resuspended in 10 ml of phosphate buffered saline, pH 7.1 (PBS) then dialyzed against PBS until samples of external fluid proved negative for ammonium ions when tested with three drops of Nessler's reagent.

The resulting fluid in the dialysis sac was adsorbed with mouse brain powder for 10 minutes at 4°C after which time the suspension was centrifuged at 3,000 rpm for 10 minutes. The supernatant was collected and tested for neutralization of Powassan virus; (see Materials and Methods, Neutralization of Virus).

The protein content of the precipitated gamma globulin was determined by the Lowry method (39), using bovine albumin fraction five as a standard. Readings were made on a "Spectronic 20" photometer at 500 mμ.

Fluorescein isothiocyanate (FITC) (Nutritional Biochemicals Corporation, Cleveland, Ohio) was weighed in the ratio of 1 mg of FITC per 50 mg of protein (27). This quantity was mixed with 1 ml of acetone and slowly added for 20 minutes to the gamma globulin employing a magnetic stirrer. The mixture was allowed to gently stir for 18 hours at 4°C following which time it was filtered through a Sephadex G-25 medium column to remove unconjugated fluorescent material (UFM) which remained at the top of the column. The conjugated gamma globulin which eluted first was collected into a beaker and activated charcoal (prepared by boiling in IN HCl), 2.5 mg per mg of protein, was added and stirred for 1 hour at 24°C which was of paramount importance to avoid tick autofluorescence. The resulting mixture was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was distributed into screw cap vials and stored at -20°C until used. All vials of conjugated antibody were tested for neutralization of Powassan virus before using for staining.

Microscopy Procedures

a. Immunofluorescence

Fluorescein-conjugated antibody was adsorbed for 10 minutes

at 24°C with acetone-dried whole-tick powder before staining sections. The following protocol was adopted as a routine. 1. Conjugated antiserum alone (staining) 2. Blocking test: unconjugated antiserum, followed by conjugated antiserum (no staining). 3. Control blocking test: unconjugated control serum, followed by conjugated antiserum (staining). 4. no treatment: slide mounted unstained to control autofluorescence (no staining). 5. Uninfected samples stained with specific conjugated serum (no staining).

Fixed sections were covered with the conjugate and kept in moist chambers at 27°C for 30 minutes. The slides were then washed with 3 changes of PBS (5 minutes each wash) and mounted wet in buffered 50% glycerol, pH 8.0 and examined under a Vickers Patholux Fluorescence Microscope employing an Osram H.B.O. 200 mercury vapour lamp. A 3 mm thick BG12 primary filter and two secondary filters (O.G. 1/1.5 mm and G G.9/1.5 mm plus clear glass/1.5 mm) were inserted.

Sections were photographed with a Vickers 35 mm camera on Kodak Ektachrome-X or Plus X Pan film magnifying 100 times and 500 times.

b. Light Microscopy

Following examination for immunofluorescent foci the cover-

slips were removed from the sections and the slides washed twice in phosphate buffer for 10 minutes. After dehydration in ethanol, sections were stained with hematoxylin and eosin (H and E) (22), and mounted with Permount. Sections of infected ticks were compared with similarly treated control ticks for the presence or absence of cytopathology. Sections stained with H and E were observed in the Vickers Patholux microscope employing an iodine quartz light source and a blue primary filter together with 1.5 mm clear glass and 1.5 mm G.G.9 secondary barrier filters.

Virus

The LB prototype strain of Powassan virus (43) in its fifth suckling mouse brain passage contained $10^{8.7}$ mouse LD_{50} per ml when titrated by intracerebral inoculation of weaned mice. This stock preparation of 20% mouse brain suspension was stored in 0.1 ml amounts in sealed ampoules at -70°C and used in all experiments.

Virus Assay Procedures

Whole blood from viremic animals was diluted 1:10 upon collection into Alsever's solution. Individual adult female ticks (approximately 0.1 ml) were ground in mortars and extracted

with 0.9 ml of 0.15 M sodium chloride solution containing 20% calf serum. These provided starting suspensions for virus assay. Five to 10 larvae, individual nymphs and dissected organs were extracted with 0.9 ml of 0.15 M calf serum saline and considered as a 1:10 dilution for starting suspensions.

Assays were performed by intracerebral inoculation of groups of 3 weaned mice aged 3 to 4 weeks with 0.03 ml quantities of serial 10-fold dilutions of starting suspensions. All virus dilutions were performed in 0.15 M sodium chloride containing 10% calf serum together with penicillin 1000 units per ml and streptomycin 0.5 mg per ml. The mouse LD_{50} titers per ml were calculated for each preparation by the method of Reed and Muench (61). All infectivity titration results were reported representing the geometric mean of 3 to 5 tick pools.

Serology

Neutralization tests (NT) were conducted by mixing 0.15 ml volumes of undiluted serum with similar volumes of serial tenfold dilutions of Powassan virus (42). The resultant mixtures were held at 24°C for 1 hour, following which 0.03 ml quantities were inoculated intracerebrally into groups of 3 mice. In the presence of neutralizing antibody, mice survived for 10 days, but in the absence of antibody, mice developed fatal encephalitis 6 to 8 days

after inoculation. The $\log_{10}$ neutralization index of each serum sample was calculated from the difference between the $\log_{10}$ mouse LD_{50} of virus in the presence of normal serum and that in the presence of test serum.

Hemagglutination inhibition (HI) antibodies were assayed by the Microtiter technique (66), employing disposable plastic plates manufactured by Cooke Engineering Company, Alexandria, Virginia, U. S. A.

Powassan virus hemagglutinin was prepared by the sucrose-acetone technique of Clarke and Casals (20). All sera were extracted twice with 10 volumes of acetone to remove non-specific inhibitors of hemagglutination, followed by adsorption with goose erythrocytes. Serial twofold dilutions of treated sera in 0.025 ml quantities were made in borate saline pH 9 in Microtiter plates, 4 to 8 agglutinating doses of Powassan virus antigen were added, and after standing for 60 minutes at 24°C, 0.05 ml of a 0.25% goose erythrocyte suspension in an appropriate virus adjusting diluent were added. The HI titer was recorded as the reciprocal of the highest dilution of serum exhibiting 100% hemagglutination inhibition.

Viremia Experiments

a. Rabbits

New Zealand white rabbits were inoculated into an ear vein

with 1.0 ml of a mouse brain suspension of Powassan virus containing $10^{8.5}$ mouse LD_{50} per ml to produce a high level of viremia instantly. Blood was collected from the ear before inoculation and tested for neutralizing antibodies to Powassan virus.

Immediately following inoculation and at selected time intervals thereafter, samples of whole blood (0.5 ml) were collected into screw cap vials containing 4.5 ml of Alsever's solution. These samples were subsequently titrated for virus content using serial tenfold dilutions. Complementary 5.0 ml samples were collected into centrifuge tubes. The serum was separated by spinning at 3,000 rpm for 10 minutes, stored at -20°C and tested for neutralizing and HI antibodies to Powassan virus.

A second dose of $10^{5.5}$ mouse LD_{50} of Powassan virus in 0.3 ml was administered on the fifty-sixth day following which blood samples were again collected and processed for HI and neutralizing antibodies to Powassan virus.

b. Hamsters

Twelve hamsters were inoculated s.c. with Powassan virus in an attempt to produce viremia and thus provide an indicator system for transmission studies. A group of 6 hamsters each received 0.03 ml of 10 LD_{50} of virus and six others each received 100,000 LD_{50}. Blood was collected by cardiac puncture before inoculation

and tested for HI and neutralizing antibodies to Powassan virus. Samples of whole blood (0.5 ml) which were collected in screw-cap vials containing 0.5 ml of Alsever's solution immediately after inoculation and at varying intervals thereafter for a total of 9 days, were assayed for Powassan virus.

Beginning at 5 days, approximately 1 ml volumes of blood were collected into centrifuge tubes and the serum tested for HI antibodies to Powassan virus. Each serum was divided equally into 0.1 ml volumes. One volume was diluted 1:10 in 2-mercapto-ethanol (0.1 M) and the other received a similar dilution in distilled water. The resulting mixtures were incubated at 27°C for 30 minutes following which time they were treated as outlined for treatment of sera for HI antibodies in Serology Techniques.

c. Guineapigs

An experiment using guineapigs instead of hamsters was performed as described above. Blood was collected by cardiac puncture and Alsever's solution was used as the anticoagulant in all collections of whole blood. Extraction with 2-mercaptoethanol was not performed on guineapig sera.

Results and Discussion

Serological Response of Rabbits to Intravenous (i.v.) Inoculation of Powassan Virus

Rabbits inoculated i.v. with $10^{8.5}$ mouse LD_{50} of Powassan virus per ml had average blood levels of virus at 1 hour of $10^{4.5}$ mouse LD_{50} per ml (Figure 5 summarizes the average response of 3 rabbits). This level decreased to $10^{1.0}$ mouse LD_{50} per ml by 6 hours and at 8 hours virus was not detected. In each of the 3 rabbits used, hemagglutinin inhibiting antibodies first appeared at 5 days (Figure 6). Average peak titers of 640 were observed at 9 days, which gradually decreased to 10 or 20 by the forty second day. Neutralizing antibodies were first detected in the serum 9 days after injection and at 7 weeks the $\log_{10}$ neutralization index was 3.0. A second dose of $10^{5.5}$ mouse LD_{50} per ml of Powassan virus in a 0.3 ml volume was injected s.c. on the fifty sixth day. Average peak antihemagglutinin titers of 640 were observed on the sixty first day, followed by a gradual decline to very low levels by 90 days. No increase of $\log_{10}$ neutralization index was observed in serum obtained 4 weeks after the second dose of virus.

Induction of viremia at high titer immediately, by intravenous injection of stock virus, was necessary because s.c. injection of virus induced titers of viremia less than $10^{2.0}$ mouse LD_{50} per ml. Rabbits are convenient animals for the feeding of all stages of D. andersoni ticks. Active blood sucking by ticks was noted 2

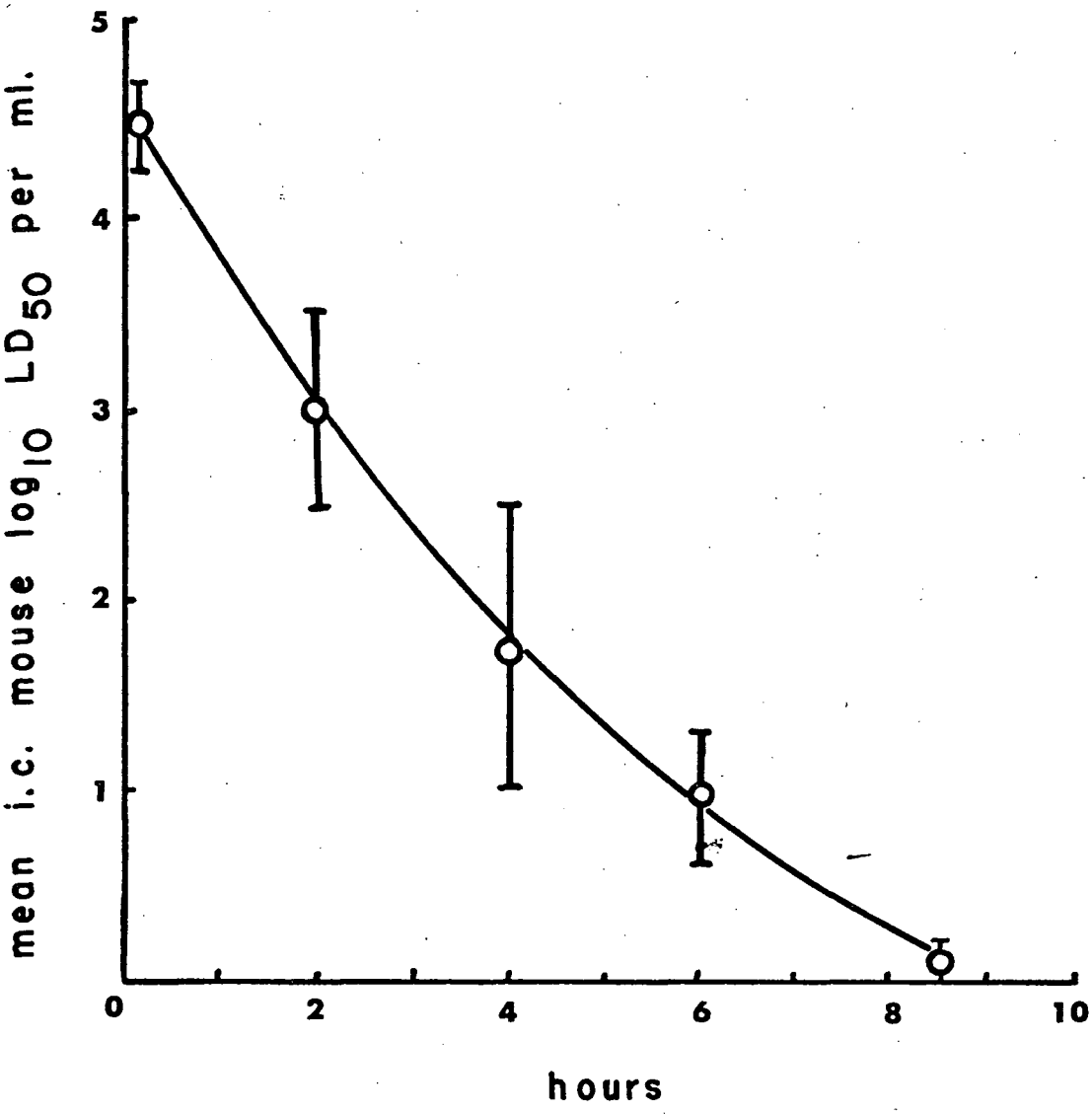


Fig. 5. Viremia in New Zealand white rabbits after intravenous injection of $10^{8.5}$ mouse LD_{50} of Powassan virus per ml.

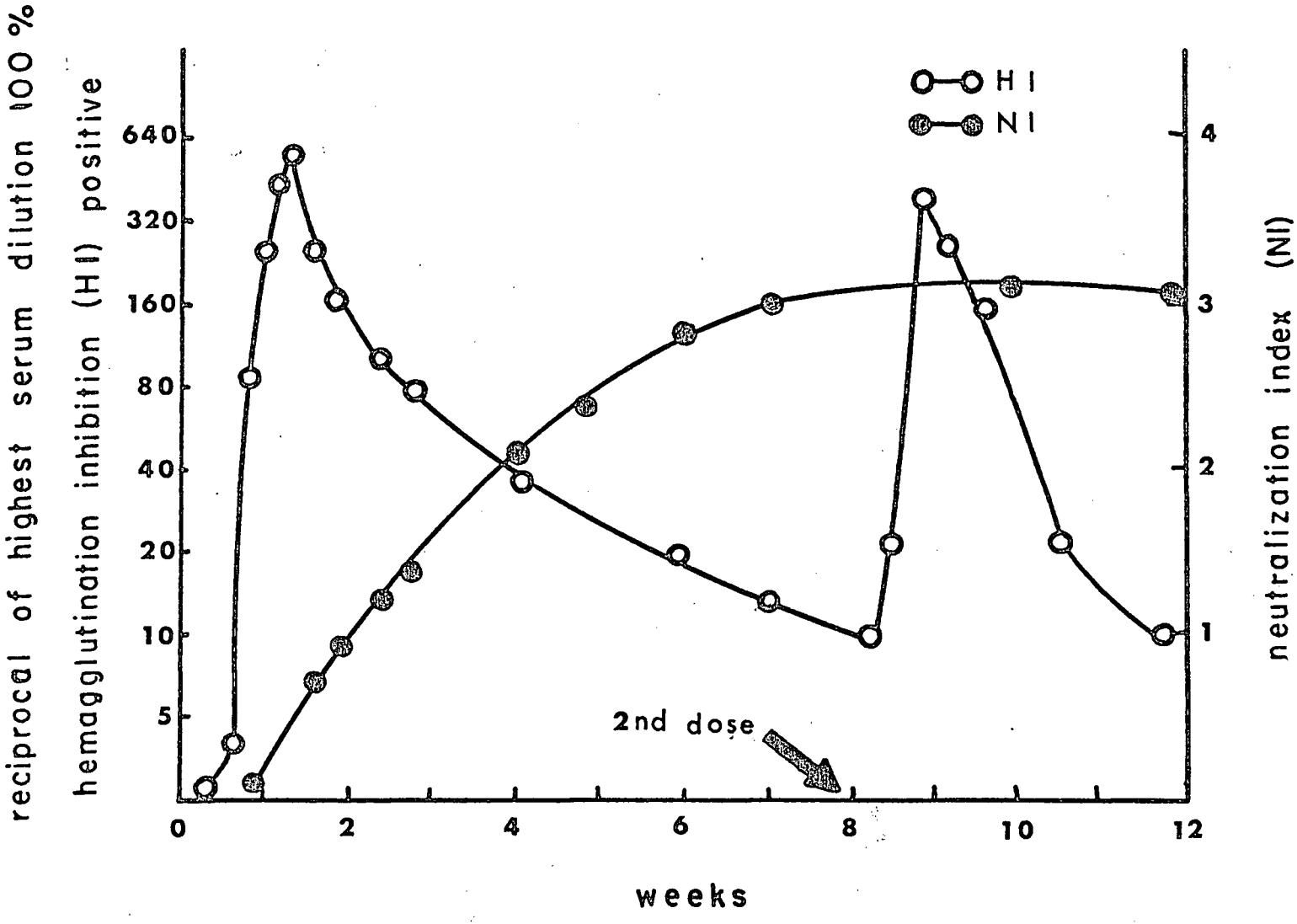


Fig. 6. Serological response of rabbits to Powassan virus following intravenous injection of $10^{8.5}$ mouse LD_{50} per ml. (Each measurement an average of 3 samples).

to 3 days after they were placed on rabbits, at which time virus was injected i.v.

This method proved to be a convenient and readily reproducible technique for infecting ticks. It offers an alternative to the following systems: (i) O. moubata fed on membranes of embryonated hen's eggs, infected with WEE virus (12); (ii) D. andersoni fed on ground squirrels circulating CTF virus in the blood following s.c. injection of virus (10); (iii) D. andersoni fed rabies virus in blood from a capillary tube placed over the hypostome (8); (iv) ixodid ticks parenterally inoculated into the hemolymph with TBE virus (64).

Since neutralizing antibodies to Powassan virus were first detected in sera of rabbits 9 days after inoculation, by which time all ticks had become detached, it seems unlikely that antibody within the vertebrate host would interfere with virus infection of the gut cells of the arthropod. This was suggested by Benda's experiments with I. ricinus which ingested TBE virus (6). Similarly it is unlikely that a sufficient concentration of antibody in a recently ingested blood meal would interfere with virus released from cells during the crushing of ticks for assay.

Serological Response of Hamsters to s.c. Inoculation of Powassan Virus

Twenty four hours after inoculating hamsters s.c. with

10 LD_{50} of Powassan virus, no infectivity was demonstrated in peripheral blood (Figure 7). However, at 48 hours an average titer of $10^{3.5}$ mouse LD_{50} per ml was demonstrated. Infectivity increased to $10^{4.5}$ mouse LD_{50} per ml by the 3rd day and a peak titer of $10^{6.2}$ mouse LD_{50} per ml was attained on the 4th day after inoculation. At 108 hours a decrease to $10^{5.7}$ mouse LD_{50} per ml was evident and a rapid decrease to $10^{3.8}$ mouse LD_{50} per ml took place by the 5th day. The average titer of virus on the 6th day was $10^{2.2}$ mouse LD_{50} per ml which decreased to zero by the 7th day.

HI antibodies first appeared at a titer of 10 on the 7th day after virus inoculation and increased to 40 by the 10th day. All of the HI antibody in these samples was in the IgM fraction since its activity was abolished by 2-mercaptoethanol treatment. Although the average HI titer of antiserum sampled on the 12th and 14th days did not increase above 40, much of the HI activity was contained in the IgG fraction. The highest HI antibody activity was demonstrated on the 17th day (HI titers $\geqslant$ 160) following which titers of 80 were demonstrated on the 19th and 24th days and 40 on the 28th day. A gradual rise in IgG antibody was evident between the 12th and 24th days, and by the 28th day all the HI activity was contributed by IgG antibody.

A strikingly different response was attained in hamsters inoculated with 100,000 LD_{50} of Powassan virus (Figure 7).

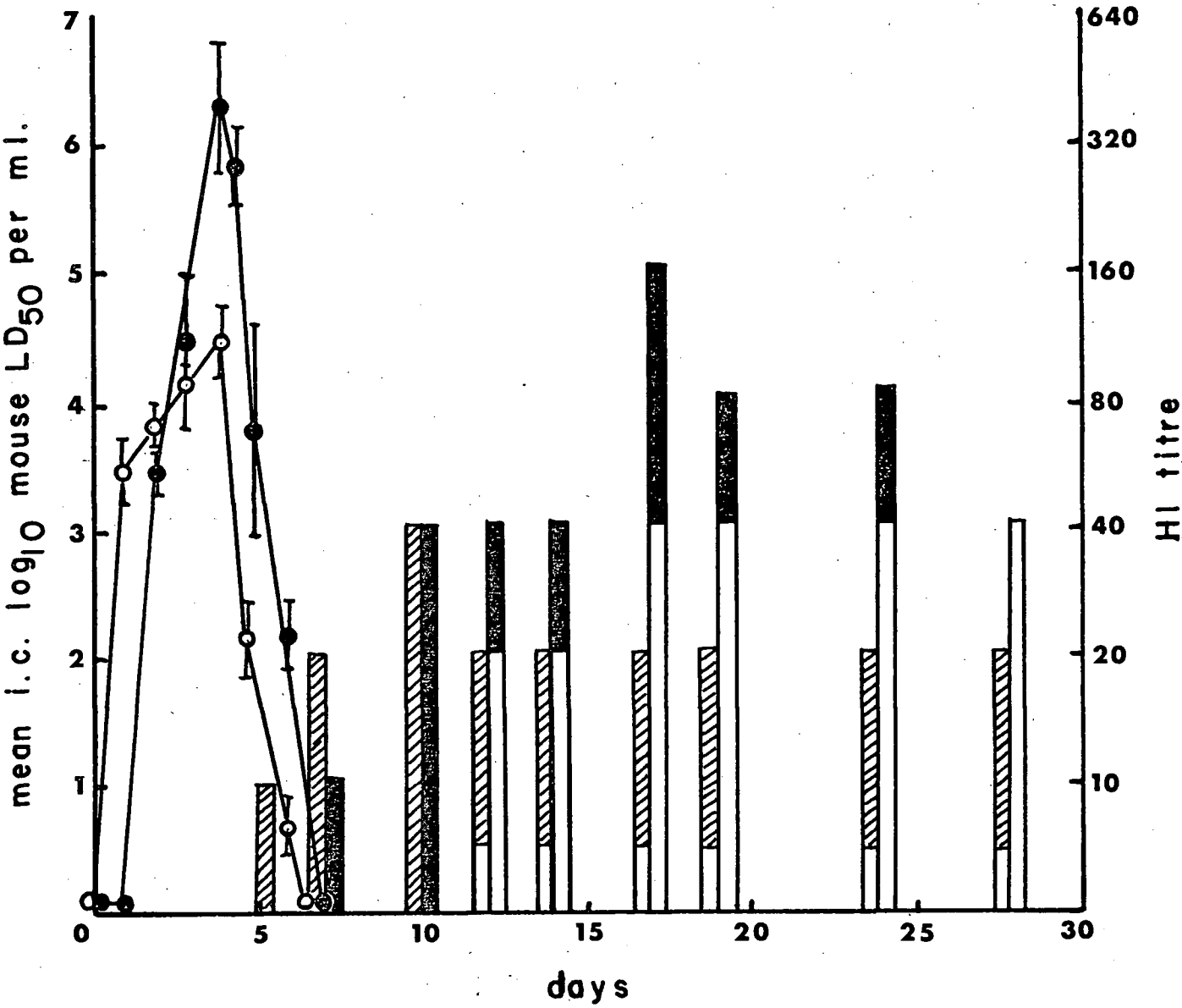


Fig. 7. Serological response of hamsters to a high and low subcutaneous dose of Powassan virus: [viremia following: (O) 100,000 LD_{50} inoculum, (●) 10 LD_{50} inoculum]; [antibody response: (▨ IgM to 100,000 LD_{50} inoculum, ■ IgM to 10 LD_{50} inoculum, □ IgG .]

Average titers of $10^{3.5}$ mouse LD_{50} per ml were found in whole blood 24 hours after inoculation. A gradual increase to $10^{4.5}$ mouse LD_{50} per ml was demonstrated on the 4th day, subsequently dropped to $10^{2.2}$ mouse LD_{50} per ml on the 5th day, and disappeared by the 7th day.

HI activity first appeared in hamsters receiving the high dose of virus on the 5th day (titer of 10) and increased to a maximum of 40 by the 10th day. All of the HI activity from the 5th to the 10th day was contributed by IgM antibody as shown by the 2-mercaptoethanol test. HI activity stabilized at 20 for the subsequent 18 days of sampling and virtually no IgG antibody could be demonstrated.

Although the viremia induced by a high dose of virus (100,000 LD_{50}) was elicited 24 hours earlier it also declined more rapidly. Similarly it failed to reach as high a peak as the low (10 LD_{50}) inoculum; (the high-dose peak averaged $10^{4.5}$ mouse LD_{50} per ml whereas the low-dose peak averaged $10^{6.2}$ mouse LD_{50} per ml).

Although the HI activity following the high dose of virus appeared as early as 5 days when virus still circulated in the blood, it never reached titers higher than 40 and most of the antibody activity resided in the IgM component. In direct contrast, the response following the low virus dose appeared 2 days later (7 days) but reached higher titers ($\geq$160) which

persisted between 40 and 80 for the duration of the experiment. The low inoculum elicited an early IgM response which was gradually replaced by an equally high IgG response by the 28th day.

The viremia results presented a meaningful observation upon which to base transmission attempts by the bite of ticks. Therefore, an isolation of virus from the blood of a hamster 4 or 5 days following the beginning of engorgement by an infected tick would provide good evidence of virus transmission. This observation, correlated with the antibody response several days later, would be conclusive.

Induction of an antibody response which had virtually no IgG component after a high dose of virus, mitigates against the use of 2-mercaptoethanol as a method of determining the approximate time of exposure of an animal to a virus, because the response of the animal is not only dependent upon time but also upon the initial immunizing dose.

Determination of the Infection Threshold of D. andersoni Adults for Powassan Virus

When varying doses of Powassan virus were inoculated intravenously into rabbits with adult ticks feeding upon them an infection threshold phenomenon was demonstrated (Table II). When rabbits circulated $10^{1.5}$ mouse LD_{50} of Powassan virus per ml

Table II: Infection threshold of Dermacentor andersoni adult ticks for Powassan virus

Log_{10} LD_{50} per ml. of Powassan virus in blood *	1.5	2.5	3.5	4.5
% ticks infected after 30 days	0(0/24)	4(2/50)	90(27/30)	96(48/50)

* Rabbits were injected intravenously 72 hours after commencement of tick feeding

Table III: Effect of time of viremia on D. andersoni adult infection

Days after ticks commenced feeding *	3	5	7
% ticks infected after 30 days	85(17/20)	50(15/30)	28(7/25)

* Rabbits were injected intravenously to create a circulation of $10^{3.5}$ LD_{50} Powassan virus per ml of blood

of blood immediately after injection, none of 24 ticks were infective when tested 30 days later. However, when $10^{2.5}$ mouse LD_{50} of virus per ml was circulating, 2 of 50 ticks (4%) became infected as did 90% and 96% at $10^{3.5}$ LD_{50} and $10^{4.5}$ LD_{50} respectively. Thus the infection threshold of viremia with Powassan virus for D. andersoni adult ticks was approximately $10^{2.5}$ mouse LD_{50} per ml. Female ticks became infected 90% more often than male ticks because the latter are inconsistent blood feeders.

A similar experiment was performed in which the infecting dose ($10^{3.5}$ LD_{50} per ml) was kept constant and the time of inoculation following the commencement of tick engorgement, was varied (3,5 and 7 days). The percent number of ticks infective after 30 days was then determined by i.c. inoculation of engorged tick homogenates into weaned mice. Eighty five percent (17/20) of ticks ingesting virus on the 3rd day were infective 30 days later (Table III), as compared to 15 of 30 (50%) imbibing $10^{3.5}$ mouse LD_{50} of virus on the 5th day. This percentage was reduced to 28% (7 of 25) when the virus was inoculated on the 7th day after feeding commenced.

This trend of reduction of numbers of individuals becoming infected ("raising the infection threshold"), beyond the 3rd day after feeding commences, does not correlate with the fact that larger amounts of blood are ingested towards the end of engorgement,

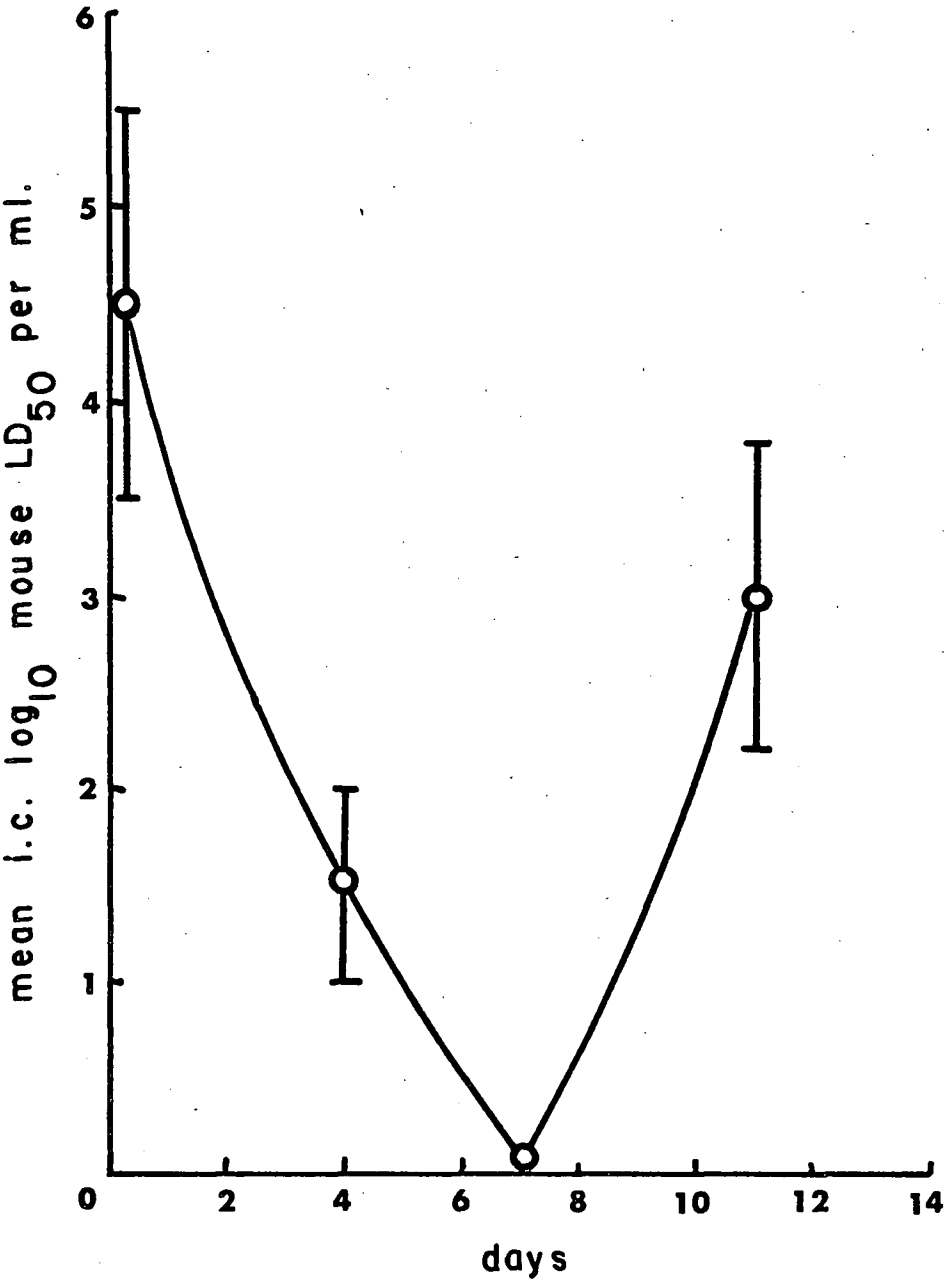


Fig. 8. Development of Powassan virus in whole D. andersoni larvae following feeding upon a rabbit injected intravenously.

and thus, presumably, larger numbers of virus particles. It is observed, however that as feeding progresses the number of viable epithelial cells connected to the membrane propria decreases significantly until there are only a few cells left by the time the tick drops from its host. Thus, regardless of the increased numbers of virus particles ingested, a decreased number of cells available for invasion may contribute to a lesser number of individuals becoming infected.

Development of Powassan Virus in D. andersoni Larvae Following Feeding Upon a Rabbit Injected i.v.

Forty eight hours after larvae attached to a rabbit's ear, the animal was injected i.v. with $10^{8.5}$ mouse LD_{50} of Powassan virus per ml. Within 8 hours an average titer of $10^{4.5}$ mouse LD_{50} per ml of virus was demonstrated in homogenates of groups of 5 to 10 whole larvae (Figure 8). On the 4th day only $10^{1.5}$ mouse LD_{50} per ml was recovered from engorged larvae and virus was not detected on the 7th day. By the eleventh day, the average titer rose to $10^{3.0}$ mouse LD_{50} per ml as larvae began to molt.

The isolation of $10^{4.5}$ mouse LD_{50} per ml of Powassan virus from whole larvae within 8 hours after the rabbit was injected was virus that was ingested during engorgement. The decrease to zero by the 7th day and subsequent increase to $10^{3.0}$ mouse LD_{50} by

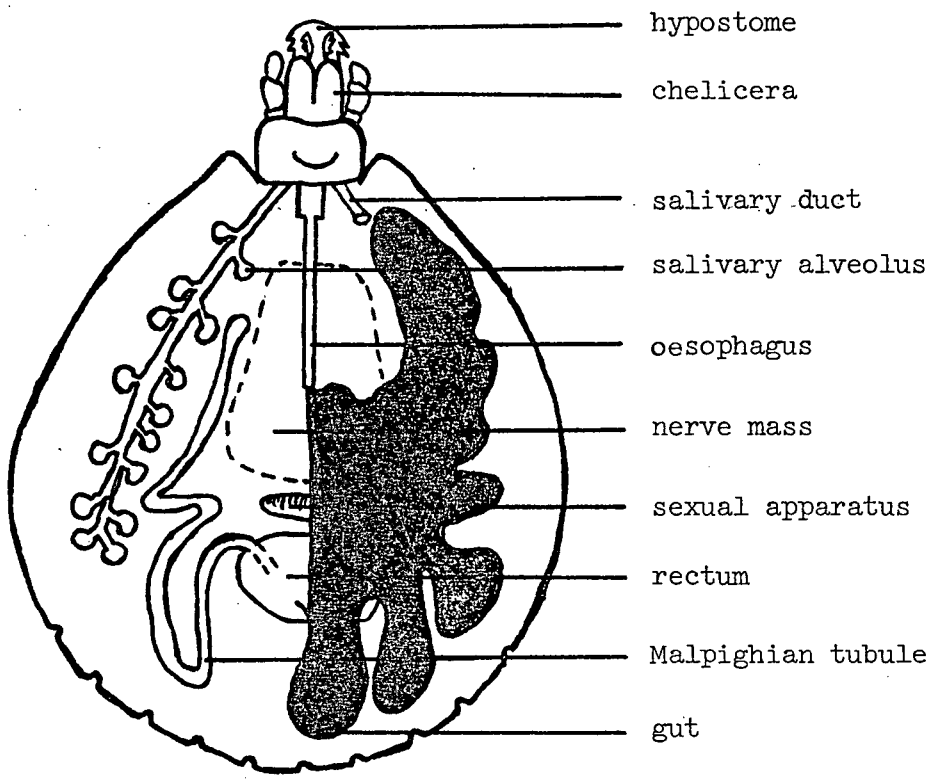


Fig. 9. Unfed larva of D. andersoni showing internal view of organs

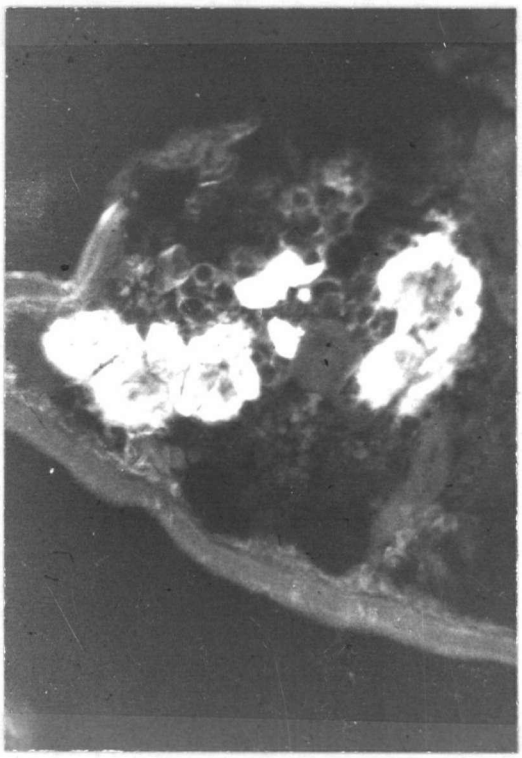


Fig. 10. Gut cells of a larva 13 days after taking a blood meal containing Powassan virus. (Section was stained with fluorescein isothyiocyanate-conjugated Powassan virus antiserum) (X100).

the eleventh day represented a possible combination of virus inactivation as well as virus entrance, uncoating, and replication in susceptible gut cells.

Longitudinal and transverse sections of whole larvae were cut and stained with Powassan virus antibody conjugated with FITC to determine the organs (Figure 9) that supported virus growth during the engorged larval stage. Specific staining was consistently observed in gut epithelial cells between the 9th and 18th days after virus inoculation. Figure 10 demonstrates specific cytoplasmic fluorescence in the gut epithelium of a 13 day larvae. Specific fluorescence was not observed in any other organs during the larval stage. H and E staining of sections depicted no cytopathological changes due to virus infection.

Development of Powassan Virus in D. andersoni Nymphs Following Feeding Upon a Rabbit Injected i.v.

At varying time intervals after infection, organs were selected for titration from individual nymphs that had engorged (Figure 11).

Within 8 hours after injection of $10^{8.5}$ mouse LD_{50} of Powassan virus per ml into the rabbit, dissected nymph gut yielded an average of $10^{2.7}$ mouse LD_{50} of virus per ml (Table IV). On the 5th day no virus could be detected from any organs. However

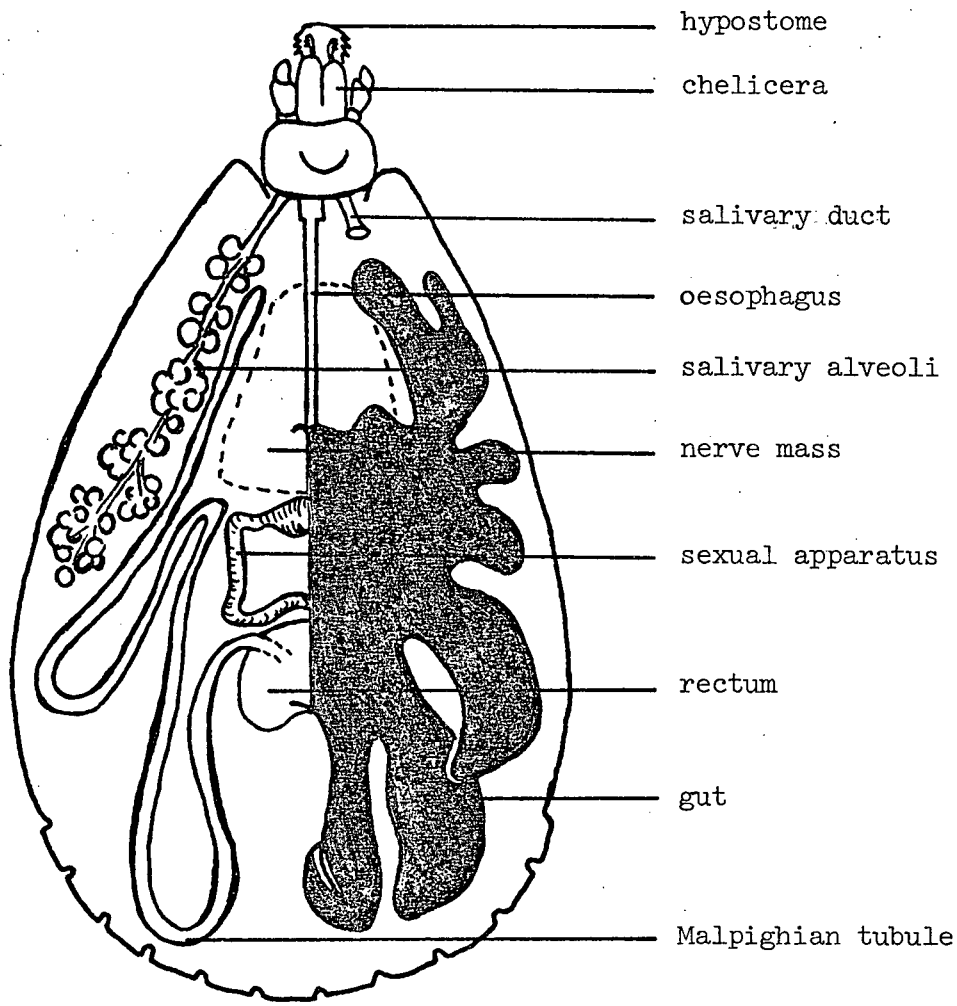


Fig. 11. Fed nymph of D. andersoni showing internal view of organs

Table IV: Development of Powassan virus in D. andersoni nymph organs after feeding upon a rabbit injected intravenously

Anatomical unit	Days after infection				
	0	5	12	19	26
Diverticula	2.7$^{\gamma}$±.25 (washings)	0*	3.0±.30	4.0±.41	4.0±.47
Malpighian tubules	0	0	0	2.5±.67	2.5±.67
Central nerve mass	0	0	0	0	0
Reproductive system	0	0	0	0	0
Salivary glands	0	0	0	3.5±.12	3.5±.30

* = virus not detected

γ = $\log_{10}$ LD_{50} per ml based upon 5 different measurements with 95% confidence limits

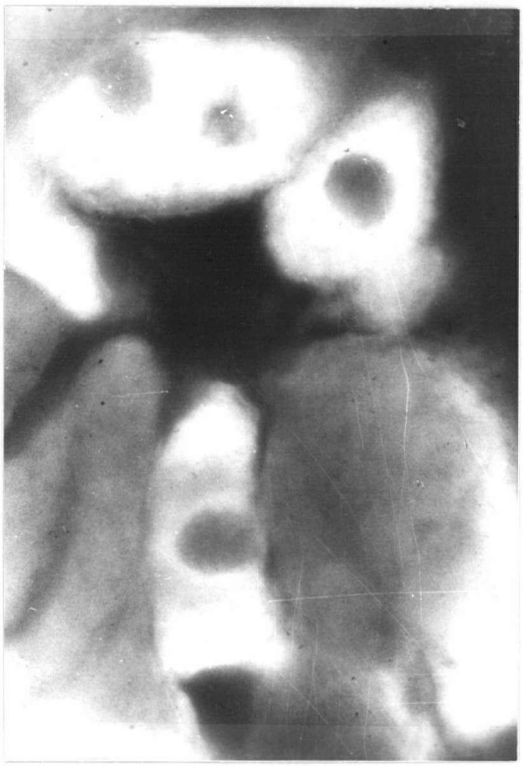


Fig. 12. Gut cells of a nymph 24 days after taking a blood meal containing Powassan virus. (Section stained with FITC conjugated Powassan virus antiserum) (X500).

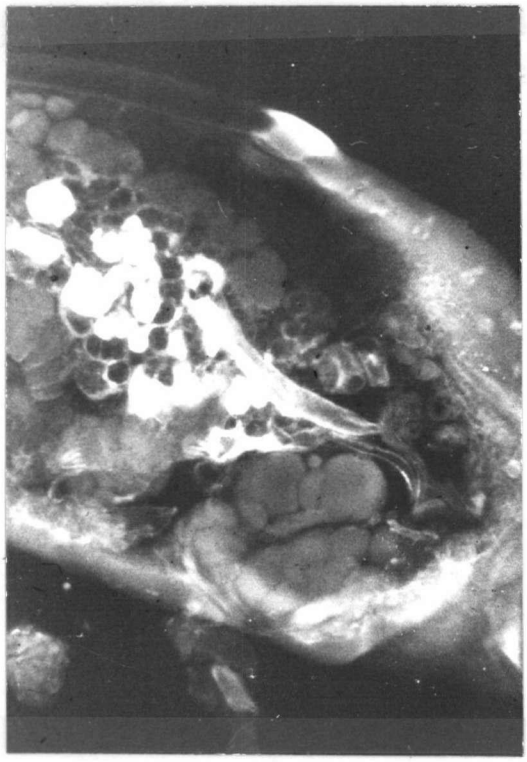


Fig. 13. Salivary gland alveoli of a nymph 24 days after taking a blood meal containing Powassan virus. (Section stained with FITC conjugated Powassan virus antiserum). (X100).

by the 12th day $10^{3.0}$ mouse LD_{50} of virus per ml was detected in diverticula and subsequently in Malpighian tubules, and salivary glands on the 19th day. Peaks of $10^{4.0}$, $10^{2.5}$ and $10^{3.5}$ LD_{50} per ml in diverticula, Malpighian tubules and salivary glands respectively on the 26th day were demonstrated when several individuals had begun their molt to adults.

A phase of infectivity-eclipse was demonstrated between the 1st and 11th days and is comparable to results obtained in the diverticula of larvae. Virus was demonstrated in gut, Malpighian tubules and salivary glands of nymphs as compared to only diverticula of larvae.

Specific cytoplasmic fluorescence was observed only in gut cells (Figure 12) and salivary gland alveoli (Figure 13). Although Malpighian tubules were infective when titrated in mice, no fluorescence or only very dim fluorescence was shown in tubule sections. It is possible that virus being released from cells is picked up by Malpighian tubules that monitor the hemolymph. This virus may become slowly inactivated in the tubule lumen as the waste passes to the rectum, thus never setting up an infection within Malpighian tubule cells.

Development of Powassan Virus in D. andersoni Adults Following Feeding on a Rabbit Injected i.v.

Figure 14 represents the organs that were assayed for virus

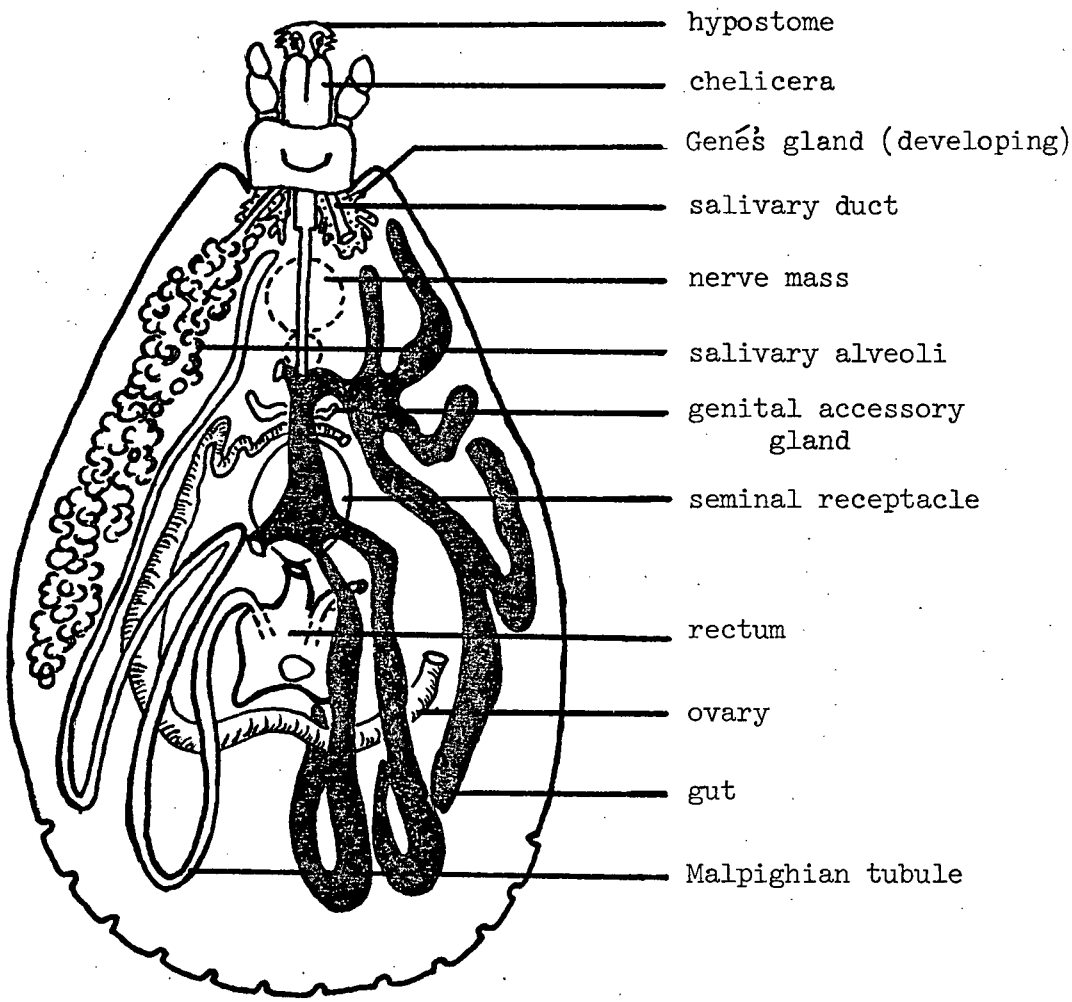


Fig. 14. Unfed adult female of D. andersoni showing internal view of the organs. (Male tick is basically identical except testes replace the sexual apparatus).

content after adult ticks were infected by feeding on rabbits injected with $10^{8.5}$ mouse LD_{50} of Powassan virus per ml.

Within 8 hours after actively feeding adults imbibed infective blood an average of $10^{3.2}$ mouse LD_{50} per ml was present in washings from diverticula (Table V). Virus was not detected in any organs at 5 days, however on the 9th day only diverticula yielded virus (average of $10^{2.5}$ LD_{50}). Virus titers rose to $10^{4.0}$ mouse LD_{50} per ml in diverticula on the 17th day and remained at that high level until the 32nd day following which a marked decline to $10^{3.0}$ mouse LD_{50} per ml was observed to the 61st day after infection.

Virus first appeared in salivary glands of females on the 17th day ($10^{3.5}$ mouse LD_{50} per ml) and increased to maximum levels of $10^{4.5}$ mouse LD_{50} per ml by the 32nd day following which a slight decline was evident to $10^{3.5}$ mouse LD_{50} per ml on the 39th day. In males, however, there was no decline in salivary gland infectivity. Maximum titers of $10^{6.5}$ mouse LD_{50} per ml were observed in salivary glands dissected on the 61st day.

Malpighian tubules and the accessory sex glands of either sex yielded variable virus titers between the 24th and 61st days, ranging from $10^{2.0}$ to $10^{3.5}$ mouse LD_{50} per ml.

Genés glands supported virus growth beginning with $10^{3.0}$ mouse LD_{50} per ml on the 24th day, increased to $10^{5.0}$ mouse LD_{50} per ml on the 32nd day and subsequently declined to an average of $10^{3.5}$ mouse LD_{50} per ml on the 61st day.

Table V: Development of Powassan virus in D. andersoni adult tick organs after feeding upon a rabbit injected intravenously

Anatomical unit	Days after infection							
	0	5	9	17	24	32	39	61
Diverticula	$3.2^{\gamma}\pm.23$ (washings)	0*	2.5±.52	4.0±.08	4.5±.20	4.0±.55	2.0±.76	2.5±.32
Malpighian tubules$^{\gamma}$	0	0	0	0	3.0±.88	3.0±.90	3.5±.63	2.5±.45
Genés glands	0	0	0	0	3.0±.09	5.0±.23	4.5±.09	3.5±.34
Accessory sex gland	0	0	0	0	2.3±.67	3.5±.87	2.5±.77	2.0±.32
Salivary glands ♀	0	0	0	2.5±.63	4.5±.12	4.5±.22	3.5±.17	4.0±.30
Salivary glands ♂	0	0	0	2.5±.33	4.5±.12	6.5±.20	6.0±.20	6.5±.09

* = virus not detected

γ = $\log_{10}$ LD_{50} per ml based upon 5 different measurements with 95% confidence limits

Heart, trachea, nerve mass, ovary, testes, and fat body did not yield virus

Heart, trachea, nerve mass, ovary, testes and fat body did not yield virus.

When sections of organs were cut and stained with fluorescent Powassan virus antiserum, specific cytoplasmic fluorescence was observed only in diverticula, Gené's glands, accessory sex glands and salivary gland alveoli.

Figure 15 is a cross section of two lobes of Gené's gland from a 28 day engorged female showing fluorescent foci located peripherally in the section. Figure 16 is a higher magnification of Gené's gland cells showing fluorescence throughout the entire cell cytoplasm. Several females demonstrated focal areas of fluorescence in the accessory gland (Figure 17), which were cytoplasmic, correlated well with infectivity assays and were quenched by prior addition of unconjugated Powassan virus antiserum (blocking-test). Figure 18 shows specific focal areas of Powassan virus infection in salivary glands. Some alveoli had only 2 or 3 cells infected, whereas some entire alveoli showed specific cytoplasmic fluorescence, as demonstrated in Figure 19. H and E staining showed no cytopathology due to virus growth in infected organs.

Similar to larvae and nymphs, Powassan virus grew first in the epithelial cells of adult diverticula and gained access to salivary glands and other organs subsequently. The fact that virus was never demonstrated in heart, trachea, nerve mass, ovary, testes or fat body indicated a definite preferential selection of certain tissues for growth.

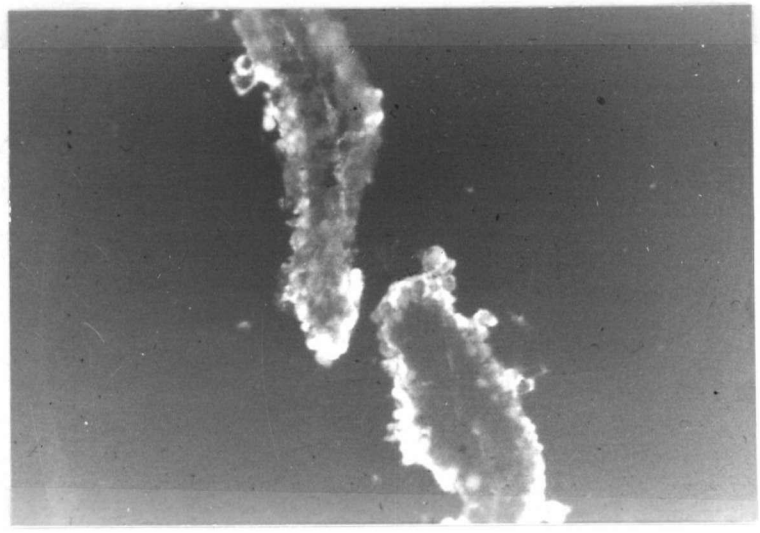


Fig. 15. Cross section of two lobes of Gené's organ gland of an adult female tick 28 days after imbibing a blood meal containing Powassan virus. (Stained with FITC conjugated Powassan virus antiserum). (X100).

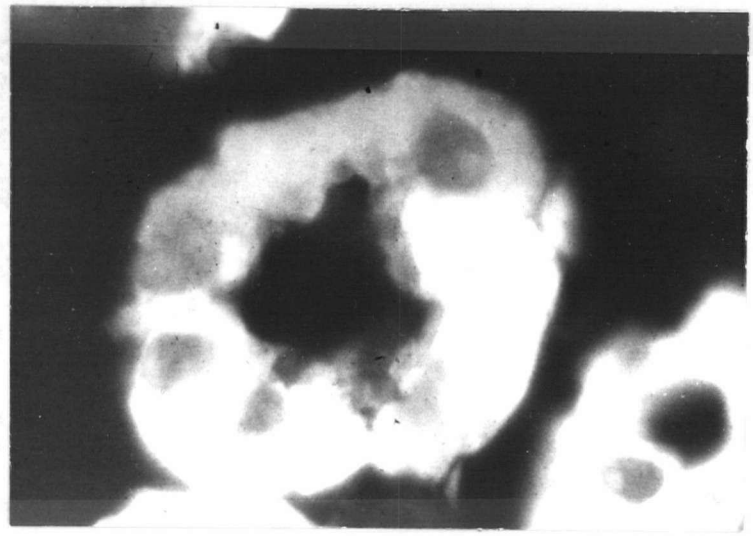


Fig. 16. Lobe of Gené's organ gland stained with FITC conjugated Powassan virus antiserum 28 days after infection by feeding (X500).

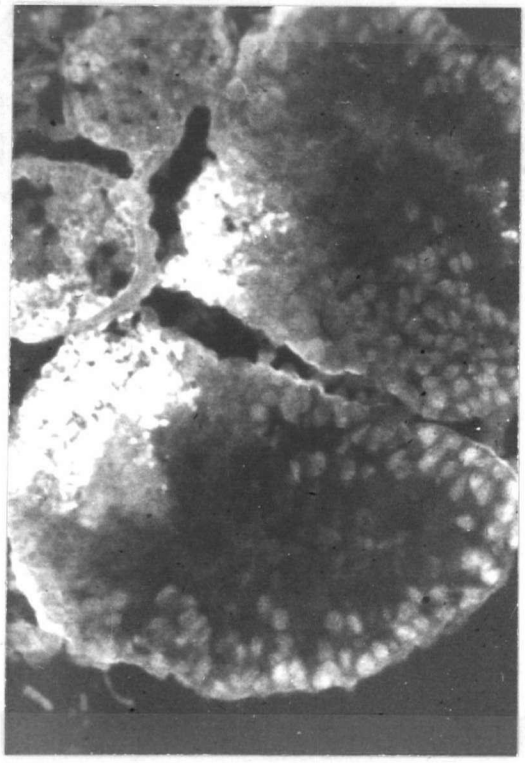


Fig. 17. Accessory gland lobes of an adult female tick 32 days after taking a blood meal containing Powassan virus (Stained with FITC conjugated Powassan virus antiserum) (X100).

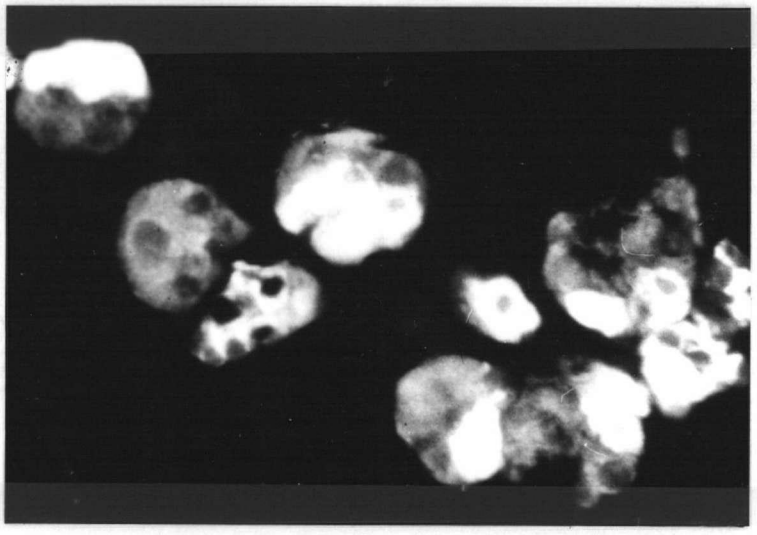


Fig. 18. Salivary gland alveoli dissected from a D. andersoni adult male tick 32 days after imbibing a blood meal containing Powassan virus (stained with FITC conjugated Powassan virus antiserum) (X100).

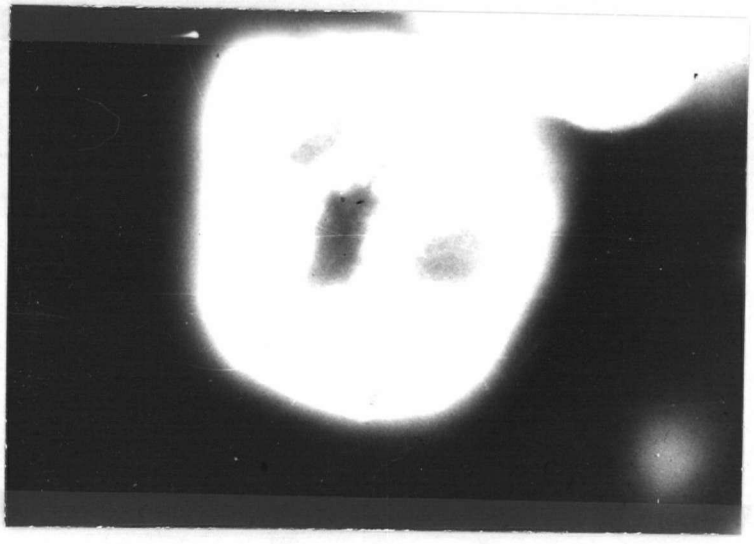


Fig. 19. Cross section of an adult tick salivary gland alveolus 32 days after imbibing a blood meal containing Powassan virus (stained with FITC conjugated Powassan virus antiserum) (X500).

By comparison, it is significant that the salivary glands of male ticks supported growth of virus, as high as $10^{6.5}$ mouse LD_{50} per ml to 61 days whereas female salivary glands reached peak titers of $10^{4.5}$ mouse LD_{50} per ml at 24 days and declined slightly thereafter. These findings correlated well with the development of salivary gland tissue in males and females during this period. The alveoli of males did not degenerate significantly after taking a blood meal and thus male adults may take several meals in a life time, and could be extremely good transmitters of Powassan virus. In direct contradistinction to males the salivary glands of replete females atrophied to a great extent after ticks dropped from their hosts and started to lay eggs. It was at this time that a tremendous surge of growth took place in the glands of Gené's organ and a switch over of virus growth and maintenance occurred from salivary gland tissue to Gené's gland tissue. This was reflected in the decline of titers in salivary glands and subsequent increase in Gené's gland between days 24 and 61.

Attempts to Isolate Virus from Oral and Anal Secretions

In contrast to frequent isolations of TBE virus (6) and EEE virus (62) from tick feces, Powassan virus was not detected in the feces of D. andersoni adults. If virus did pass into

the rectum and out the anus as indicated by frequent isolations from Malpighian tubules it probably became inactivated before or soon after leaving the anus.

Although arbovirus transmission has been successfully achieved for TBE by I. ricinus (5), and CTF by D. andersoni (10), infectivity has not been demonstrated in tick saliva. Following the collection of saliva from D. andersoni adult females that had been infected with Powassan virus in the larval stage, serial tenfold dilutions were made and inoculated i.c. into weaned mice. Table VI demonstrates the large variation of virus titer in 3 secretions from 3 different ticks, collected into capillary tubes within 1 hour after removing from a rabbit. In each case the tick was allowed to attach and engorge for 8 hours before removal, except in the first collection when the ticks were allowed to engorge for 4 days.

An increase in virus content in saliva was demonstrated in all 3 ticks from the first feeding to the last. This may be due to an induction of virus release from alveolar cells into the lumen and/or a mechanism of virus concentration as saliva accumulates. The titers of virus in saliva collected after the first 4 days of feeding ranged from $10^{3.5}$ to $10^{4.5}$ mouse LD_{50} per ml. Two days later when saliva was again collected the titers of virus ranged from $10^{5.0}$ to $10^{6.0}$ mouse LD_{50} per ml. A similar range was present on the third feeding. At each feed the donor

Table VI: Powassan virus titers determined from the salivary secretions of three adult ticks

Tick no. \ Dates	4 days	(4 + 2) days	(4 + 2 + 2) days
1 (female)	3.5±.23*	5.0±.61	6.5±.07
2 (female)	4.5±.36	6.0±.36	5.5±.42
3 (female)	4.5±.12	5.5±.24	6.5±.50

* = mean i.c. $\log_{10}$ LD_{50} of Powassan virus per ml with 95% confidence limits

rabbit used had an HI antibody conversion from negative to positive, which indicated that virus transmission had occurred.

Transstadial Development of Powassan Virus from Larvae to Nymphs (Infected as Larvae)

The development of Powassan virus in D. andersoni ticks has resembled some aspects of the growth of an unrelated arbovirus, CTF virus, in this tick species (65). Maximum titers of $10^{3.0}$ mouse LD_{50} per ml of Powassan virus were attained in engorged larvae as compared to $10^{4.5}$ mouse LD_{50} of CTF virus. Due to the difficulty in determining exactly when a larva began its molt, representative quiescent samples were pooled and tested for infectivity as well as for fluorescent foci. As demonstrated in Figure 20, the infectivity of Powassan virus in molting larvae dropped sharply from $10^{3.0}$ mouse LD_{50} per ml to undetectable amounts on the 16th day; then increased very rapidly to reach a maximum of $10^{6.0}$ mouse LD_{50} per ml on the twenty third day, as nymphs began to emerge. Slightly lower titers (average of $10^{5.0}$ mouse LD_{50} per ml) were evident when the molt was complete, and in the subsequent 3 weeks an average level of $10^{3.0}$ mouse LD_{50} per ml was present.

It was evident when slides were stained with FITC conjugated Powassan virus antibody, that only gut cells supported virus

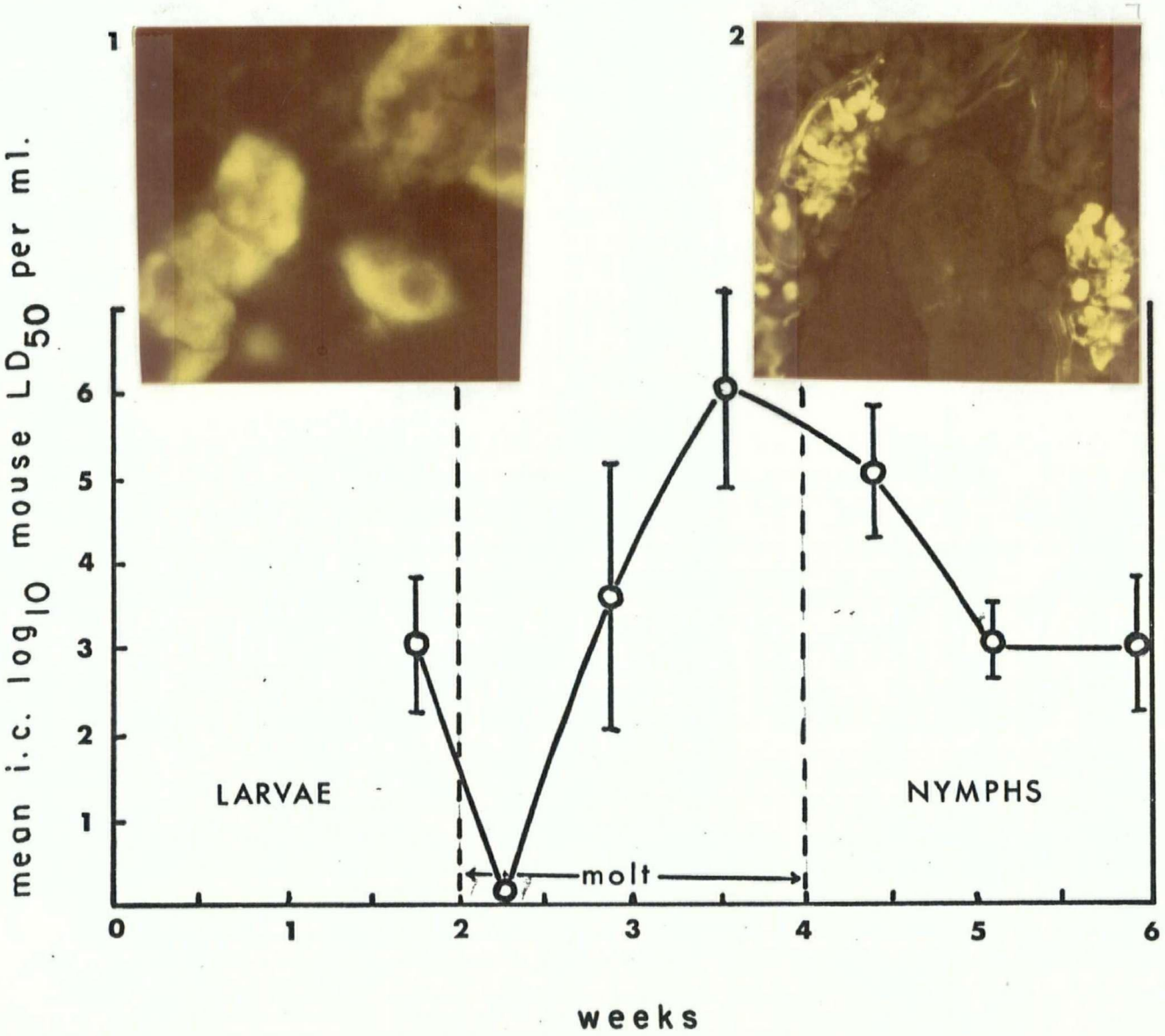


Fig. 20. Development of Powassan virus transstadially from larvae to nymphs, (1) larvae gut cells (X500); (2) nymph salivary glands (X100).

growth from the day of infection until the 13th day when larvae began to molt (Figure 20; insert 1). The rapid decrease in virus titers in the first few days of ecdysis may have been due to infected cells being detached and floating in the lumen. Virus particles may have escaped into the lumen to be quickly inactivated.

During the 14 days of ecdysis, larval tissue was rapidly removed by phagocytes and new nymphal tissue grew from embryonic growing areas to replace it. By this time virus had grown in gut cells and was transported either free or bound to phagocytes into the hemolymph and then to other organs such as salivary glands to gain access for infection. Sections cut and stained during the third week after infection showed an increasing amount of salivary gland tissue which had greater numbers of alveoli infected (Figure 20; insert 2). As individuals emerged into nymphs the majority of virus growth took place in salivary glands and nymph diverticula showed a lesser proportion of cells infected.

A tremendous amount of waste material, predominantly in the form of guanine crystals was present in the Malpighian tubules and rectum. During the molt an average of $10^{3.0}$ mouse LD_{50} per ml of virus was demonstrated in isolated Malpighian tubules. However, specific FITC staining was never demonstrated in cross sections. As the nymphs emerged from the larval skin large amounts of guanine were expelled from the anus. None of the excretions contained virus.

Transstadial Development of Powassan Virus from Nymphs to Adults (Infected as Larvae)

D. andersoni nymphs that contained $10^{3.0}$ mouse LD_{50} per ml of Powassan virus, and had obtained this infectivity as larvae, were placed upon a non-infected rabbit to feed. During engorgement, average virus titers dropped rapidly to $10^{0.4}$ mouse LD_{50} per ml following repletion and detachment from the host (Figure 21). This finding was in direct contrast to titers as high as $10^{5.0}$ mouse LD_{50} per tick of CTF virus in engorged D. andersoni nymphs (65).

During ecdysis a gradual increase in virus titers were observed which correlated well with the development of new adult tissue growth. By the first week after molting began, an average of $10^{1.5}$ mouse LD_{50} of virus was demonstrated in whole ticks which increased to $10^{3.5}$ mouse LD_{50} per ml upon emergence of adults. Ticks contained $10^{4.5}$ mouse LD_{50} per ml one week after emergence, and maximum levels of $10^{6.0}$ mouse LD_{50} per ml were attained during the next 2 weeks. This finding closely paralleled titers of $10^{5.6}$ mouse LD_{50} of CTF virus in flat adults (65).

During engorgement, and for 3 days thereafter, most cells within the salivary glands of nymphs underwent necrosis, leaving only isolated cells unaffected. Fluorescent alveolar foci

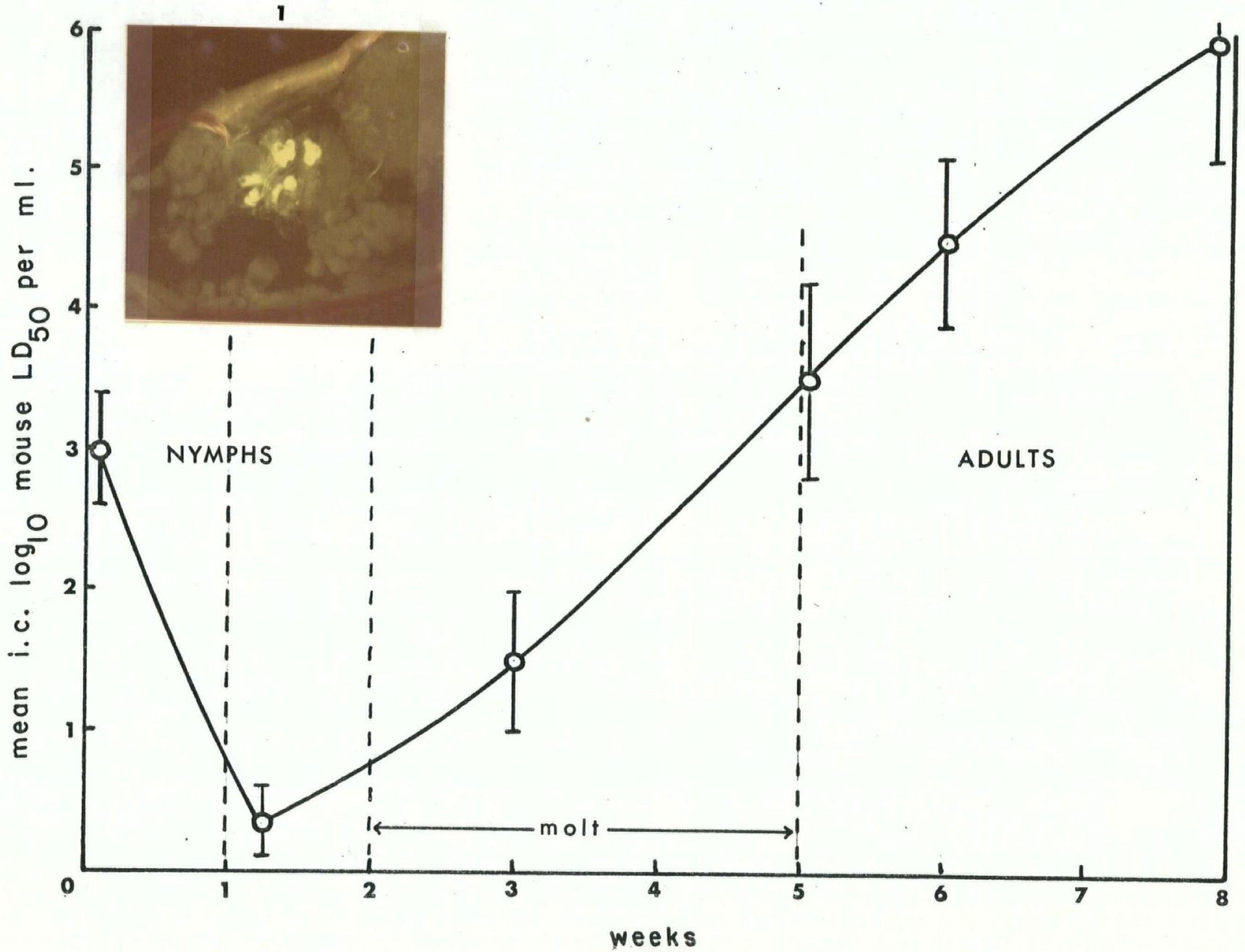


Fig. 21. Development of Powassan virus transstadially from nymphs to adults (1) nymph salivary glands (X100).

became minimal during this time and concomitantly virus titers dropped substantially (Figure 21; insert). Progressive replacement of nymphal by adult tissues occurred within one week after commencement of ecdysis until 2 weeks after emergence of adults, and this was accompanied by increasing size in the salivary glands. Virus replication occurred principally in the salivary glands, whereas gut tissue yielded only residual amounts of virus (average $10^{1.0}$ mouse LD_{50} per ml). Sections of adult gut rarely showed bright fluorescence when cut and stained during this molt.

Although Malpighian tubules and rectum became greatly distended during the molt and yielded average titers of $10^{2.5}$ mouse LD_{50} per ml of virus, fluorescent foci were not observed in tubule cells or lumen contents in cross sections. A great deal of guanine excretion took place upon emergence and for 1 week subsequently. None of the excretions contained infective virus.

When adults were placed at $4^{\circ}C$ one week after emergence, the average virus titer was $10^{5.6}$ mouse LD_{50} per ml. During the ensuing 8 weeks, individual ticks contained an average of $10^{6.0}$ mouse LD_{50} per ml of virus. Virus was not found in other organs during this time by titration and FITC staining procedures. When adults were removed from the $4^{\circ}C$ incubation chambers, salivary glands contained $10^{6.0}$ mouse LD_{50} per ml of virus and

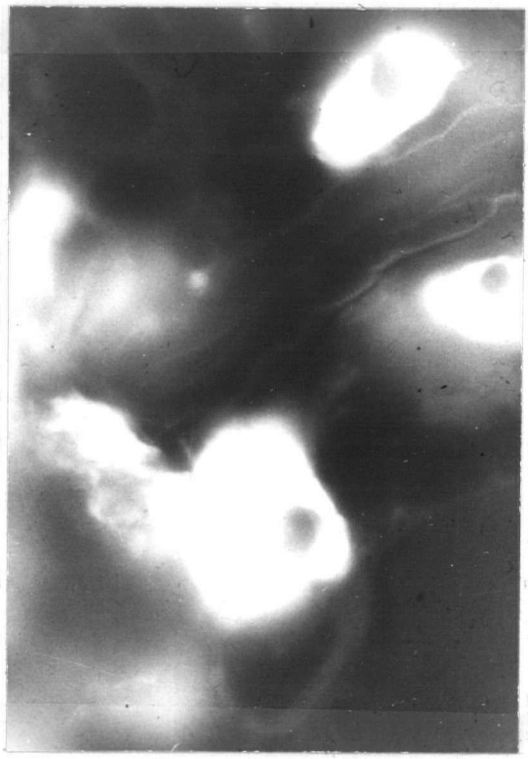


Fig. 22. Cross section of salivary gland alveoli dissected from a flat adult tick after 2 months at 4°C following infection as a larva. (Section stained with FITC conjugated Powassan virus antiserum) (X500).

stained cross sections were highly fluorescent (Figure 22). H and E sections yielded no cytopathology due to virus infection. It was significant that lowering of extrinsic temperatures from 27°C to 4°C for 8 weeks had no significant effect upon virus titers in salivary glands.

Development of Powassan Virus in Engorging Adults (Infected as Larvae)

Flat adults were transferred from 4°C to 27°C for 1 week before placing them upon an uninfected rabbit to engorge. Dissected individual ticks had an average of $10^{6.0}$ mouse LD_{50} per ml of Powassan virus in their salivary glands and the majority of cells of individual alveoli possessed cytoplasmic fluorescence when examined as cross sections. During engorgement, individual alveoli became very large, increasing in size from 30 μ to 80 μ, however virus titers did not increase. During the final 3 days of repletion and the ensuing 2 weeks a dramatic difference in organs supporting virus growth was evident in the separate sexes. During these 3 weeks (Figure 23) the virus content in male salivary glands remained at an average of $10^{6.0}$ mouse LD_{50} per ml and no other organs demonstrated virus infectivity. Four of the several hundred that were cut and stained showed small focal areas of specific staining in the

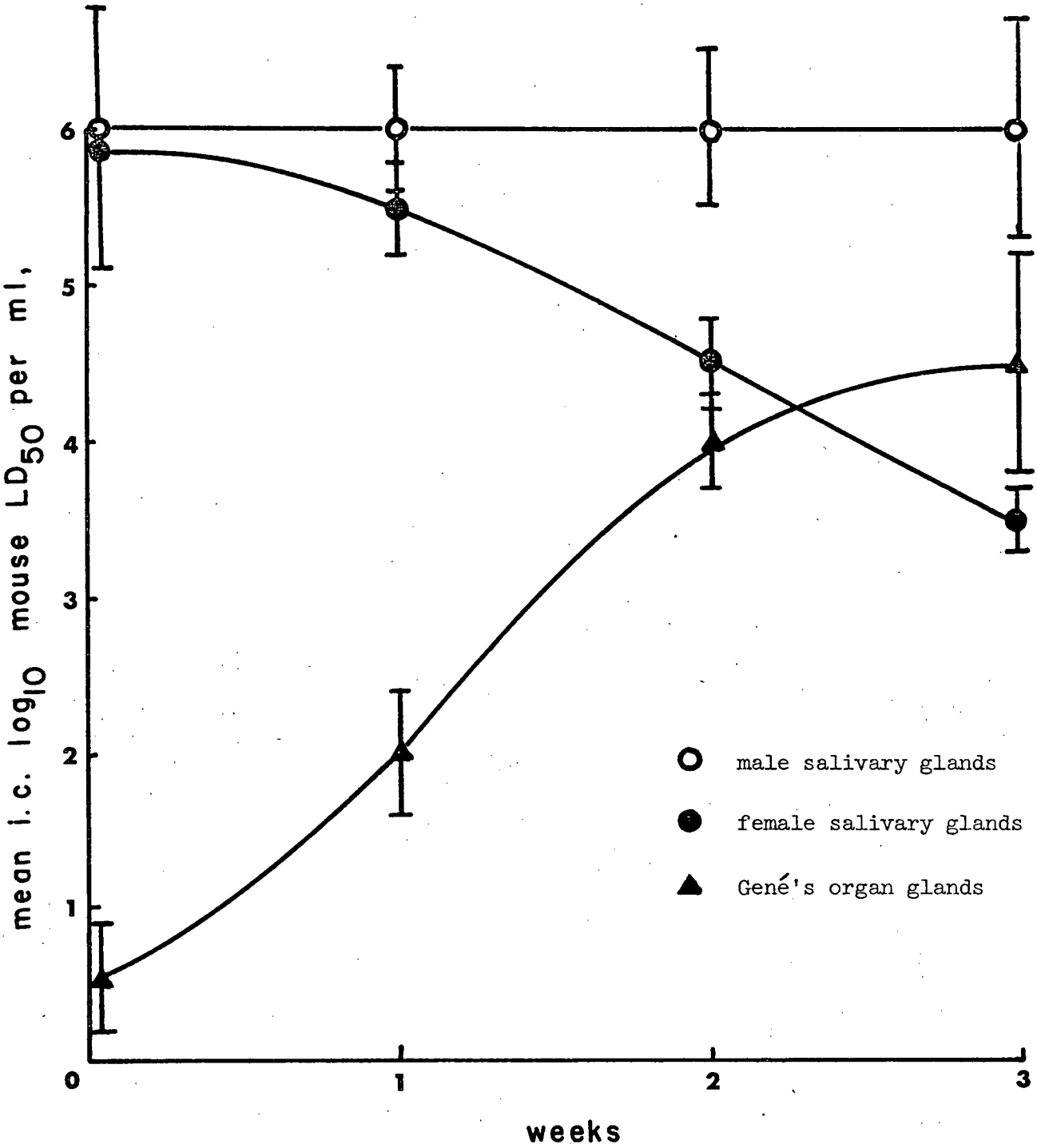


Fig. 23. Powassan virus titers in salivary glands and Gené's organ glands of adults after feeding. (Ticks acquired infection as larvae).

in the sex accessory gland. These areas of infection were always peripheral and were assumed to be incidental invasion of susceptible cells.

In females, salivary gland virus titers declined to $10^{3.5}$ mouse LD_{50} per ml during this time, however the glands that provide a wax secretion to Gené's organ became infected with virus as they increased in size. Both infectivity of $10^{4.5}$ mouse LD_{50} per ml and cytoplasmic fluorescence of Gené's gland cells were demonstrated during this period. The accessory sex gland of a few females demonstrated average maximal titers of $10^{3.0}$ mouse LD_{50} per ml 3 weeks after dropping from the host. Fluorescent foci were observed peripherally in accessory glands of females. It is probable that while salivary gland tissues degenerated and were removed, many virus particles gained access to the transporting system in the hemolymph and were conveyed to other organs such as Gené's organ glands and the accessory gland where they replicated. In contradistinction to these organs, ovary, testes, brain mass or Malpighian tubules showed no ability to support Powassan virus infection.

These findings differ from those reported by Pavlovsky and Soloviev with TBE virus in I. persulcatus adults (54). They detected high titers of $10^{6.0}$ mouse LD_{50} in the gut, salivary glands and ovaries, and smaller amounts of virus in Malpighian tubules, brain and Gené's organ 1, 10, 20 and 25 days after

infection. In the present studies with adults, infected either as adults or as larvae, virus was never detected in ovaries or brain by assay or fluorescent methods and only in Malpighian tubules by the assay technique.

Transovarial Transmission Studies

In a preliminary experiment 6 of 32 D. andersoni egg clusters contained Powassan virus (average $10^{3.5}$ mouse LD_{50} per ml). This infectivity was reduced tenfold when the eggs were washed with 0.15 M sodium chloride solution. Several egg clusters which demonstrated Powassan virus infectivity were allowed to hatch and the resulting larvae were tested at weekly intervals for 5 weeks. No virus was recovered from any sample tested.

An experiment was designed to determine the effect of Gené's organ on Powassan virus content of eggs during egg laying. Figure 24 shows the functioning of Gené's organ as it extrudes forward and downward to pick up the eggs individually from the vagina. Replete, mated adult females were gently embedded in plasticine ventral side up. They were allowed to lay eggs in the dark with their hypostome and chelicerae free and their Gené's organ free to extrude and coat each egg individually with it's waxy secretion. During this period of time, eggs were

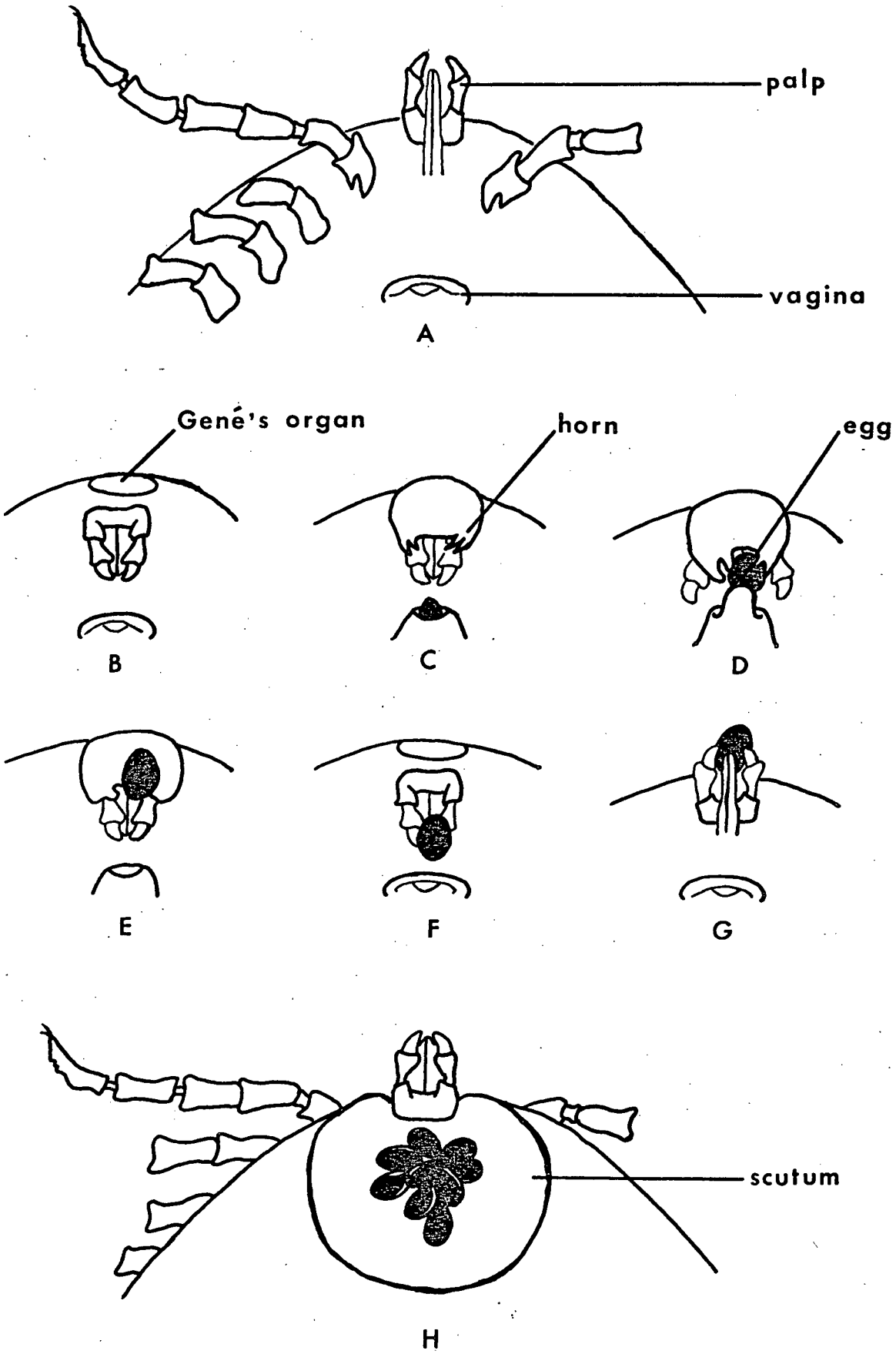


Fig. 24. Diagramatic representation of the functioning of Gené's organ in egg laying. Adapted from Till, 1961 (79).

collected, counted and tested for infectivity. Table VII summarizes this data.

When Gené's organ was uncovered for 72 hours ticks 4, 5, 6 and 7 laid 363, 402, 314 and 268 eggs respectively. All of the egg clusters contained virus. When larvae which emerged from these eggs were tested for virus, no infectivity was demonstrated.

The hypostome and chelicerae of each female was then held down with tape so that Gené's organ could not be employed to grasp the eggs from the vagina as they were laid. Eggs were collected from the ventral surface of the tick and tested for infectivity. Ticks 4 and 7 laid 100 and 91 eggs respectively during this 72 hour period but no infectivity was found. Ticks 5 and 6 laid 52 and 78 eggs respectively, and only a trace of infectivity ($10^{0.5}$ mouse LD_{50} per ml) was demonstrated. This virus may have been picked up from the secretion of an infective accessory gland. No virus was isolated from the few larvae which hatched from uncoated eggs.

After releasing Gené's organ, ticks laid reduced numbers of eggs (between 57 and 106 in 72 hours). However all of the clusters tested yielded virus (average $10^{2.5}$ mouse LD_{50} per ml). Larvae which hatched from these eggs did not yield virus. Gené's glands dissected from all 4 females contained virus.

Transovarial transfer of virus has been reported for RSSE virus in I. ricinus, I. persulcatus, D. nutalli, H. dromedarii,

Table VII: Effect of Gené's organ on Powassan virus content of eggs during egg laying by infected D. andersoni females

Tick Number	uncovered		blocked		uncovered	
	eggs	hatched	eggs	hatched	eggs	hatched
4	+(363)*	-	-(100)	-	+(57)	-
5	+(402)	-	±(52)	-	+(82)	-
6	+(314)	-	±(78)	-	+(106)	-
7	+(268)	-	-(91)	-	+(104)	-

\+ = Powassan virus present

\- = Powassan virus absent

* = values in parentheses denotes numbers of eggs laid

H. turkmeniense, and H. asiaticum (18). Benda (6) estimates that this phenomenon occurs in only 6% of I. ricinus ticks infected with TBE virus. However the following evidence points towards the lack of true transovarial transfer of Powassan virus in D. andersoni ticks: (i) the reduction of virus titers of egg clusters following washing; (ii) the removal of infectivity from egg clusters by blocking Gené's organ; (iii) the failure to detect virus in the larvae hatched from infective eggs; (iv) repeated isolations of virus and fluorescent foci of infectivity in dissected Gené's organ glands. These data agree with findings by Eklund (24) who has never obtained transovarial transfer with LI virus, RSSE virus or CTF virus under laboratory conditions. Furthermore, CTF virus has seldom been recovered from D. andersoni larvae collected in nature (24).

Transmission of Powassan Virus to Mammals by Feeding Ticks

Figure 25 summarizes the development of Powassan virus in whole D. andersoni throughout the tick's life cycle. Nymphal or adult ticks infected as larvae transmitted Powassan virus by biting hamsters or guineapigs after carrying virus through a molt. When hamsters were employed as indicators, whole blood samples collected on the 4th day contained Powassan virus, and tick salivary glands were shown to possess virus by infectivity or by

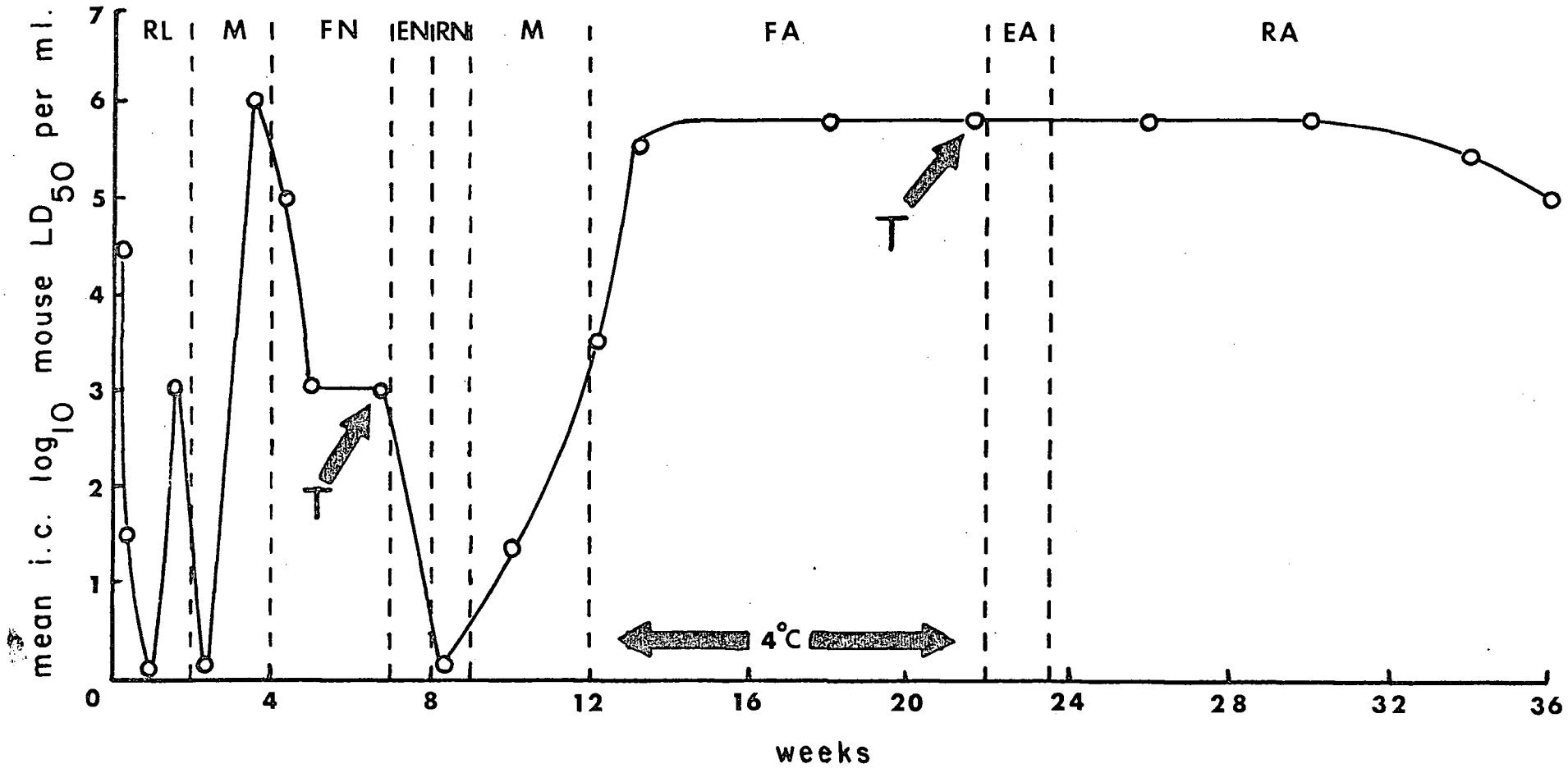


Fig. 25. Development of Powassan virus throughout the life cycle of D. andersoni ticks and the transmission of virus to healthy hosts. (RL - replete larvae; M - molt; FN - flat nymphs; EN engorging nymphs; RN - replete nymphs; FA - flat adults; EA - engorging adults; RA - replete adults). (Each measurement an average of 5 samples).

fluorescent techniques. Transmission of Powassan virus by tick bite parallels the transmission of TBE virus by I. ricinus ticks (5) and of CTF virus by D. andersoni adults (9) and nymphs (10).

Guineapigs were used because they do not normally produce detectable viremia following exposure to Powassan virus (unpublished data), but served as good transmission indicators by the elevation of HI antibodies in their serum 17 days after infected D. andersoni nymphs or adults engorged upon them. Titers of virus in flat adult ticks reached an average of $10^{5.5}$ mouse LD_{50} per ml after feeding as nymphs and passing through a molt. This finding contrasts with Benda's hypothesis (6) that successive stages of I. ricinus require a blood meal on hosts which are capable of producing a substantial viremia for TBE virus in order to insure reinfection and high virus titers in the tick.

General Discussion

The fact that Powassan virus invades susceptible tissues of D. andersoni and multiplies there, fulfils a biological criterion of an arbovirus. Increments of virus contents in larval, nymphal and adult stages of D. andersoni are noted only following an extrinsic incubation period. The virus is transmitted trans-stadially from larvae to nymphs, and from nymphs to adults (Figure 25). Furthermore, transmission of Powassan virus to hamsters and guineapigs by bites of nymphal and adult D. andersoni substantiates the capacity of this tick species to be an efficient virus vector. This abundant evidence of biological transmission contrasts sharply with mechanical transmission of myxoma virus (a Poxvirus) to rabbits by the bite of Anopheline mosquitoes (25), or transmission of the plant virus tobacco mosaic virus (TMV) by the contaminated mouthparts of the grasshopper (Melanoplus differentialis (84).

True transovarial transmission of Powassan virus has not occurred in D. andersoni. This provides a natural break in the pattern of virus replication throughout the life cycle of this tick species. This is substantiated by the following evidence: (i) ovaries do not contain virus when ticks are infected as larvae, nymphs or adults; (ii) any shedding of virus during egg laying is confined to the surface of the eggs, and is not conveyed to the ovum inside. Thus, infectivity of whole eggs is reduced by washing or by warm external temperatures; (iii) eggs that are

laid by the mediation of an infected Gené's organ gland and are covered with infective secretion yield viable virus, however, when Gené's gland is not allowed to function, the eggs laid are non-infective; (iv) dissected Gené's organ glands have shown both virus infectivity and foci of specific immunofluorescence; (v) larvae hatched from infective eggs never contained virus.

The kinetics of growth for this tick-borne arbovirus in gut cells are different from the growth of mosquito-borne viruses in several mosquito species. Whereas the average time of Powassan virus infectivity-eclipse and return to titers originally ingested is approximately 9 to 12 days in D. andersoni ticks, mosquito-borne viruses such as MVE virus (40), Japanese B encephalitis (JBE) virus (35) and CE virus (15) require only 4 to 6 days to attain high titers in Culex annulirostris (Skuse), Culex pipiens (L), and Aedes vexans (Meigen) respectively.

Only gut cells of larvae become infected after taking a virus meal, because salivary glands degenerate shortly after engorgement and the larvae begin molting before sufficiently high virus titers develop in gut cells to become transferred throughout the tick via the hemolymph to other organs. During the molt, newly developing nymphal tissue including salivary glands become invaded with virus. Similarly during the molt from nymph to adult, newly forming salivary glands become highly infective with virus. It is significant that Gené's organ gland and accessory sex glands

which are in the adult female, also become infected during rapid proliferation of their parenchymal cells.

The method of placing ticks on a rabbit and allowing engorgement to commence before injecting Powassan virus intravenously to create an instant viremia, should provide a convenient and simple method for future studies on the development of arthropod-borne viruses in ticks. It is possible that this method may be extended to viruses transmitted by mosquitoes or sandflies. The titer of viremia can be conveniently controlled by adjusting the titer of inoculum and calculating the dilution factor from a knowledge of the blood volume of the animal used.

An infection threshold of $10^{2.5}$ mouse LD_{50} per ml of Powassan virus for D. andersoni adult ticks implies that animals which circulate virus at levels below $10^{2.5}$ mouse LD_{50} per ml would not serve as natural reservoirs of infection for this tick species. It is possible, however, that such animals may provide a natural source of infection for any other tick species that possess a lower infection threshold for Powassan virus.

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