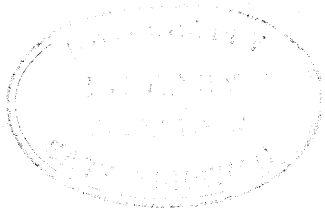
TRANSMISSION OF WESTERN EQUINE ENCEPHALITIS VIRUS BY SASKATCHEWAN MOSQUITOES AND BEHAVIOR OF THE VIRUS IN SELECTED LABORATORY VERTEBRATES IN RELATION TO EPIDEMIOLOGICAL STUDIES

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of
Doctor of Philosophy
in the Department of Veterinary Microbiology
University of Saskatchewan

by
Launcelott Barrington Hayles
Saskatoon, Saskatchewan
April, 1971



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THESIS ABSTRACT

For laboratory experiments on transmission of Western equine encephalitis (WEE) virus by Saskatchewan mosquitoes, a local virus isolate of human origin, strain 1540-1544 Regina 66, was selected. The half-day old chick which was used as the principal vertebrate host in these experiments, showed a consistent response to subcutaneous inoculation with this strain of WEE virus. After infection with moderate doses, viremia was maximal at 18 to 24 hours, when virus concentration was within the range of 10^8 to 10^9 intracerebral three-week old mouse LD_{50} (ic mouse LD_{50}) per 0.03 ml of blood. Most infected chicks showed inactivity followed by prostration before death; very few displayed overt clinical signs of neurologic disturbance. In comparative titrations, chicks were more sensitive than weanling mice for detection and titration of virus.

An indirect fluorescent antibody test applied directly to brain sections from WEE infected mice, was satisfactory for diagnostic purposes. Sections were fixed in Carnoy's fluid and transferred through chloroform to paraffin. The use of Evans blue, of absorbed antisera to WEE virus, and the inclusion of negative and positive control preparations, ensured specificity of staining

reactions in the tests. The technique could not be applied to brain sections from chicks infected subcutaneously, despite the presence of large amounts of virus in this tissue. Confirmation of WEE infection in chicks which were bitten by infected mosquitoes was achieved by passage of virus to mice, from which brain sections served for diagnosis by immunofluorescence. These initial investigations formed the basis for the design and interpretation of detailed quantitative transmission experiments with mosquitoes.

Nine species of local mosquitoes were examined for their ability to transmit WEE virus by bite. Culex tarsalis was a very efficient transmitter at 69 and 75°F. This mosquito transmitted from four days after infection and remained infective for up to 44 days, which was the longest period of observation. Throughout this period, infection rates remained at 100% or close to this level at both temperatures, but at 69°F, transmission rates declined after about two weeks. At the higher temperature, almost all infected mosquitoes transmitted after the first week of incubation, and this trend continued throughout. The minimum concentration of virus required to infect 50% of C. tarsalis was $10^{2.5}$ ic mouse LD_{50} per 0.03 ml of donor blood, and for the same level of infection in Culiseta

inornata, a concentration of $10^{4.0}$ was necessary. The latter did not transmit WEE virus by bite. However, infection rates were maintained between 63 and 88% from the second to the sixth week of incubation, and the quantities of virus in individual mosquitoes were comparable to those in C. tarsalis.

Anopheles earlei and Aedes vexans failed to support viral growth, while Aedes campestris, A. flavescens and A. spencerii maintained infection for close to two weeks at 69°F. Infected A. dorsalis did not survive beyond the first week of incubation, at which time, individual mosquitoes had minimal amounts of virus. Apart from C. tarsalis, A. fitchii was the only other mosquito which transmitted the infection, and it did so after an extrinsic incubation period of nine days.

All the mosquitoes, except for C. tarsalis, were reluctant to feed on the chick. In an attempt to provide an alternative laboratory host for future transmission studies, a short investigation was undertaken to determine the susceptibility of the Mongolian gerbil (Meriones unguiculatus) to peripheral infection with WEE virus. Four-week old animals responded by exhibiting uniform viremia patterns to a standard dose of virus, and small doses regularly caused death. Resistance to infection

increased with age but ten-week old gerbils were as susceptible as three-week old mice. Brains from gerbils which died within ten days after infection, contained large amounts of virus.

ACKNOWLEDGEMENTS

I am grateful to my supervisors, Dr. J.R. Saunders and Dr. J. McLintock for the guidance they have given me throughout the study. I thank the other members of my committee, Drs. C.H. Bigland, A.N. Burton, J.R. Allen, J.H.L. Mills, G. Dempster and D.L.T. Smith, and also Dr. J.G. Rempel, for advice and constant encouragement.

Miss M. Kroeger and Mr. H. Weegar assisted with servicing mosquito traps and mosquito colonies, and Mrs. E. Angielski patiently typed the thesis. To each, I extend my sincere appreciation.

Funds for this project were provided by Canada Department of Agriculture E.M.R. Grant No. 6904. A Medical Research Council Fellowship provided additional financial support during the final year of the study.

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CHAPTER 1

REVIEW OF THE LITERATURE

THE GROUP A ARBOVIRUSES: A REVIEW OF VECTOR-VIRUS RELATIONSHIPS WITH PARTICULAR REFERENCE TO WESTERN EQUINE ENCEPHALITIS IN CANADA

INTRODUCTION

The World Health Organization Study Group on Arthropod-Borne Viruses set out certain criteria for the recognition of an arbovirus vector (10). These criteria may be summarized as follows.

1. Recovery of virus from wild-caught specimens of the arthropod, free from visible blood.
2. Demonstration of ability to become infected by feeding on an infected vertebrate host or artificial substitute.
3. Demonstration of ability to transmit the virus by bite, following a period of viral multiplication within the body of the suspect vector.

Vector status is considered confirmed, if, in addition to the above, there is evidence of a significant association of the infected arthropods with the appropriate vertebrate population in which the infection is occurring.

The main purpose of this review is to examine the extent to which the criteria listed above, have been fulfilled in the case of the group A arboviruses. In keeping with this objective the review stresses mosquito-virus relationships. Problems and new techniques associated with virus isolation from mosquitoes and with identification of the viral isolates are briefly mentioned.

Aspects of the performance, interpretation and limitations of virus transmission studies with mosquitoes, and new findings in the area of mosquito infection are presented in some detail. A fairly extensive discussion follows, in which these matters are related to field observations on the group A arboviruses in their mosquito hosts.

An attempt is made to present in tabular summary the pertinent data concerning isolations of these viruses from mosquitoes, throughout the many areas in which the viruses occur. In this summary (Table 2, page 48) not all viruses are treated to the same extent. Emphasis is placed on those which are either known or suspected to cause disease in man or domestic animals, and for which in addition, there is incomplete knowledge of their epidemiology in a particular area. For example, the epidemiology of Western equine encephalitis (WEE) and Eastern equine encephalitis (EEE) in the United States has been extensively investigated, and between 1950 and 1960 there were several excellent reviews on aspects of these diseases (35,46,66,67,103,141,150). The paper written by Ferguson (67) presented the detailed entomological aspects up to 1952. Throughout the text and in the tabular summary presented here, findings before that time are very much condensed for WEE and EEE in the

United States, and newer developments are emphasized. On this basis WEE in Canada receives particular attention. In addition, this emphasis provides a base for evaluating the results of transmission studies reported here.

With regard to virus isolations listed in table 2, no attempt is made to assess their validity from original or from subsequent reports. In some instances, the original report was unavailable so that omissions, which are certain to occur in the table, are due largely to this fact. A more critical appraisal has been made in the case of original reports of transmission studies. Where conclusions are indefinite, this is indicated in the table and further elaborated in the text.

Finally, the review examines briefly, the factors which must be considered in assessing the relative values of laboratory and field observations. Such epidemiological correlations play an essential part in meeting the general goal of the workers in this field, which is to clarify the manner in which mosquito-borne viruses are maintained and transmitted in nature.

VIRUS ISOLATION FROM MOSQUITOES AND IDENTIFICATION OF ISOLATES

Isolation Methods

The infant Swiss mouse was introduced for the isolation of arboviruses by Hammon and coworkers in 1942 (87) and it is now the most widely used laboratory host for this purpose. The fact that more than 70% of the original arbovirus isolates were made in mice (212) indicates their range of susceptibility and their value to arbovirology. In practice, the isolation of virus from wild-caught mosquitoes involves the intracerebral or intraperitoneal inoculation of 0.02 ml of a mosquito suspension into each member of a litter of one to three-day old mice. These are observed for up to 14 days for signs of arbovirus infection which include tremor, ataxia, prostration, paralysis, alopecia (219,221) and death. For the preparation of mosquito suspensions, up to 100 individuals are pooled according to species, area of collection and anticipated infection rates, then ground in suitable diluent. Due to the lability of several arboviruses certain precautions are observed during mosquito processing. Dead mosquitoes are kept chilled during the identification process and suspensions are prepared in small amounts of cold diluent, pH 7.4-7.6, containing protein stabilizers,

usually 0.75% bovine albumin fraction V or 25% normal serum, and antibiotics. Mosquitoes or their suspensions are stored in sealed vials at -70°C. Tissues from sick or dead mice are removed for virus identification, but on occasion, one or more blind passages may be necessary to obtain a sufficiently high virus titer for performance of serologic tests. Reisolation from the original suspension is frequently attempted to ensure absence of cross-contamination of samples.

Although a number of vertebrate cell culture systems are susceptible to a range of arboviruses (12,19,97,157, 169,176,201), none exhibit the broad susceptibility of suckling mice. Comparative studies have indicated that of the commonly used systems, baby hamster kidney cells and the PS-Y15 line of porcine kidney have the widest range of sensitivity (19,119,129,167,187,202). They are probably less sensitive than most to the non-specific toxic effects of mosquito tissues (129,223). Nevertheless, where several viruses occur within a particular area, cell culture systems are not normally used as the sole means for initial isolation. The recent development of continuous lines of invertebrate cell cultures (26,79,80) and their adaptation to growth in hemolymph-free media (144,195,196), have stimulated investigations on their possible value for arbovirus isolation from field materials.

It has been shown that mosquito cells, particularly the established lines of Aedes aegypti and Aedes albopictus, readily support the growth of many mosquito-borne viruses, and support poorly most of those transmitted by other arthropods (27,69,111,174,175). In addition, the line from A. albopictus shows cytopathogenic effect when infected with several group B viruses (161,194,207) and plaques under agar can be produced in these cells with Japanese encephalitis (JE) virus (161). Further investigation will undoubtedly determine whether dependence may be placed on these systems for primary isolation and preliminary characterization of mosquito-borne viruses (194). For general information, the results of recent investigations on group A arboviruses in arthropod cell cultures are presented in table 1, page 8.

Identification of Isolates

Hemagglutination (HA), complement fixation (CF) and neutralization tests are routinely used for presumptive and confirmatory identification of the group A arboviruses. It is pertinent to mention here, some of the problems associated with their performance and interpretation, and the newer developments aimed at eliminating some of the problems.

The sucrose-acetone method for extracting HA and

TABLE 1

GROUP A ARBOVIRUSES IN PRIMARY AND ESTABLISHED ARTHROPOD CELL CULTURES

Virus	Results	References
BEBARU	No growth in established line from Aedes aegypti.	175
CHIKUNGUNYA	Growth in established lines from A. aegypti and A. albopictus.	27
	Growth and attenuation for infant mice in established line from A. albopictus. Loss of mouse virulence by 35th passage.	11
EASTERN EQUINE ENCEPHALITIS	Growth in primary cultures from Hyalomma dromedarii.	174
	Growth in primary cultures from A. aegypti embryos; maximum titer 4-7 days, persistent infection up to 60 days.	163,164
	Growth in established lines from A. aegypti and A. albopictus.	27
	Growth in primary larval cultures of A. aegypti.	111

Table 1 (Continued)

SEMLIKI FOREST	No growth in established line from *A. aegypti*.	175
	Growth in primary cultures from *Hyalomma dromedarii*.	174
	Growth in primary cultures from *A. aegypti* embryos. Maximum titer 2-4 days; prolonged infection up to 60 days.	163,164
	Growth in established lines from *A. aegypti* and *A. albopictus*.	27
	Inapparent persistent infection in subcultured *A. aegypti* cells derived from embryos; 0.01 to 8% of cells support infection from 2 days after inoculation. Cells susceptible to infectious RNA.	165,166
SINDBIS	No growth in established line from *A. aegypti*.	175
	Growth in primary cultures of *Hyalomma dromedarii*.	174
VENEZUELAN EQUINE ENCEPHALITIS	Growth in established lines from *A. aegypti* and *A. albopictus*.	
	Growth in primary larval cells from *A. aegypti* and *A. triseriatus*. No change in mouse virulence or plaque size for up to 10th passage.	111

Table 1 (Continued)

WESTERN EQUINE ENCEPHALITIS	Growth in primary cultures of Hyalomma dromedarii.	174

CF antigens from suckling mouse brain suspensions of arboviruses is well established, and though normally satisfactory, it is laborious. Palmer et al. (154) demonstrated that the method can be applied to EEE and WEE infected chick embryos to obtain a considerably greater quantity of antigen compared with that obtained from mouse brain. They believed that this high yield compensated for slightly lower titers compared with those obtained in mouse brain. Other recent methods employ gamma irradiation of chick embryo fluids (168), borate saline-beta propiolactone extraction of mouse brain (24) and fluorocarbon extraction of mouse brain or tissue culture fluids (162,170). The use of acetone or kaolin for removal of non-specific HA inhibitors continues, but recent studies have indicated that dextran sulphate or rivanol may be satisfactorily employed (65,82,145).

Antigenic variations between prototype virus strains and new isolates, and cross reactivity between viruses within a subgroup are among the main problems associated with serological identification of group A arboviral isolates. The use of highly specific antisera and very sensitive techniques, eg plaque neutralization tests, are perhaps necessary as final diagnostic procedures if some of these problems are to be avoided. However, the influence of species of laboratory host, the

immunization schedule, time of bleeding and the nature of the antigen used, are probably more critical than for most systems in the preparation of arbovirus immune sera (98,184). With regard to the nature of the antigen, the influence of the host and passage level on the antigenic characteristics of VEE and WEE viruses are well known (98,100,105,118). Karabatsos et al. (118) and others (99), provided evidence that in epidemiological investigations, the incidence of positives in serological tests depended to a great extent on the particular virus strain used in each. This was true even in neutralization tests employing relatively recent isolates. Clearly, there is a need for standardization of antigens, reagents and methods, for, at this time, it does not appear that any criteria exist for designating a new isolate (Group A) as a strain variant or as a new serotype. This is not to imply that there is a lack of awareness of these problems. Efforts are constantly being made to examine immunological relationships, particularly between new isolates, to avoid the proliferation of "distinct" arboviruses which differ only slightly in immunological relationship (25,112,216). It should be mentioned however, that the complexity of factors involved in arbovirus maintenance not only gives rise to antigenic variations between geographic areas,

but within a particular area from time to time (98,112). This further complicates any effort at standardization. Except in relation to alteration in virulence and the selection of candidate strains for possible attenuated vaccine production (15,212), it seems that little thought has been given to antigenic variation which may occur as a result of prolonged mosquito infection. Since various vertebrate hosts appear to be selective in propagating different antigenic sub-populations of a particular virus eg WEE (184), Chikungunya (218), Middelburg (123), it is conceivable that a similar process could occur in different mosquito hosts.

The practical aspects of group A arbovirus antigenic variations in time and space, and the close immunological relationships within subgroups are numerous. They are associated with such matters as vaccine production, protective value of neutralizing antibody, serological surveys and others, but the aspect which concerns us here is the relationship to definitive identification of group A arboviral isolates. A number of examples can be cited. Shope and coworkers in 1964 (191) emphasized the close relationship between the viruses of the Venezuelan equine encephalitis (VEE) complex: Pixuna, Mucambo and VEE. Other workers (186) demonstrated that after infection

with one of these agents, neutralizing antibodies were usually elaborated against all three, so that in surveys of virus activity in nature, great caution must be exercised in areas where these viruses, as well as Mayaro (186) and Una (34) are encountered (See table 2, p. 48). Other group A viruses which exhibit close immunological and geographic relationships and are known to cause problems of differentiation in large scale surveys, are the Ross River-Bebaru-Getah subgroup (61), Chikungunya and O'nyong-nyong (95) and to a lesser extent, Sindbis and Whataroa (132).

Particularly for those viruses which cause disease in man and domestic animals, it is desirable that rapid and reliable procedures exist for definitive identification. However, the preparation and employment of batteries of specific antisera, standardized antigens, and the performance of plaque reduction tests are likely to be beyond the scope of most laboratories involved in the processing of thousands of mosquito pools and the frequent passage of tissues. The latter procedures are routine in arbovirus surveys of wild mosquitoes and they consume a considerable amount of time and labour. Nevertheless, the more widespread use of miniaturized serological and cell culture

methods (22,94,130,202,209) may allow for more thorough routine processing of isolates. This is likely to result in the application of other highly sensitive methods, eg immunofluorescence (57,92) for diagnostic purposes, and it may also act as the major stimulus towards standardization of methods, even if local viral isolates must continue to be used for comparative purposes.

LABORATORY TRANSMISSION STUDIES

General Considerations

The vectorial ability of a mosquito species is not indicated by the mere isolation of virus from wild caught individuals. Nevertheless, such mosquitoes become suspected vectors (10) and the degree of suspicion will depend on the circumstances of virus isolation from them. Vectorial ability can only be confirmed by transmission experiments in which infected mosquitoes are allowed to feed on susceptible vertebrate hosts under controlled conditions, and before this is done, the indescriminate use of the term "vector" should be avoided. Although the prime objective of transmission experiments is to determine whether or not a suspected vector can transmit virus, evidence of transmission based on the mass feeding of mosquitoes on a single host is of limited value. Use of this method is particularly justified in instances where suspicion is minimal, but, should it yield positive results, this should be regarded merely as the first step towards further characterization of the vector. This involves precise laboratory control and quantitative determinations of vector infection levels and transmission efficiency (142).

Determination of Transmission Efficiency

Wild-caught or laboratory reared mosquitoes are held in an insectary provided with adequate controls for regulation of lighting, temperature, and humidity. Mosquitoes are infected by allowing them to feed on a host circulating virus at various levels, to cover where possible, the range of viremia exhibited by natural hosts of the virus. Great care is required in handling infected mosquitoes. The investigator must ensure his own safety and that of other individuals by adherence to proper laboratory techniques and provision of adequately screened doors and windows on insectaries. He must avoid any unnecessary procedure which may cause accidental escape of infected mosquitoes, for such accidents have been known to occur (155,203). Drastic interference with the mosquitoes' physiological processes is avoided, so that facilities for egg laying and sugar solutions for maintenance, are provided. Gentle handling of mosquitoes avoids excessive mechanical damage and helps to prolong survival. During the experimental period, observations are made at different temperatures within the range in which mosquitoes are active in the local area, according to the following protocol.

1. Virus levels in whole mosquitoes and in selected tissues.
2. The proportion of mosquitoes which maintain infection; ie infection rates.
3. The time taken from mosquito infection to first transmission; ie extrinsic incubation period, and its association with virus levels in whole mosquitoes, salivary glands or other tissues.
4. The proportion of infected mosquitoes which transmit the infection; ie transmission rates.
5. The minimum quantity of virus required to infect a given percentage of mosquitoes under standard laboratory conditions; ie infection threshold.

Additional observations that may be made during transmission studies include;

1. A measure of the amount of virus inoculated by transmitting mosquitoes.
2. Pathological effect of virus on the mosquito host.
3. Association between infection or transmission and physiological processes eg feeding, gonadotrophic cycle and oviposition.
4. Transovarial transmission of virus.

Interpretation of Transmission Experiments and Difficulties Encountered

Experiments of the type outlined above require reasonable laboratory facilities. They are expensive and often laborious to perform, largely because individual mosquitoes must be used for several measurements. While positive findings provide a measure of comparative transmission efficiency between mosquito species, they must be interpreted finally, in relation to conditions existing in nature. The abundance of each species and relative infection levels, natural host range, frequency of blood feeding and chance of obtaining an infected blood meal, and probability of survival in nature are only some of the factors which bear on the assessment of laboratory findings. Negative findings too, must be interpreted with caution, for differences in virus strains, mosquito populations (16) eg between wild and laboratory generations may account for such results. This is particularly important if one finds that a given species which is known to transmit in one region of its area of distribution does not appear to transmit in another. For strict appraisal, all findings must be related to mosquito source, laboratory conditions and virus strain.

The inability of mosquitoes to survive for long

periods and their refusal to feed at least twice on a live host, are problems often encountered in transmission studies, even from the earliest recorded attempts (101). Artificial infection on blood-virus-sugar mixtures from cotton pledgets, through skin or artificial membranes provide a reasonable alternative (53,56,153,159). The use of sugar-virus mixtures only, may give misleading results since such meals are deposited in the diverticula which may differ in susceptibility from the midgut to which blood is normally diverted. Similarly, the parenteral inoculation of virus, in bypassing the gut, gives no indication of the ability of the mosquito to support infection under natural conditions, and positive transmission findings are not meaningful from an epidemiological point of view. The method is more suitable for demonstrating that a particular virus can or cannot multiply in mosquito tissue, but, with the advent of mosquito cell cultures it is now hardly necessary. Difficulties of mosquito feeding and survival justify to some extent the use of mass feeding techniques for demonstrating transmission. In this connection, the availability of mosquito colonies and the methods used for establishment and maintenance should be carefully reviewed before too much effort is spent on empirical methods (17,49,77,117, 125).

The vertebrate host selected for transmission studies must be one whose response to the virus has been adequately studied, and preferably one which dies as a result of infection. Newly hatched chicks have been extensively used for WEE and EEE on the basis of intensive study but their reaction to different strains of virus may vary (39). The same is true for the other hosts normally employed eg suckling and weanling mice, guinea pigs, hamsters, rabbits, monkeys, so that preliminary investigation of the host is usually required before transmission studies begin. That infection occurs following the bite of an infected mosquito is best confirmed by virus isolation and identification at least at intervals during a transmission series. At other times, development of characteristic symptoms or death with a given period normally suffices.

Studies of the type outlined have yielded considerable information on mosquito infection and transmission and this is reviewed in the next section.

VECTOR INFECTION AND MECHANISMS OF TRANSMISSION

The Infection Cycle

The natural infection of mosquitoes by ingestion of infected vertebrate blood involves several processes which must succeed before transmission of virus can occur. Since blood is drawn into the lumen of the gut, gut cells must be infected before virus can be distributed through hemolymph to other tissues, including salivary glands, where secondary multiplication occurs.

Gut Infection

In Culex tarsalis infected with WEE virus, multiplication first occurs in the gut where virus content remains at comparatively high levels throughout mosquito infection (214). A similar finding was reported for JE in C. pipiens (153) and in C. tritaeniorhynchus summorosus and C. pipiens pallens (154,155). These observations were made in separate studies using titration techniques on the one hand (128,214) and immunofluorescence methods on the other (62,63). They are limited in breadth but they suggest that infection of natural mosquito vectors is similar with both group A and group B viruses. On this basis, it is reasonable to pool, as far as the gut is concerned, the cytological observations provided by immunofluorescence, and the quantitative

findings of titration techniques in order to obtain a composite picture.

Virus invades the epithelial cells of the midgut shortly after ingestion of infected blood (128,214) but mature virus may not be detectable until after a lag period extending up to seven days (62,63). As far as can be ascertained, no electron microscopic studies have been made on gut cells of mosquitoes infected naturally with arboviruses. However, such studies following a normal blood meal (21) or following parenteral infection (20), may be considered in relation to the types of investigations already mentioned. They provide further evidence of the general similarity of mosquito infection by several arbovirus types and allow a more detailed examination. In starved, sugar-fed or water-fed Aedes aegypti and A. togoi females, Bertram and Bird (21) observed the endoplasmic reticulum of the midgut cells to be in the form of distinctive whorls restricted to the perinuclear zone. Following a blood meal and during its digestion the whorls unfold to ramify throughout the cytoplasm for a period of about 48 hours. Akov (4) has shown that protease activity in the gut of A. aegypti is reduced to barefly detectable levels 48 hours following

a blood meal and this has been confirmed by Gooding (78). Based partly on such comparisons, Bertram and Bird associate the morphological change in the endoplasmic reticulum with the digestive process; primarily with proteolytic secretion into the gut lumen. The suggestion can be made here that viral infection of gut cells occurs in the midst of intense metabolic activity related to blood digestion. Due to the early infection of the gut, this appears true at least for WEE in C. tarsalis (214). However, the data are limited and no sweeping generalizations can be made. Moreover, unlike group A viruses, several group B viruses, including JE, are inactivated by proteolytic enzymes (50) and one would expect a significant difference between the groups in initial infection of the gut. The inability of Doi et al. (63) to demonstrate JE in mosquito gut cells up to four days after an infective blood meal suggests that only a fraction of the total virus ingested succeeds in infecting the gut. Perhaps it is that portion which escapes inactivation due to early infection or protection by blood proteins. This argument tends to be reinforced by Boorman's claim that only about 0.1% of Uganda S (Gp B) virus passes the gut of A. aegypti and that successful infection of the mosquito is limited to a period of ten minutes to three hours after feeding

(23). My argument is not supported by LaMotte's recovery of JE in individual guts within hours after feeding (128) nor by Boorman's ability to recover Uganda S in high titer from whole mosquitoes at the same time that gut infection remained minimal. The suggestion by the latter investigator that the peritrophic membrane presents a physical barrier to gut infection may more reasonably account for his findings. Experiments of the type in which mosquitoes are infected by parenteral inoculation of virus and multiplication observed by titration of whole individuals (45) do not help to clarify the situation. Clarification must await further studies combining cytological, virological and perhaps biochemical observations.

There appears to be considerable structural, though not necessarily functional uniformity between the epithelial cells of the midgut of the female mosquito (21), and the suggestion that viral multiplication is not restricted to any particular area, though possibly concentrated in the posterior portion (63,128) is in accord with such findings. Midgut cells are not known to exhibit any type of pathological degeneration as a result of virus infection and multiplication (128) and it can be reasoned from studies of vesicular stomatitis virus in A. aegypti mosquitoes, which were infected parenterally (20), that

reinfection of these cells occurs after systemic infection is established. Immunofluorescence studies also suggest its occurrence (63). Vesicular stomatitis (20) and Sindbis (149) viruses have been shown to be excreted in the anal discharge of A. aegypti during the course of infection. This is thought to occur via the gut or malpighian tubules as part of the normal excretory process. There is, therefore, little reason to doubt that this could be of widespread occurrence.

The susceptibility of the mosquito midgut to virus infection is one of the main factors in determining its importance as an arbovirus vector. Infection by a particular virus may not be established with equal facility in two or more mosquito species under the same conditions and this has been attributed to the presence of a "gut barrier" to infection. It is probably related to the physiological and anatomical factors discussed above, but, in its applied aspect where it is measured in terms of "infection threshold", it is more significant in relation to "species susceptibility". Accordingly, it is discussed in relation to this topic in a following section.

Distribution of Virus in Hemolymph, Salivary Glands and Other Tissues

Although only small amounts have been recovered

from the hemolymph (23,128) of mosquitoes, virus is generally assumed to spread by this means from the gut to other tissues and body regions. Thomas (214) reported that WEE virus in C. tarsalis was distributed during the first week of infection to all body regions with the largest amounts remaining in the abdomen and the least occurring in the wings. From his examinations of specific tissues, he found that the highest concentration per unit weight of tissue was in the salivary glands although the actual quantity varied considerably between individuals over a six-week period. The above observations were later confirmed in LaMotte's study on JE (214), and amplified by Doi and coworkers (62,63). The large amount of virus in the abdomen appears to result from massive invasion of the gut and of fat body cells and the latter may play a significant role in virus distribution, for in them, infection seems to be rapidly established even in those between thoracic muscles (62,63). Following infection of hemolymph and fat cells, salivary gland infection is the next stage closely followed by other organs such as ovaries, malpighian tubules, nervous tissue and eyes. Although virus multiplication has been amply demonstrated in mosquitoes by the recovery of several thousand times more virus than was originally ingested

(44), doubt has been expressed as to whether virus grows preferentially in any tissues or organs. It seems clear from the above observations that preferential growth takes place in gut, fat, and salivary glands, but that other tissues and organs also contribute to the total content.

On the basis of their study of Chikungunya virus in A. aegypti, Janzen et al. (110) presented a detailed hypothesis on the replication of this virus in salivary glands. They believe that precursor particles form in the nucleus and are released, probably by rupture of the nuclear membrane, into the cytoplasm where they attach to membrane-bound vesicles. The particles then bud through the vesicles thus acquiring an envelope and are released from the cell by "reverse phagocytosis". There was a suggestion that different areas of the gland varied in their potential to produce virus but no definite association with metabolic activity in these areas was established. The proximal portion of the lateral lobes appeared to be most productive despite an apparent inability of mature virions to negotiate the ducts and channels in the area. Based on this observation, Janzen et al. suggested that transmitted virus originates from lateral and median lobes where the structure of the

ducts and channels offered no physical barrier to the passage of virus. They reported that no pathological changes were observed within infected cells. It is not certain to what extent other group A viruses follow this pattern of replication in the salivary glands of their vectors. Apart from a lack of association with the nucleus, several, including WEE, EEE, VEE and Semliki Forest (146,150,200) are known to replicate by forming precursor particles. In addition, they acquire envelopes by budding into cytoplasmic membrane-bound vesicles, so that a fairly general pattern may emerge with further study. Unlike the situation with virus distribution throughout organs and tissues, no analogy can be made with the members of group B as far as replication is concerned, since the latter do not form precursor particles (150).

The pattern of viral multiplication in whole mosquitoes is generally followed throughout the course of transmission experiments, by titration techniques. The growth curves obtained in this manner exhibit several phases. Firstly, titers are high shortly after the blood meal and this is followed by a decreasing and perhaps an eclipse phase, after which titers increase again, to be

followed by a "stationary" phase. The time intervals vary with different viruses in different mosquito species but usually the stationary phase is reached within a week after infection of a reasonably efficient vector. In such cases too, transmission may be effected in the early phases. The stationary phase is attributed to the continuous multiplication of virus in the tissues balancing the progressive inactivation and possibly excretion of older particles. Infection often persists in this phase throughout the life of the mosquito but titers may show a slow steady decline over the period, barely suggesting that as the mosquitoes age, viral multiplication becomes less efficient. It is not yet clear whether viral multiplication in mosquitoes proceeds continuously or cyclically in relation to particular physiological phases. Virus growth curves of the pattern outlined were observed in studies with the following group A viruses in mosquitoes: EEE in A. aegypti and A. triseriatus (37,42); WEE in A. aegypti (37) and C. tarsalis (44,214); Chikungunya in A. albopictus, A. polynesiensis and A. aegypti (188); Semliki Forest in A. aegypti (153).

Mechanisms of Virus Transmission

The concepts of mechanical and biological transmission of microorganisms by hematophagous arthropods

are well established. They apply to mosquito transmission of arboviruses as a means of causing epidemics and of maintaining virus in nature, but each type has peculiar biological characteristics and significance. In mechanical transmission, the vector is merely a carrier of the organism presumably by contamination of mouth parts. Theoretically, its importance in group A arbovirus transmission would depend on virus stability and concentration on mouth parts, and on the degree to which a mosquito is likely to probe or take a number of blood meals from different vertebrates in rapid succession. Other factors which would influence the success of mechanical transmission include temperature and humidity at the time of virus transfer and the susceptibility of the vertebrate host to infection. Biological transmission on the other hand depends on infection of salivary glands (as discussed under Vector Infection above) and the inoculation of virus with salivary secretions at time of feeding. In transmission of this type the arthropod plays an essential role, and compared to mechanical transmission, there is overwhelming evidence that it is of far greater epidemiological significance in the spread of arboviruses. The factors on which biological transmission depends are numerous, but in the first place they are related to

successful arthropod infection. These facors are discussed in the next section of the review and here, further concern can be given to mechanical transmission and other possible means of virus dissemination by mosquitoes.

Mechanical transmission of arboviruses has been demonstrated in the laboratory. Aedes triseriatus transmitted EEE in this manner from three to six days after infection (42) and WEE was similarly transmitted by Culex tarsalis by means of interrupted feedings on chickens (13). No field observations on arboviruses have established the epidemiological role of mechanical transmission by mosquitoes but it is well known to be a potent factor in the spread of myxomatosis among rabbits in Australia. In this connection too, recent work related to the possible spread of reoviruses by mosquitoes, is worth noting. These viruses, particularly type 3, are widely distributed in nature and the latter has been isolated from mosquitoes (142). In addition, its isolation from several cases of Burkitts tumor in East Africa (7,8) and the suggestion that the tumor may be caused by a mosquito-transmitted agent, stimulated the performance of transmission studies (7,8,142). The investigations provided evidence of mechanical transmission by C. quinquefasciatus (142). Chamberlain and Sudia (46) in their assessment of the possible significance of mechanical

transmission of arboviruses in nature, suggested that even in an area of intense virus activity, it would serve only as a supplementary means of virus transfer.

The possible spread of arboviruses, eg Sindbis (149), vesicular stomatitis (20) in anal discharges of infected mosquitoes, the ingestion of infected mosquitoes by susceptible vertebrate hosts, are other possible means of virus dissemination by mosquitoes. At present they are not known to be of profound epidemiological significance.

FACTORS AFFECTING VECTOR INFECTION AND TRANSMISSION; VECTOR EFFICIENCY

Species and Tissue Susceptibility to Infection; Effect of Virus Content of Blood Meal

The importance of the susceptibility of the mosquito midgut to infection has been mentioned. In this connection, the amount of virus ingested has an important bearing on the subsequent progress of infection, and several factors are in operation. The virus content of the blood meal depends on the size of the meal and the level of viremia in the vertebrate host supplying it. As far as the establishment of mosquito infection is concerned, the situation may be summarized as follows. Groups of individuals of a given species are allowed to feed on blood infected with a particular virus. The response, in terms of infection rates is proportional to the "dose" of virus in the blood meal, within a certain range of doses. However, the maximum infection rate obtained is not necessarily 100% even with further increase in virus dose. When two or more mosquito species are compared, the amount of virus required to establish a given infection rate within the range of quantal response, may differ. This means that for each species a certain minimum quantity of a given virus must be ingested in order to establish infection,

and this is the infection threshold. For example, about 100,000 times more virus is required to infect C. salinarius and C. quinquefasciatus with EEE compared to A. aegypti (41). C. tarsalis requires a relatively low concentration of WEE virus for infection (13,44,214) whereas A. vexans and C. restuans require in the region of one to ten million times more, and C. quinquefasciatus is rarely infected at this level (41). Jupp and McIntosh (115,116) reported that 10^3 LD_{50} of Sindbis virus infect 10% of C. univittatus while C. pipiens fatigans requires 10^5 LD_{50} for infection at the same level.

Several hypotheses attempt to account for differences in species susceptibility. Differential virus inactivation by digestive juices has been suggested and this has already been discussed. Impermeability of the peritrophic membrane could prevent virus adsorption to gut cells (23) and if this is the case, variations in time of formation between species could be important. Bertram and Bird (21) mentioned that in A. aegypti the membrane is completely formed within half-an-hour after a blood meal but its development continues for up to 15 hours later. At this time it is discrete and compact. Similarly, Davidson (58) observed progressive changes in composition and thickness of the membrane in honey bee

larvae, but concluded that it was not a factor in determining susceptibility to bacterial infection. Zhuzhikov (225) has shown that colloidal gold particles of up to 9 mμ diameter permeate through the peritrophic membrane of A. aegypti larvae. Since the arboviruses range in size from 20 to 50 mμ they may therefore be retained. However, this mechanism would not account for high susceptibility of some mosquito species to infection with one virus and a low susceptibility to another, which implies that there is some degree of selectivity and specificity. Zhuzhikov (225) claims that the permeability of the peritrophic membrane to non-ionic substances is not merely dependent on pore size, but, to a considerable extent on the interaction between the matrix of the membrane and the permeating substance. This could be an important factor in the process of mosquito infection with arboviruses.

Chamberlain and Sudia (46) proposed a theory to account for species differences in susceptibility. They suggested that there may be specific receptor sites for different viruses on the epithelial surface of the gut. Some measure of support for this theory has been provided by recent findings of Pattyn and De Vleeschauwer (160). These investigators observed that diethyl-amino-ethyl

(DEAE) dextran enhanced the infectivity of Semliki Forest and two plaque mutants of Sindbis viruses for A. aegypti, which is susceptible to these viruses under normal conditions. It did not alter the infectivity of Sindbis for Anopheles stephensi which is known to be refractory to this virus, and the same applied to Central European encephalitis and 17-D Yellow Fever viruses for A. aegypti. The negative findings conform to the selective nature and specificity of mosquito susceptibility. Additional findings that the activity of DEAE-dextran was quantitative rather than qualitative led these investigators to conclude that its most probable action was "on the virus receptors" of the epithelial cells. Despite the knowledge that DEAE-dextran enhances the infectivity of several viruses in vivo and in vitro, (121,157,160,166) its mode of action is unknown; similarly, the presence of virus receptor sites on mosquito midgut cells has not been established. The conclusions of Pattyn and De Vleeschauwer (160) could therefore be somewhat premature, for the effect of dextran could well have been exerted intracellularly. Kim and Sharp (121) employed the electron microscope in their study of the enhancing effect of DEAE-dextran on vaccinia infection, and indicated that dextran particles entered cell vesicles along with virus. They

suggested that dextran may exert a protective effect on viral nucleic acid during replication, for earlier studies had demonstrated a marked stabilizing effect on the infectivity of purified nucleic acid, without indicating the possible mode of action. Related investigations have been performed in mosquito cells in vitro. Peleg (166) was unable to infect A. aegypti cells with intact virions or with infectious RNA from polio and encephalomyocarditis viruses. DEAE-dextran failed to improve infectivity of these viruses but it did so with the arboviruses which he used. These were Semliki Forest and West Nile. Since it is unlikely that the infectious RNA from the first two viruses was selectively barred from penetrating the cells, the action of dextran is probably related to intracellular factors rather than to adsorption and penetration. In effect, the susceptibility of mosquito cells to arboviruses may depend partly on the ability of infectious RNA to reach the site within the cell where replication takes place, and partly on the presence of the necessary machinery for replication. Pattyn and De Vleesschauwer's results (160) become even more difficult to interpret when one recognizes that dextran did not markedly enhance infectivity of infectious RNA of Semliki Forest and West Nile viruses in mosquito cells (166); that it improved,

though slightly, the adsorption of pox viruses to monolayers (121); and that its enhancing effect on virus infectivity is known to be inhibited by heparin (121) which these investigators used in their infectious blood meals for A. aegypti (160). Several factors may therefore work together to determine the susceptibility of the mosquito midgut to infection with arboviruses, which is reflected in variations in susceptibility between species.

Reference has already been made to the apparent preferential growth of arboviruses in fat cells and salivary glands of mosquito vectors. There are some important factors related to infection of the latter and to subsequent transmission. In a highly susceptible vector it is not uncommon to obtain 100% infection rates in the laboratory (13,44) but transmission rates at this level are rarely encountered (13). Failure to detect transmission following the bite of an infected mosquito may be due to inoculation of insufficient virus in relation to the susceptibility of the vertebrate host. It could also be attributed to absence or insufficiency of salivary gland infection. The matter becomes of particular importance where a mosquito species fails to transmit but maintains high infection rates and relatively high virus titers for several weeks. For example, 79%

of Anopheles quadrimaculatus maintained infection with EEE virus for 13 days yet failed to transmit (41). Evidence has been presented that not unlike the gut, a minimum quantity of virus is necessary to infect salivary glands. Within certain limits above this level, there is a direct relationship between the virus content of the blood meal and the appearance of increasing quantities of virus in the glands. This in turn is related to the rate of increase in transmissions and a shortening of the extrinsic incubation period to a fixed minimum. These relationships have been demonstrated in C. tarsalis infected with WEE virus (214).

Finally, when virus is inoculated into arthropods rather than fed to them, the range of species susceptible to infection becomes very wide. Hurlbut and Thomas infected cockroach, grasshopper, moth, housefly, mosquito, bedbug, beetle and tick with a range of arboviruses and only the cockroach failed to support multiplication of any of them (107,108). Arthropod tissue in vitro also shows broad susceptibility to arboviruses but this tends to be restricted to the mosquito-borne viruses in the case of mosquito cells (See table 1) while a broader spectrum is exhibited in cells from ticks (174).

Amount of Virus Inoculated by Transmitting Mosquitoes

The results of several experiments show that the amount of virus inoculated by a mosquito is highly variable. A. aegypti inoculated between one and 100,000 mouse LD_{50} doses of EEE (38) in experiments designed to investigate this aspect of transmission efficiency. Anopheles albimanus inoculated from 300 to 10,000 (mean 400) mouse LD_{50} of Semliki Forest virus while feeding through a membrane (53) and A. aegypti inoculated from ten to 200 chick embryo tissue culture plaque forming units of Middelburg virus through mouse skin (159). Hurlbut (106) observed that the concentration of St. Louis encephalitis virus in the saliva of Culex pipiens changed significantly within very short periods, even as short as one hour. This may account in part for the great variation in amounts of virus inoculated.

Persistent Infection in Mosquitoes; Effect of Aging.

Brief mention has already been made of these factors in relation to mosquito infection. Their relationship to virus dissemination concerns the probability of transmission for long periods, the possible overwintering of virus in temperate regions, and effects on the virus. In general, transmitting ability decreases after prolonged infection (42,44) and is probably due to less

efficient production of virus or of salivary secretions, general weakness and lowering of metabolic activity. In any event, the fact that a mosquito may remain infective for life, means that transmission lasts as long as conditions are favourable to allow normal activity in nature.

Investigations on the role of mosquitoes in maintaining virus throughout winter have been related primarily to the North American encephalitic arboviruses. Bellamy et al. (15) showed that C. tarsalis females could maintain WEE infection under conditions of simulated winter (California) hibernation. After eight months they were able to transmit by bite, but there was evidence of virus attenuation over the period. Alterations in virulence have also been observed in persistent infection of arthropod cells in vitro (See table 1) and after prolonged infection following parenteral inoculation (107,108). These findings suggest that alternate passage of virus between arthropod and vertebrate hosts is an essential requirement for maintaining the stability of arboviruses in nature. With regard to field observations, WEE has been isolated from C. tarsalis during winter (51,172) but other studies indicate that this mosquito requires a carbohydrate rather than a blood meal for successful hibernation (14,18), and is therefore not likely to be

an important overwintering host. It appears that little work has been done in connection with the role of mosquitoes in maintaining virus throughout adverse conditions, eg drought, in tropical areas. Although it is generally assumed that arboviruses can maintain themselves in these areas by continuous cycling between vertebrates and arthropod vectors, recent findings indicate that other mechanisms are also in operation. For example, VEE can be maintained between cotton rats (Sigmodon hispidus) in Panama and Mexico by extra-mosquito mechanisms (226) and EEE and WEE may be transported to tropical areas by migratory birds (179). In general, evidence is lacking that mosquitoes play an essential role in maintaining virus throughout long periods (eg winter, drought) that are adverse to the usual rapid transmission cycles (172,182,199).

Transovarian Transmission and Trans-stadial Infection

WEE virus has been isolated from eggs of infected C. tarsalis (214) and A. triseriatus (122). Chamberlain and Sudia (46) cite several isolations of other arboviruses but emphasize that there were no instances in which infected progeny resulted. A number of investigators (46,52,55,214) have demonstrated that infection of intermediate mosquito stages is possible and that in a small proportion of individuals virus persists to maturity. Transmission of

St. Louis encephalitis virus by adults infected as larvae (52,55), was minimal and suggested that this mode of infection is of limited epidemiological significance.

The reliability of experimental procedures used in testing the possible significance of transovarian transmission has been questioned (46). Criticism is based on the fact that in most instances only the first egg batch from infected mosquito lots were tested for virus. This may be a valid criticism for it is known that virus is usually isolated from ovarian tissue rather late in the infection cycle (128,214). On the other hand, cognizance should be given to the fact that Doi et al. (62,63) were unable to demonstrate the presence of virus in developing eggs by immunofluorescence. However, virus was present in ovariole sheath and in the wall of the oviducts and the first situation could account for virus isolation from ovarian tissue by other workers. The second could result in contamination of mature eggs during oviposition. Since transovarian passage of virus could be an important mechanism for long term maintenance of virus, the issue warrants further investigation.

Dual Infections; Pathologic Effects of Virus in Mosquito

Naturally occurring infections of mosquitoes with

two or more different viruses is a real possibility. In Thailand where both Dengue and Chikungunya are endemic, A. aegypti has been implicated in the simultaneous transmission of both viruses (85). Apart from confusing the epidemiological picture, dual infections could theoretically result in varying degrees of interference between viruses and this could alter viral properties, the response of the vector to infection, and subsequent transmission. C. tarsalis infected with EEE and WEE transmitted both viruses concurrently without difficulty and without any apparent effect on virus or vector (43). A similar observation was made for A. aegypti infected with various combinations of Sindbis, West Nile and Murray Valley encephalitis viruses (126). The combinations included Semliki Forest virus for up to ten days after infection. Earlier studies indicated that in A. aegypti this virus causes cytological changes in salivary glands which result in decrease in secretory activity, difficulty in obtaining blood and inability to transmit (127,143). These changes were maximal three weeks after infection but they did not affect the life span of the mosquito. A. aegypti is not a natural vector of Semliki Forest virus and as far as can be ascertained, no natural vectors of group A arboviruses suffer from disease as a result of infection.

Effect of Virus Strains

The attenuation of virus in mosquito tissues has been mentioned. Although this may be regarded as evidence for selection of strain variants, it is dependent on laboratory conditions which allow prolonged infection either in vitro (See table 1) or in vivo (15,107,108), unlike conditions normally expected to occur in nature. Evidence for selection of strain variants within the normal period of infection is provided by the behavior of two plaque mutants of Middelburg virus in A. aegypti (158). A large-plaque variant considered representative of the wild type virus, and more pathogenic for infant mice than its small-plaque relative (156), multiplied to higher titers in comparative studies in A. aegypti. Since plaque size provides a convenient marker, a study of the behavior of plaque mutants in natural mosquito hosts might provide some understanding of the antigenic variations which are known to occur among some arboviruses. On account of their important pathologic properties, the North American encephalitis viruses would be of particular importance in this respect (93,192,224). It may be mentioned here that evidence for selection of strain variants of West Nile virus by C. pipiens molestus has also been provided (181).

Climatic Factors

Several laboratory studies provide evidence that the temperature of incubation has a marked influence on development of infection in salivary glands (13,37,42,86, 182). Within the temperature range of mosquito activity, increases in temperature by about $10^{o}F$ result in shorter extrinsic incubation periods and higher transmission rates. The effect on total virus content of whole mosquitoes is not readily apparent and may be reflected simply in a more rapid advance towards the stationary phase of the growth curve and less variation in titers between individuals.

Variations in photoperiod may affect infection and transmission indirectly by influencing such cyclic activities as blood feeding, and secretory activity of salivary glands. Cates and Huang (33) claim that continuous light is more favourable for rapid development of infection and high transmission rates of JE virus by C. tritaeniorhynchus summorosus. They compared this with continuous darkness and half-day light. Their findings, even if true for other virus-mosquito systems, have little epidemiological significance in temperate and tropical regions and their methods cannot be easily employed as routine laboratory procedures in large-scale surveys.

TABLE 2

ISOLATIONS OF GROUP A ARBOVIRUSES FROM MOSQUITOES IN NATURE,

AND RESULTS OF TRANSMISSION TRIALS†

Virus	Clinical Disease	Mosquito Species Virus Isolation and Transmission	Locality
AURA		Aedes serratus (34)	Brazil
		Culex (Melanoconion) sp. (34)	Brazil
BEBARU		Aedes butleri complex	Malaya
		Culex (Lophoceratomyia) spp.	Malaya
			Australia (183)
CHIKUNGUNYA	Fever, joint pains, rash in man (59,71)	Aedes aegypti (7,85,+110,+118)	E. Africa, Thailand
		A. africanus (7,+9,188,218)	E. Africa, Thailand
		Culex fatigans (-188)	E. Africa, Thailand
		C. gelidus (85)	E. Africa, Thailand
		C. quinquefasciatus (85,-188)	Thailand
		C. tritaeniorhynchus (85)	Thailand

Table 2 (Continued)

CHIKUNGUNYA (Continued)		Mansonia africana (7)	East Africa
		M. fuscopennata (7,221)	East Africa
		* Aedes albopictus (+188)	
		* A. apicoargenteus (+9)	
		* A. calceatus (+),(188)	
		* A. furcifer taylori group (+155)	
		* A. polynesiensis (42)	
		* Anopheles gambiae (-155)	
		* Anoph. stephensi (-)	
		* Culex pipiens (-)	
EASTERN EQUINE ENCEPHALITIS	Fever, sometimes encephalitis in humans and equines	Isolations from, and transmission by several species in the United States.	U.S.A.
		Aedes (+), Anopheles (+), Culex, Culiseta (+), Mansonia (35,36, 41,42,44,47,48,66,67,88,103,141, 215,206)	
		Culex nigripalpus (199)	Trinidad, ? Jamaica
		C. pipiens quinquefasciatus	Thailand
		C. taeniopus (199)	Trinidad, Brazil
		C. taeniorhynchus	Brazil

Table 2 (Continued)

GETAH		Aedes amictus amictus (61)	Australia (183)
		Culex bitaeniohynchus (61)	Australia
		C. (Culex) gelidus	Malaya
		C. (Culex) tritaeniorhynchus	Malaya
MAYARO	Human illness Brazil and Guyana (3)	Aedes serratus (83)	Columbia
		Culex (Melanoconion) vomerifer (+74)	Panama
		Haemagogus spp. (199)	Brazil
		Mansonia venezuelensis (3,199)	Trinidad
		Psorophora ferox (74,75,83)	Panama, Columbia
		Several mosquito spp. harboured the virus for up to 12 days after intrathoracic inoculation. Among them, Aedes scapularis only one which transmitted. (2,3)	
MIDDELBURG	Fever in lambs experimentally (123)	Aedes caballus (+123)	S. Africa
		Sheep probably involved in transmission cycle. (123)	

Table 2 (Continued)

MUCAMBO	Fever in man and horse experimentally (61)	Culex mojuensis	Brazil
NDUMU		Aedes (Neomelanoconion) circumulteolus (124)	S. Africa
		Mansonia (Mansonioides) uniformis (124)	S. Africa
O'NYONG-NYONG	Illness in man	Aedes gambiae (+),(7,95,219)	E. Africa
		Anopheles funestus (+),(7,95)	E. Africa
PIXUNA		Anopheles (Stethomyia) nimbus (191	Brazil
ROSS RIVER	Possibly epidemic polyarthritis in man (61)	Aedes vigilax (61)	Australia (183)
SEMLIKI FOREST		Aedes (Aedimorphus) abnormalis (7,54)	E. Africa
		A. (Aedimorphus) argenteopunctus (54)	
		Anopheles (Cellia) funestus (96)	E. Africa

Table 2 (Continued)

SEMLIKI FOREST (Continued)		* Aedes aegypti (+54)	
		* A. togoi (+54)	
		* Anopheles albimanus (+54,56)	
		* Anoph. quadrimaculatus (+54,56)	
SINDBIS	Human illness, including neurologic disorders (1)	* Aedes aegypti (+149)	
		A. butleri complex (60)	Malaya
		A. (Neomelaniconion) circumluteolus (221)	E. Africa
		A. (Aedimorphus) cumminsii (221)	E. Africa
		A. normanensis (60,61)	Australia
		Anopheles amictus amictus (60)	Australia
		Culex annulirostris (60,61,221)	Australia (183)
		C. attenatus	Egypt
		C. bitaeniorhynchus (60)	Australia, Philippines
		C. gelidus (60,193)	Malaya
		C. molestus (151)	Israel
		* C. (Culex) pipiens fatigans (+115)	

Table 2 (Continued)

SINDBIS (Continued)		C. pseudovishnui (193)	
		C. (Lutzia) tigripes (221)	E. Africa
		C. tritaeniorhynchus (60,193)	Malaya
		C. univittatus (95,+116,221)	Egypt, S. Africa
		Mansonia (Mansonioides) africana (221)	E. Africa
		M. annulifera (193)	
		M. fuscopennata (7,149,221)	E. Africa
UNA		Aedes leucocelaenus (34)	Brazil
		A. (Ocherotatus) sp. (75,167)	Panama
		A. serratus (34,113)	Trinidad, Brazil
		Culex (Melanoconion) caudelli (34)	Trinidad, Brazil
		Culex ybarmis (113)	Trinidad
		Psorophora albipes (34,75)	Panama
		P. ferox (34,75,113,167)	Trinidad, Brazil
VENEZUELAN EQUINE ENCEPHALITIS	Fever, sometimes encephalitis in man and equines	* Aedes aegypti (+203)	
		A. atlanticus tormentor (204)	U.S.A.

VENEZUELAN EQUINE ENCEPHALITIS (Continued)

Table 2 (Continued)

A. (Ochlerotatus) sp. (67,75)	Panama
A. serratus (83,114)	Trinidad, Brazil
A. taeniorhynchus (+40,48,204)	U.S.A.
* A. triseriatus (+40)	U.S.A.
Anopheles crucians (204)	U.S.A.
Culex accelerans (114)	Trinidad
C. amazonensis (114)	Trinidad
C. nigripalpus (114,204)	Trinidad, U.S.A.
C. (Melanoconion) sp. (48,67,68, 204)	Brazil, U.S.A.
C. pipiens quinquefasciatus (67, 75,-203)	Panama, Venezuela
C. portesi (+114)	Trinidad
* C. quinquefasciatus	
C. taeniopus (67,74,75,114,167)	Panama, Trinidad
C. vomerifer (67,74,75,114,167)	Panama, Trinidad
Deinocerites sp. (+67)	Mexico
Haemagogus sp. (83)	Brazil
Limatus flavisetosus (114)	Trinidad

Table 2 (Continued)

VENEZUELAN EQUINE ENCEPHALITIS (Continued)		* Mansonia perturbans (+40)	
		M. titillans (+40,114)	Trinidad
		M. venezuelensis (114)	Trinidad
		Psorophora ferox (+40,83,114,167)	Brazil, Trinidad, Panama
		Sabethini (83)	Brazil
		Wyeomyia medioalbipes (114)	Trinidad
WHATAROA		Culex pervigilans (132)	New Zealand
		Culiseta tonnoiri (132)	New Zealand
WESTERN EQUINE ENCEPHALITIS	Fever, sometimes encephalitis in man and equines	Isolations from, and transmission by several species in the United States.	
		Aedes (+), Anopheles, Culex (+), Culiseta (+), Psorophora. (13,15,35,36,41,44,47,51,66,67, 86,89,91,99,103,122,141,205,215,220)	
		Aedes campestris (137)	Canada
		A. dorsalis (137,197)	Canada
		A. flavescens (137,197)	Canada
		A. spencerii (137)	Canada

Table 2 (Continued)

WESTERN EQUINE ENCEPHALITIS (Continued)	*A. vexans* (137,189)		Canada
	Culex restuans (152)		Canada
	C. tarsalis (135,137,189,197)		Canada
	Culiseta inornata (137,189,197)		Canada
	Aedes nigromaculis	J. McLintock and A.N. Burton, unpublished information.	Canada
	Anopheles earlei		

† (+) or (-) preceeding reference number indicates successful or unsuccessful transmission of the virus as reported in the reference cited. Absence of either of these notations indicates virus isolation only, since all mosquitoes from which isolation attempts were negative, are exluded. Absence of a reference number indicates that data were obtained from reference (212) which has been used as a subsidiary source of information.

* It has not been possible to ascertain whether isolations of the virus have been made from these mosquitoes in nature.

EPIDEMIOLOGICAL CORRELATIONS

The occurrence of group A arbovirus disease in man and domestic animals depends, among other things, on the existence of reservoir hosts of the virus, on the presence of a sufficiently large population of mosquito transmitters, and on the degree of intimacy between hosts and vectors. Those mosquito species from which the group A arboviruses have been isolated in nature, and those with which vector transmission studies have been performed, are listed in table 2. It is clear that it is virtually impossible to conduct such studies on all of them, so that other factors must be taken into account in order to assess the epidemiological importance of a suspect vector. Indeed, they ought to determine to a large extent, whether or not elaborate transmission experiments are conducted in the first place. For a given mosquito species, these factors concern observations on its biology and on the relative abundance of infected individuals in nature. This review, in concerning itself with vector-virus relationships cannot consider the many aspects of mosquito biology which contribute to the epidemiological analysis of group A arboviral disease. For general details on this matter, the reader is referred to the recent review by Reeves (173), and for examples of application in specific situations, the following

references cited throughout the text may be consulted (9,85,88,136,137,210,221). Here, one may simply indicate that factors affecting vector population size and distribution (81,85,123,210), vector-vertebrate host (32,102, 113,133,213) and vertebrate host-virus (61,76,90,95,186) associations, are as important as those concerned with vector-virus relationships.

WESTERN EQUINE ENCEPHALITIS IN CANADA

1935-1941: Outbreaks in Man and Horses

The first recorded major outbreak of WEE among horses in Canada occurred in Saskatchewan in 1935 (70) and although there are no reported estimates of the number of animals involved, there is evidence that it seriously affected the farming economy of the province. This evidence presents itself in the tremendous efforts made at the time, particularly by Dr. J.S. Fulton of the Animal Diseases Laboratory of the University of Saskatchewan to isolate and characterize the causative agent of what was then regarded as a new and strange disease of horses. The efforts of Fulton and his colleagues resulted in a better understanding of the pathogenesis of the disease, provided methods for isolation and identification of the virus and at a later date, the production of a chick embryo vaccine for horses (70,71,72). In addition, these workers in Saskatchewan were able to recognize in retrospect that the disease had existed in endemic form for several years. Reports of clinical cases among horses in Manitoba which were made by a "particularly alert" veterinary practitioner as early as 1932, then became of some substance (185).

In 1937 WEE recurred in epidemic form in Saskatchewan

(70) and the Provincial Veterinarian's report for that year mentions that there was "considerable mortality" among horses (5). In the following year, also an epidemic year, it was estimated that 14,000-16,000 horses died from the disease. These losses can be placed in perspective if it is realized that at the time, horses accounted for the major portion of the value of all livestock in the province; so that, the efforts of the Department of Agriculture to educate the public, to encourage the production and the (subsidized) sale of chick embryo vaccine can be appreciated (6). It is of passing interest that the vaccine was manufactured by Dr. Fulton and his colleagues in the Animal Diseases Laboratory of the University of Saskatchewan. The "J.S. Fulton Laboratory" which today stands on the Saskatoon Campus and forms part of the Department of Veterinary Microbiology is a constant reminder of the tremendous importance of this disease, not only to the farming economy of earlier years, but also to the human population then, as it is at present.

Human cases of encephalitis appeared in Saskatchewan during the period of the equine epidemics (70,71) and Fulton's laboratory was called upon to assist in the diagnosis of these cases. In 1939, a year after Beatrice Howitt had isolated WEE virus from the brain of a child

(104), Fulton achieved the same feat (71) and was able to show that the same agent was responsible for the human and equine disease. At about the same time, the virus of Eastern equine encephalitis (EEE) was also isolated from human cases of encephalitis in Massachusetts (217). Following these developments, a major outbreak of WEE occurred among human beings in Manitoba and Saskatchewan in the summer of 1941. It was associated with epidemics in Minnesota and North Dakota (table 3), and the

TABLE 3

HUMAN CASES OF ENCEPHALITIS IN EPIDEMIC AREA, 1941 (64)

Province or State	Clinical Cases	Deaths
Saskatchewan	543	44
Manitoba	509	78
Minnesota	639	86
North Dakota	1101	138

appearance of few equine cases in the epidemic area was attributed to the extensive use of chick embryo vaccine (6). Jackson (109) estimated the loss of over 200,000 man hours in Manitoba alone, and since most of the cases occurred in adult males at the peak of the harvest season,

the loss in farm income and the damage which the epidemic caused to the war effort were considerable. Accordingly, vaccine trials were undertaken in man in the following year (109). It is interesting to note in this connection, that the Animal Diseases Research Institute in Hull, Quebec, was called upon to examine the sera from vaccinated persons and in this manner, played its part in assisting in the control of this important zoonosis.

1942-1970: Mosquito Hosts; Disease in Man and Horses

Human vaccination marked the end of a phase of intense activity in relation to WEE in Canada. During the period some thought was given to the possibility of insect transmission of the virus; for, in addition to overwhelming circumstantial evidence (64,70) the investigators in Manitoba and Saskatchewan were aware of successful laboratory transmission experiments performed in the United States with both EEE and WEE viruses. They were particularly aware of the work of Kelser (120) who was the first to demonstrate as early as 1933, that an encephalitic virus (WEE) could be transmitted by mosquitoes (Aedes aegypti) from guinea pigs to horses. That A. dorsalis and A. nigromaculus could also transmit the virus was also known since 1936 (131). These and other early reports from the United States stimulated investigations aimed at identifying the possible vectors of WEE

in the endemic and epidemic areas of Canada. There were however, some misgivings. In fact, Fulton, who by now was regarded as an expert on the disease, had dismissed at the time of the earlier outbreaks, the probability of mosquito transmission and was inclined to incriminate Stomoxys calcitrans as the epidemic vector (70). It must be emphasized that his opinions were based on firm scientific observations and even so, he was particularly cautious in suggesting S. calcitrans. It is not known to what extent these opinions discouraged the performance of vector studies in Saskatchewan but in Alberta and Manitoba such studies were in progress in the early 1940's (84,134) and as a consequence, WEE virus was isolated from wild caught Culex restuans and C. tarsalis (135,152).

Although the disease continued to be important on the prairies between 1942 and 1959 (See table 4), the South Saskatchewan River Dam Project was the factor which further stimulated the study of the natural history of the disease around 1960 (J.G. Rempel, personal communication). In 1962 virus was isolated from wild caught Culiseta inornata, Culex tarsalis, Aedes dorsalis and A. flavescens in Saskatchewan (197), and following this, two serious epidemics occurred in the province in 1963 and 1965. In table 4 the numbers of confirmed cases are

TABLE 4

INCIDENCE OF WEE IN SASKATCHEWAN (MAN AND HORSES)

1942-1970*

Year	WEE Cases
1942-46	Low incidence, scattered human and equine cases mainly in southern region. (59)
1947	68 human, mainly central region. (59)
1948-52	Low incidence.
1953	46 human, mainly central region. (59)
1954-62	Low incidence, human and equine. 7 equine in 1962. (59)
1963	38 human, 3 deaths. (136) 279 equine, 47 deaths. (136)
1964	1 human (confirmed). 41 equine.
**1965	77 human (confirmed), 9 deaths. 106 equine, 7 deaths.
1966-70	3 human: 1966, 2 (confirmed); 1967, 1 fatal. 84 equine: 1966,8; 1967, 10; 1968,9; 1969, 40; 1970,17 (2 confirmed).

* Information obtained from the references shown, from the Saskatchewan Dept. of Agriculture annual reports, and from the records of the Dept. of Veterinary Microbiology, University of Saskatchewan.

** Alberta 1965: 136 equine (63 confirmed) cases, with 5 confirmed deaths and possibly 30 others (148).

presented. The figures do not indicate the true severity of the epidemics, for it is known that during August and September of 1965, 490 human cases of presumed encephalitis were treated in Saskatchewan hospitals (140). Furthermore it is estimated that hundreds of affected individuals were not hospitalized. In a follow-up study on 85 human cases from these epidemics, it was found that ten children and two adults suffered permanent disabling defects and another 30 showed defects of a non-disabling character. This confirmed earlier reports on the disabling effect of WEE in man (73). Apart from the personal problems involved, and the fear and anxiety created by the disease, the magnitude of the situation can be grasped by noting that the support of one disabled child throughout its life costs about $100,000.00 (140). Consequently, studies on various aspects of the disease continue. Those concerning the identification of mosquito and vertebrate hosts and behavior of virus in the latter (28,29,30,31, 139,171,178,180,189,197,198), the seasonal and geographic distribution of these hosts in relation to virus activity during epidemic and interepidemic periods (136,137,138, 147,148,177,190), have been in progress in Alberta and Saskatchewan for some time. Elsewhere, investigations are nearing completion on the production of a suitable vaccine

for use in man (C.H. Bigland, personal communication) and it may be mentioned here that limited vaccine trials were performed in Saskatchewan in 1963 (59).

The known mosquito hosts of WEE virus in Canada are listed in table 2. In this connection, McLintock et al. (137) recently presented a detailed analysis in relation to the 1963 and 1964 epidemics (See table 4) and the intervening non-epidemic period in Saskatchewan. In the two epidemic years there were larger infected populations of C. tarsalis than of any other mosquito species, whereas in the non-epidemic year the majority of WEE isolations were from other mosquitoes. Based on these findings and on supporting evidence related to distribution of clinical cases, to virus isolations from vertebrates and to known mosquito-vertebrate host relationships, they arrived at a number of conclusions. Firstly, that unlike the situation in the western United States (35), C. tarsalis seemed to be the primary epidemic vector of WEE in Saskatchewan while Aedes spp. and Culiseta inornata appeared to be involved in maintaining the enzootic status of the disease. Secondly, that Culiseta inornata might be a significant transmitter of the virus to horses, particularly in northern areas of the province where C. tarsalis is rare. Finally, that the early Spring aedines might be responsible

for transferring infection from overwintered vertebrate hosts to birds. This would initiate the bird-*C. tarsalis* transmission cycle which occurs during summer.

In order to confirm the above, laboratory transmission studies were conducted. They were designed to investigate the behavior of WEE virus in known mosquito hosts of the province; to determine the ability of these mosquitoes to transmit the virus from one susceptible vertebrate host to another, and to assess the efficiency of virus transmission wherever appropriate.

SUMMARY AND CONCLUSIONS

Studies on the behavior of arboviruses in their mosquito hosts play an important part in understanding the mechanisms which allow for arbovirus maintenance and dissemination in nature. The group A viruses which are all mosquito borne, are important in this respect because they are widely distributed throughout the world and several cause serious disease. It has been clearly established that transmission of these agents depends for the most part on an intimate association between virus and mosquito vector, in which the latter plays an essential biological role. The factors which influence the development of mosquito infection and subsequent transmission are complex. However, in laboratory investigations where a number of parameters can be altered in turn, several have been elucidated and many conform to observations in the field. On the basis of these investigations it is possible to determine the vectorial ability of suspect mosquito hosts, and quantitative measurements allow a comparison of transmission efficiency between species. Knowledge of vector-virus relationships influence to a great extent the laboratory procedures which may be reasonably employed for the recovery and identification of viruses from mosquitoes. This is

important because in this manner it is possible to assess the degree to which man and his domestic animals are exposed to the pathogens within the group at a given time. Great significance must therefore be attached to the development of new and reliable procedures for these purposes.

Although the study of vector-virus relationships has already contributed much to the understanding of the epidemiology of arboviruses, several important considerations demand that investigations continue. In the first place, many problems remain unsolved, and in the second, recognition must be given to the fact that local situations constantly change. For example, there is sudden exposure to new or altered viral strains as new areas are cultivated or developed in some manner, and new problems arise. Particular importance must be attached to those agents which cause clinical disease in man and domestic animals, for as more information becomes available, a more rational approach can be taken towards the control or elimination of the diseases which they cause.

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CHAPTER 2

DEMONSTRATION OF WESTERN EQUINE ENCEPHALITIS VIRUS IN MOUSE BRAIN BY IMMUNOFLUORESCENCE

SUMMARY

Brains of mice infected with Western Equine Encephalitis (WEE) virus were fixed in Carnoy's fluid at 4°C and transferred through chloroform to paraffin. The procedure gave excellent preservation of structural architecture and viral antigen. The latter was demonstrated in sections by the indirect fluorescent antibody technique in which Evans blue was used as a counterstain. The technique proved to be rapid, simple and reliable for the identification of WEE virus in mouse brain.

INTRODUCTION

The usefulness of immunofluorescence techniques in a number of specific research problems with arboviruses has been demonstrated. For example, detailed studies on intracellular virus localization (3,4,5,9,11), on pathogenesis (6,7) and on differentiation of viral strains (2,8) have employed fluorescent antibody methods. Despite these successful applications, the techniques have not found wide use in routine diagnostic work. As a step in this direction, a method was sought for the rapid identification of Western Equine Encephalitis (WEE) virus in mouse brain by the fluorescent antibody technique (FAT). This report describes the failure to identify the virus on impression smears and the successful application of a

paraffin embedding process. The latter was a modification of the procedure originally employed by Albrecht et al. (1) for the study of a number of neurotropic viruses.

MATERIALS AND METHODS

Virus

WEE virus, strain 1540-1544 Regina 66, which had undergone three suckling mouse brain passages and was in its sixth chick embryo passage was used. It was originally isolated from the brain of a human being (JR) who died of the disease in Saskatchewan in 1966. The stock material of 20% chick embryo allantoic fluid was stored in sealed containers at -70°C. It contained $10^{5.8}$ intracerebral mouse LD_{50} per 0.03 ml when titrated in weaned mice.

Preparation of Smears and Paraffin Sections

Weaned white mice were inoculated intracerebrally with approximately 1 to 1000 LD_{50} virus. Each dose was contained in 0.03 ml of the appropriate dilution of stock virus in beef infusion broth, pH 7.4. Brains were removed shortly after death, or from animals sacrificed when showing paralysis or other clinical signs of encephalitis.

Impression smears were prepared by applying a microscope slide to the cut surface of the brain dissected sagittally. They were fixed in acetone for periods varying

from 10 to 60 minutes at 4°C and at -20°C, after which they were air dried and stained.

For paraffin embedding, blocks of brain approximately 3 mm thick were fixed in two changes of Carnoy's fluid for a total of five hours at 4°C. They were then transferred to chloroform (reagent grade) in which they remained overnight at room temperature. Infiltration in two changes of paraffin followed, for a total of three hours at 56°C. Each change of medium represented at least a 20-fold volume of the tissue block. Sections 6 μ thick were cut at room temperature, floated on water at 40°C and spread on microscope slides which were then dried at 37°C for 30 minutes. Slides were held at 4°C until ready for staining when paraffin was removed with xylene and the sections hydrated by transfer through 96%, 70% and 40% ethanol to distilled water. Each change lasted 2-3 minutes.

Immunofluorescent Staining

The indirect FAT was used. Antiserum obtained from rabbits immunized with WEE virus was absorbed with acetone-extracted mouse brain powder for 30 minutes at 37°C. It was diluted in phosphate buffered saline (PBS), pH 7.4, just before use. Goat antirabbit globulin conjugated with fluorescein isothiocyanate (FA conjugate) was

obtained from a commercial source*, and diluted in 0.02% Evans blue in PBS just before use.

Table 1 gives details of the staining protocol. For each of the steps 1 and 2, smears or sections were covered with the material listed and the slides incubated in a moist chamber for 30 minutes at 37°C. After step 1, they were washed for 10 minutes in two changes of PBS and air dried. On completion of step 2, they were washed as before and mounted wet in 90% glycerol in PBS.

In the initial stages of the investigation, the entire staining protocol was used with each brain examined. This was to establish the value of Evans blue as a counterstain, to allow evaluation of different batches of antisera and to compare the staining specificity of absorbed with that of unabsorbed preparations. In the later stages, protocol stages 5 to 8 were routinely used with all specimens and stages 1 to 4 were included for each new preparation of counterstain, antiserum or FA conjugate.

Microscopy and Photography

Sections were examined and photographed with a Zeiss Universal microscope equipped with an Osram HBO

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* Microbiological Associates

200 W lamp and an Asahi Pentax 35 mm camera. Exposure time was 60 seconds on Kodak "Tri X Pan" film. Filters were BG12 (4 mm thick) and BG28 (2.5 mm thick).

RESULTS

Smears

Faint fluorescent foci were observed in several positive preparations (Table 1, Protocols 4 and 5). Although they were absent from control samples (Protocols 6, 7 and 8), they were not consistently observed in all positive smears with the methods used for fixation and staining.

Paraffin Sections

Paraffin sections gave excellent preservation of structural architecture and viral antigen. In positive preparations (Protocols 4 and 5) there was brilliant specific fluorescence in neurons and ground substance of the brain stem (Figures 1, 7). Groups of fluorescent neurons were observed in the pyramidal layer of the cerebrum (Figures 3, 9) but they were not as numerous as in the brain stem. Neurons of the purkinje layer of the cerebellum also showed bright fluorescence and dendrites passing through the molecular layer were often brilliantly outlined (Figures 2, 8).

Fluorescence was consistently absent from normal serum and normal tissue controls (Figures 10, 11, 12, Protocols 7 and 8), but complete inhibition of specific fluorescence throughout all areas of the section was not always achieved in the inhibition controls (Figures 4, 5, 6, Protocol 6). Although the degree of suppression varied with different batches of antisera, specific fluorescence, when observed in the inhibition controls was always very faint and interpretation presented no difficulty when compared to positive preparations. This was observed in 25 infected samples examined in groups of five at different times, with three batches of WEE antisera.

DISCUSSION

The results demonstrate that the indirect FAT may be applied to infected mouse brain for the identification of WEE virus. Apart from the difficulty in obtaining a sheet of intact nerve cells on the microscope slide, no complete explanation can be offered for the failure of impression smears. Failure may have been due partly to the diffuse cellular localization of viral antigen as opposed to confinement in a localized area.

On the other hand, the excellent results obtained, confirmed the report by Albrecht et al. (1) on the applicability of the technique for demonstration of arbovirus

antigens in brain tissue. As emphasized in that report, we can also confirm that widely differing times for fixation, chloroform treatment and paraffin infiltration may be reasonably employed. The method reported here is that which consistently gave maximum specific fluorescence together with satisfactory preservation of structure and minor shrinkage of tissue. For more rapid diagnosis, however, the procedure can be completed within half the time reported. This requires several changes of media and is therefore more laborious when several samples are to be examined at the same time. In addition, fluorescence is less intense and structural architecture is poor.

The staining protocol which was employed, ensured satisfactory elimination of non-specific fluorescence and confirmed the value of Evans blue as a counterstain as previously reported (6,10). The employment of elaborate measures for the removal of possible contaminating fluorescent substances from the media (1) was therefore unnecessary and this served to simplify the procedure. In addition, the reliability of the technique makes it suitable not only for the rapid identification of WEE virus isolated in mouse brain, but also for studies related to the pathogenesis of the disease in the central nervous system.

TABLE 1

STAINING PROTOCOL

Protocol Stage	Tissue (Smear or Section)	Treatment		Purpose for each stage of protocol
		Step 1	Step 2	
1	Infected	PBS	PBS	Autofluorescence control.
2	Infected	PBS	Evans blue in PBS	Suppression of autofluorescence.
3	Infected	Antiserum in PBS	FA conjugate in PBS	Establish extent of total (specific and non-specific) fluorescence.
4	Infected	Antiserum in PBS	FA conjugate in Evans blue	Suppression of portion of non-specific fluorescence.
5	Infected	Antiserum absorbed with normal tissue powder and diluted in PBS.	FA conjugate in Evans blue	Suppression of all components of non-specific fluorescence; remaining fluorescence largely or entirely specific.

TEST PREPARATION

Table 1 (Continued)

Protocol Stage	Tissue (Smear or Section)	Treatment		Purpose for each stage of protocol
		Step 1	Step 2	
6	Infected	Antiserum absorbed with normal tissue powder and diluted in PBS. Further absorbed with 10% infected tissue just before use.	FA conjugate in Evans blue	Suppression of all fluorescence. INHIBITION CONTROL
7	Infected	Normal rabbit serum in PBS.	FA conjugate in Evans blue	No fluorescence. NORMAL SERUM CONTROL
8	Normal	Antiserum absorbed with normal tissue powder and diluted in PBS.	FA conjugate in Evans blue	No fluorescence. NORMAL TISSUE CONTROL

Figures 1 to 6

Figures 1 to 6 Indirect fluorescent antibody stained sections of mouse brain infected with WEE virus.

Figure 1 Specific fluorescence in neurons of brain stem, 100 X.

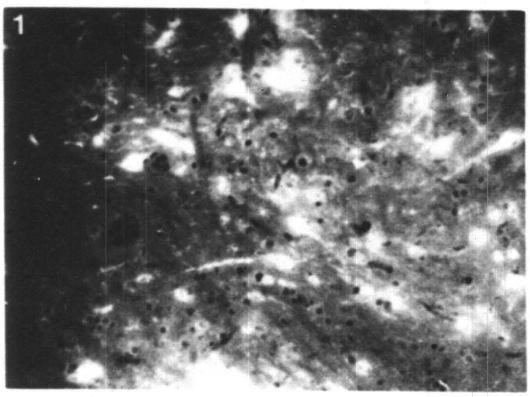
Figure 2 Specific fluorescence in neurons of purkinje layer of cerebellum, 63 X.

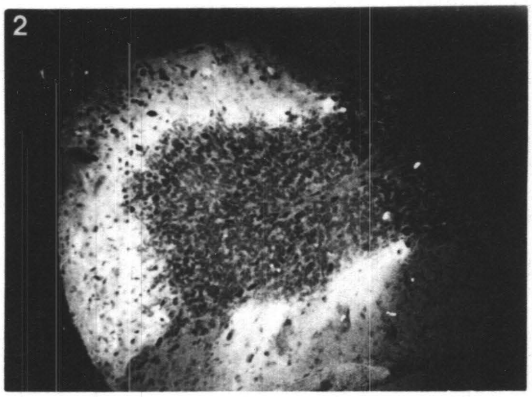
Figure 3 Specific fluorescence in neurons of pyramidal layer of cerebral cortex, 100 X.

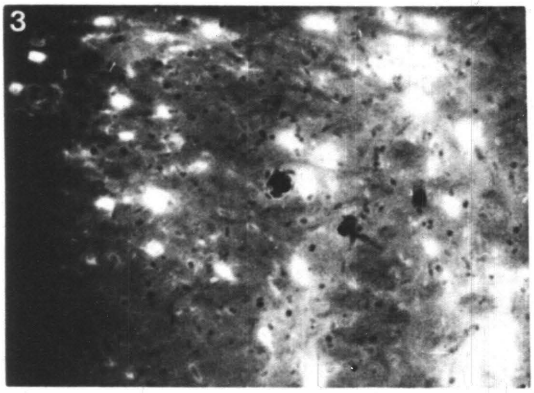
Figure 4 Inhibition control, brain stem, 100 X.

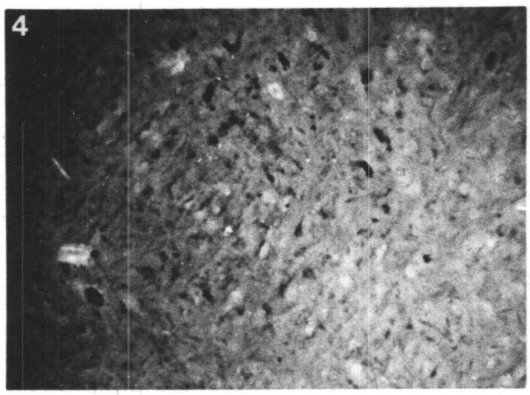
Figure 5 Inhibition control, cerebellum, 100 X.

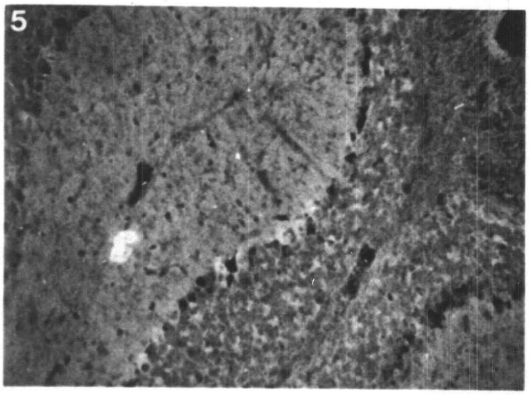
Figure 6 Inhibition control, cerebral cortex, 100 X.

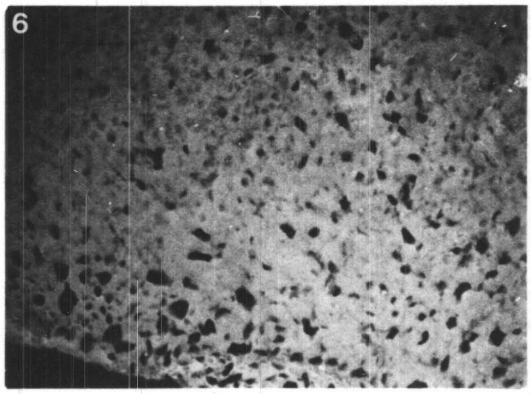












Figures 1 to 6

Figures 7 to 12

Figures 7 to 10 Indirect fluorescent antibody stained sections of mouse brain infected with WEE virus.

Figure 7 Specific fluorescence, brain stem, 400 X.

Figure 8 Specific fluorescence, purkinje layer of cerebellum, 400 X.

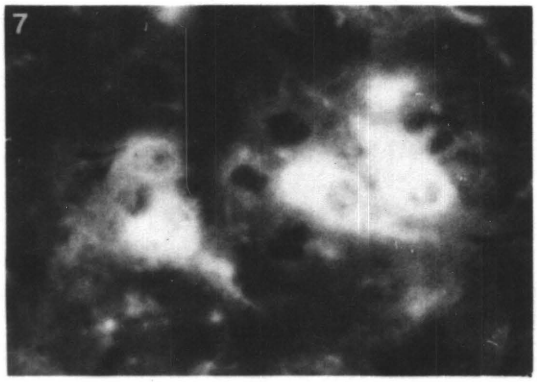
Figure 9 Specific fluorescence in neurons and ground substance of cerebral cortex, 160 X.

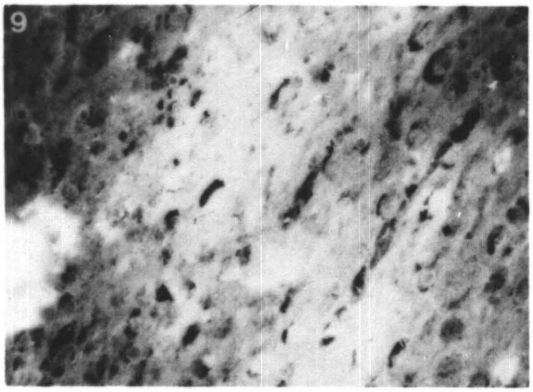
Figure 10 Normal serum control, brain stem, 100 X.

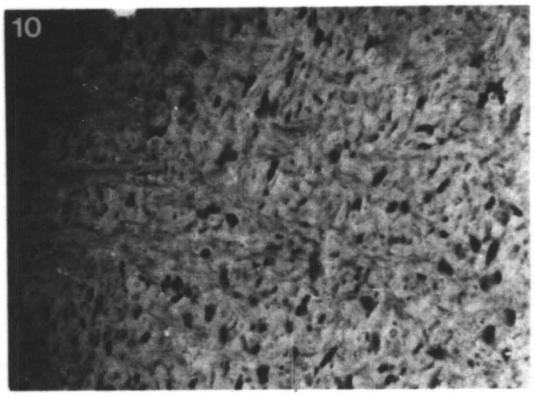
Figures 11 and 12 Indirect fluorescent antibody stained sections of normal mouse brains (normal tissue controls).

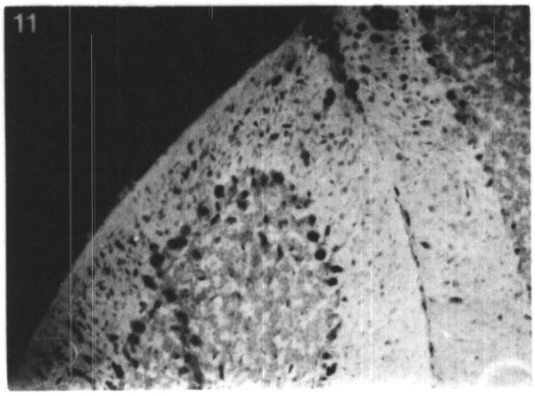
Figure 11 Cerebellum, 100 X.

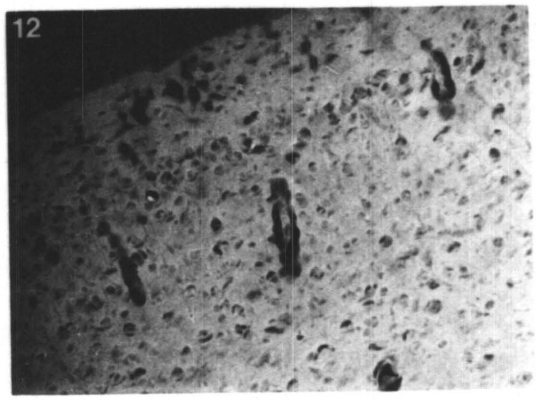
Figure 12 Cerebral cortex, 100 X.











Figures 7 to 12

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CHAPTER 3

DIAGNOSTIC APPLICATIONS OF IMMUNOFLUORESCENCE, AND USE OF CHICKS IN LABORATORY STUDIES ON TRANSMISSION OF WESTERN EQUINE ENCEPHALITIS

SUMMARY

The response of chicks to western equine encephalitis virus, strain 1540-1544 Regina 66, was investigated. Half-day old chicks showed viremia from at least eight hours after infection, and died when viremia levels were within the range of 10^8 to 10^9 intracerebral 3-week old mouse LD_{50} per 0.03 ml of blood. In comparative titrations, chicks were two to three log units more sensitive than mice for detection and titration of virus.

Immunofluorescence studies confirmed that western equine encephalitis virus could be identified without difficulty in the brains of mice showing clinical signs or dying from the disease. Differences in distribution of viral antigen within the brain were noted between chicks and mice inoculated subcutaneously and intracerebrally. These differences were related to clinical signs, histopathological brain lesions and virologic studies. In mice, neurons showing degenerative lesions or those close to inflammatory sites were not always infected and many of those infected were morphologically normal under light microscopy. Brains of chicks which may yield high titers of virus in suspension, may show neither morphological abnormality nor intracellular virus. For these reasons, the fluorescent antibody technique was without value as

a diagnostic tool when applied directly to brains of chicks which died after subcutaneous infection by western equine encephalitis virus. In transmission experiments, the technique may be used to confirm infection in such chicks by passage of virus to 3-week old mice, from which brain sections are used for immunofluorescence diagnosis. In a limited trial, cryostat sections of normal and infected chick brains showed a high degree of non-specific fluorescence and poor preservation of structure.

The results of the investigation formed the basis for the design and interpretation of laboratory experiments on transmission of western equine encephalitis virus by Saskatchewan mosquitoes.

INTRODUCTION

In transmission experiments with western equine encephalitis (WEE) virus, measurements such as mosquito transmission and infection rates, infection thresholds and virus titers must be made repeatedly. Such data are obtained by using individuals or small groups of mosquitoes at different times after infection. On many occasions, the amount of virus in each sample is very small and difficult to detect or to measure. Consequently, the vertebrate host which is selected for transmission studies must be highly sensitive to the virus and must give a consistent

and uniform response.

It is known that chickens are highly susceptible to WEE virus. LaMotte (12) investigated the use of adult chickens as sentinels for the virus and found that 3 to 8 month old birds developed viremia which persisted for approximately two days. In experiments with younger chickens, Hammon and Reeves (5) indicated a relationship between dose of virus and persistence of viremia. Chamberlain et al (4) demonstrated that chicks up to 24 hours old were more sensitive for titrating WEE virus by subcutaneous inoculation than were 3-week old mice inoculated intracerebrally. These investigations demonstrated the value of chicks for detection and titration of particular strains of WEE virus.

The objective of this study was to provide information on the response of newly hatched (wet) chicks to infection with the strain of WEE virus selected for experiments on transmission by Saskatchewan mosquitoes. In chicks, virus doses covering the estimated range of that inoculated by infected mosquitoes were used. The results were expected to form the basis for the design and interpretation of transmission studies. For this reason, the confirmation of WEE infection in chicks which died after they were bitten by infected mosquitoes was also of some importance. Confirmation is normally accomplished by

serologic tests. These procedures are time consuming and expensive, particularly so in transmission studies where they must be performed repeatedly. An investigation was therefore undertaken to determine whether a fluorescent antibody method could be employed to identify the virus in chick brain. It had previously been shown that such a technique could be used on brain sections of mice dying after intracerebral inoculation of WEE virus (6). Consequently, it was also important to determine whether the mode of infection of mice and chicks might influence the results and interpretation of the fluorescent antibody technique (FAT), applied directly to brain sections from these animals.

MATERIALS AND METHODS

Virus

WEE virus, strain 1540-1544 Regina 66, was obtained from Dr. A.N. Burton of the Western College of Veterinary Medicine. It was originally isolated from the brain of a human being who died of the disease in Saskatchewan in 1966. On receipt, it had undergone three suckling mouse brain passages and was in its sixth chick embryo passage. "Primary seed" virus stock was prepared from suckling mouse brain harvested at 48 hours after intraperitoneal inoculation. The brains of several mice were pooled, homogenized

as a 20% suspension in diluent and centrifuged lightly to remove tissue debris. The supernatant was then freeze-dried in 0.5 ml amounts and stored in sealed glass ampules. For preparation of "secondary seed" virus, the contents of three ampules of primary seed were pooled and inoculated into the allantoic cavity of fertile ten-day old chick embryos. Allantoic fluid was harvested from dead embryos at 48 hours, pooled, centrifuged lightly and the supernatant stored as a 20% suspension in 0.5 ml amounts in sealed containers at $-70^{\circ}C$. The secondary seed material contained between $10^{5.5}$ and $10^{6.3}$ intracerebral LD_{50} per 0.03 ml when titrated in weaned mice.

Beef infusion broth, pH 7.4 with 0.75% bovine albumin fraction V, was used as diluent for all virus containing material. It contained 500 units of penicillin and 2.5 mg of streptomycin per ml.

Virus Assay in 3-Week Old White Mice and Wet Chicks

Virus was titrated by making serial 10-fold dilutions and inoculating 0.03 ml of each dilution intracerebrally (ic) into five mice. Inoculations were done under light ether anaesthesia, and mice were observed for 12 days. For comparing susceptibilities, virus titration in chicks was performed in parallel with mice. Chicks were inoculated subcutaneously (sc) with 0.03 ml of each dilution and deaths

were recorded over a five-day period. At the time of each titration, two tubes of thioglycollate broth were inoculated with an aliquot of the virus suspension. They were incubated at 37°C for two days, and they served to confirm bacterial sterility of each suspension.

The LD_{50} titer is expressed as the end-point dilution of a virus suspension which gave 50% mortality, as calculated by the method of Reed and Muench. As such, it is the dilution which contained one infectious unit (ic 3-week old mouse LD_{50}) per 0.03 ml.

Viremia in Chicks

Two experiments were performed at different times. Each consisted of two groups of 16 white leghorn chicks which were removed from the incubator when 12 to 15 hours old. Each member of a group was inoculated sc with a given dose of virus (approximately one and 1000 LD_{50} respectively), contained in 0.03 ml of the appropriate dilution of secondary seed virus. The chicks were housed in subgroups of four in covered cardboard cages. Two cages, each containing four uninoculated chicks were placed among the others to test for aerosol spread of infection. (Aerosol controls). Two other control groups were inoculated with diluent only (Diluent controls) and all cages were placed in a room maintained at 80-85°F. Four chicks from each group, ie all members of one subgroup, were

bled by cardiac puncture at eight hours post inoculation. At four hour intervals thereafter until death, blood was obtained from all members of each subgroup alternately, so that no animal was bled twice within a 16 hour period. At each bleeding, 0.1 ml of blood was taken from each chick and the four samples pooled. The pooled sample was then stored at -70°C as a 10% suspension in diluent containing 20 USP units of heparin per ml. Blood from the control groups was taken at 24 and 48 hours post infection, and the chicks in these groups were observed for five days for evidence of infection.

Immunofluorescence Studies

All virus doses used are expressed as mouse ic $LD_{50's}$, and each computed dose was contained in 0.03 ml of the appropriate dilution of secondary seed virus. All intracerebral inoculations were performed under light ether anaesthesia. The procedures for fixation, paraffin embedding and sectioning were as previously reported (6). So too, was immunofluorescent staining except that acetone-extracted mouse brain and chick brain powders were used for absorption of antisera. Table 1 which gives details of the staining protocol is included for easy reference. Microscopy and photography were as previously reported (6), and paraffin sections for histopathology were stained with

hematoxylin and eosin.

Experiment 1. Comparison of Cryostat and Paraffin Sections

Each of 20 chicks was inoculated sc with approximately 1000 LD_{50} doses of virus. Brains were removed shortly after death, which occurred in all cases between 20 and 30 hours after inoculation. For cryostat sectioning they were quick frozen onto the microtome chuck with a few drops of water. Sections 6 to 8 μ thick were transferred to slides precooled in the cryostat chamber. They were allowed to thaw and dry at room temperature after which they were fixed in acetone at -20°C, 4°C or at room temperature for periods varying from ten to 60 minutes. Immunofluorescent staining was then performed.

Experiment 2. Effect of Route of Inoculation

Chicks were inoculated sc and ic and mice ic with approximately 1000 LD_{50} doses of virus. Doses between 10,000 and 100,000 were used for sc inoculation of mice. Brains were removed from chicks as previously stated, and from mice on post inoculation days five (ic group) and eight (sc group). These were the days on which deaths first occurred in the respective groups. Some samples were therefore obtained from dead mice and others from mice showing varying clinical signs of WEE infection, ranging from anorexia and lethargy to locomotor paralysis.

Experiment 3.

To examine whether the FAT could be successfully employed on brains of chicks which died later than 30 hours after infection, chicks were inoculated sc with virus doses ranging from 0.001 to 0.1 LD_{50}.

Experiment 4.

As a final check on the FAT, a correlation was sought between the results of this test and neutralization indices. Neutralization tests were performed in mice as a means of confirming WEE infection in laboratory hosts which died in preliminary transmission experiments. An index of 1.5 logs or more was considered to provide evidence of infection. The FAT was performed on the brains of mice which were inoculated ic with a 10% suspension of host tissue used in the neutralization tests.

In experiments 2, 3 and 4, paraffin sections were employed for immunofluorescence studies.

RESULTS

Viremia in Chicks

The viremia patterns in chicks inoculated with one and 1000 LD_{50} doses of virus are shown in figure 1, where data from both experiments are pooled. The response to each dose was fairly uniform between experiments, with

differences in titer at any time remaining close to one log dilution of virus. In those chicks receiving 1000 LD_{50} there was rapid development of viremia between eight and 16 hours post inoculation, followed by a marked decline in rate for the remaining eight hours during which the chicks were alive. A similar pattern was observed in those receiving one LD_{50}, but there was a six to eight hour lag in response, compared to the former group. All chicks died when viremia levels were within the range of 10^8 to 10^9 mouse LD_{50} and virus concentration in brain and liver ranged from $10^{4.6}$ to $10^{5.5}$ LD_{50}. None of the control animals showed virological or clinical evidence of infection.

Clinical Signs and Virus Titration in Chicks

The majority of chicks showed inactivity followed by prostration for two to eight hours before death. Very few, as indicated in table 2 showed mild neurologic signs. These consisted of coarse tremors of head and neck, incoordination and stupor which lasted for eight to 20 hours before death.

That chicks are two to three log units more sensitive than mice for titration of virus is indicated in table 3.

Evaluation of Tissue Absorption of Antisera and Use of Counterstain in the FAT

The WEE antisera used in the initial stages of the investigation consisted of material prepared with either primary seed (suckling mouse brain) or secondary seed virus (allantoic fluid). Both stocks were relatively crude virus suspensions containing large amounts of host tissue. Accordingly, antisera prepared from such materials and used for fluorescent antibody staining could be expected to give rise to an excessive amount of unwanted fluorescence in homologous tissue. Although both types of antisera were satisfactory, it was desirable to select one type for routine use. In this manner, possible differences in avidity between them was avoided, and comparisons between chick and mouse brains could be more easily made. Antiserum prepared with secondary seed virus was selected because of higher neutralization titers and availability of larger quantity. Particular care had to be taken therefore, to eliminate unwanted fluorescence from chick brains. Figures 2 and 3 show the value of tissue absorption and use of counterstain in achieving this in homologous tissue.

Evaluation of Cryostat and Paraffin Sections

Despite the measures taken to ensure easy recognition of a specific reaction, cryostat sections showed a

very high degree of non-specific fluorescence, making interpretation difficult. This occurred with both types of antisera. In addition, only about 30% of these sections showed reasonable preservation of histological structure, and for these reasons they were abandoned after a number of trials on chick brain.

Paraffin sections gave excellent preservation of structure. Preservation of viral antigen was also satisfactory as judged from the reactions occurring in mouse brains. (Figs. 4 and 5).

Effect of Route of Inoculation

Table 4 summarizes the results of immunofluorescence on brains of mice and chicks after sc and ic inoculation of WEE virus. Specific fluorescence was almost always absent from the brains of chicks dying within 20 to 30 hours after sc inoculation of virus (Fig. 2). In those dying within the same period of time after ic inoculation, viral antigen was largely restricted to the meninges, choroid plexus and ependyma (Fig. 3). On the other hand, in mice dying or showing clinical signs after ic or sc inoculation, viral antigen was present in neurons and ground substance of brainstem, cerebral cortex and cerebellum (Figs. 4 and 5). There was some difference between the two groups of mice. In those inoculated ic, specific

fluorescence was considerably more widespread than in those of the other group, and in the cerebellum of both, viral antigen was most concentrated in neurons of the purkinje layer, as compared to neurons of the molecular or granular layers.

Finally, despite the facility with which virus could be reisolated from the brains of chicks which died later than 30 hours after infection with minimal amounts of virus, specific fluorescence did not develop in the brains of these animals (Table 5).

Histological Lesions in Brains

No lesions were detected in the brains of chicks inoculated ic or sc. In mice inoculated ic, neutrophils were present and scattered throughout the cerebrum, while in those inoculated sc, there was extensive neuronal degeneration and fragmentation with perivascular cuffing by mononuclear cells.

Correlation Between Neutralization Tests and FAT

Satisfactory correlation was observed between neutralization tests and the FAT for confirmation of WEE infection in laboratory hosts which died in transmission experiments (Table 6). The previous experiments (See Materials and Methods, Expts. 1, 2 and 3) had indicated

that chick brains could not be employed for immunofluorescence diagnosis. Accordingly, mouse brains were used throughout for this purpose, after ic passage of the host tissue listed in table 6.

DISCUSSION

Published information on the response of the chick to infection with WEE virus cannot be considered applicable to all strains and passage levels of the virus. Several investigators (4,7,8,11,17,21) have shown that there are differences in antigenic and pathologic properties between freshly isolated and laboratory passaged strains. For this reason it was necessary to select and characterize a suitable strain of virus before transmission studies began. Strain 1540-1544 was selected because it was a recent isolate from a naturally occurring case of the disease and it had not yet undergone a large number of laboratory passages. Moreover, it was known from other studies to exhibit satisfactory pathogenicity for ten-day old chick embryos and a number of cell cultures, in addition to mice and young chicks. The first step towards characterization involved the preparation of adequate stocks of virus at a fixed passage level and the method employed was that usually practised for vaccine manufacture. This involved the preparation of primary seed stock from

which a large amount of secondary seed was made. The investigation which followed established the value of wet chicks for detection and titration of strain 1540-1544 of WEE virus. For these purposes, chicks could be particularly useful in instances where minimal amounts of virus are present in mosquito samples. Information on viremia patterns should allow for infection of mosquitoes at known periods of time after chick inoculation.

The other aspect of the investigation requires closer scrutiny since immunofluorescence methods have found limited use in the diagnosis of WEE or in research on the disease (14,19). This is surprising in view of the extensive studies which have been performed on the biology of the virus in nature. It is conceivable that in such studies the use of immunofluorescence might reduce the cost and time required for virus identification which is normally achieved by more elaborate serologic tests (3,13,15). Although more closely related to the requirements of transmission studies, the results of this investigation extend earlier findings on the suitability of Carnoy's fixation and paraffin embedding for identification of WEE virus (1,6). An important consideration in adapting a technique for diagnostic application, is that it should be relatively simple to perform. If it employs materials

which are readily obtained, this in an added advantage. These are the reasons emphasis was placed on absorption procedures of antisera prepared with relatively crude virus suspensions, rather than on techniques for antigen purification. Similarly, the use of Evans blue as a counterstain provided a simple and effective means of helping to remove unwanted fluorescence. Provided adequate precautions are taken to ensure specificity of staining, the method is reliable and interpretation is simple.

Despite the inability to detect, by the FAT, viral antigen in the brain of chicks inoculated sc, fairly large quantities of virus can be recovered from suspensions of the same tissue. No doubt, this represents virus in transit through the brain at time of death, which occurred from causes other than encephalitis. This view is supported in part by the observation that death occurs close to the peak of viremia, by the relative absence of neurologic signs and of histopathological lesions in the brains of these animals. In addition, an early report (22) indicated that the main pathologic change which occurred after infection of "young chicks" with the "virus of equine encephalomyelitis" was extensive myocardial degeneration with accompanying aneurysm-like ballooning of the heart. The early detection of viral antigen in meninges, choroid

plexus and ependyma of chicks inoculated ic, is in accord with the report by Johnson and Mims (10) regarding the spread of viruses inoculated by the intracerebral route.

Two important considerations arise in examining the results of mice inoculated ic. Firstly, there was wide distribution of viral antigen as revealed by the FAT, and an absence of degenerative lesions in these brains. These observations suggest that the clinical signs referrable to neurologic disturbance as displayed by these animals, arise from an initial disturbance of cellular function, not associated with obvious alteration of structure. In other words, cells which are morphologically normal under light microscopy, are infected. Perhaps the extent of functional disturbance of these cells determines whether or not death occurs at an acute phase of infection. The second consideration concerns the polymorphonuclear cell reaction in the brain. This type of reaction is commonly observed in man and animals dying at an acute phase of infection from eastern equine encephalitis (2,9, 16). The reason it does not appear to have been reported for WEE may be due to the fact that in natural infection with these viruses, the latter usually has a more protracted clinical course.

In contrast, mice died after a longer period of

infection following subcutaneous inoculation of WEE virus. This more closely resembled natural infection, and in accord with the usual findings (15,18) their brains displayed degenerative and inflammatory lesions. Viral antigen was not as widely distributed as in the brains of those inoculated intracerebrally. It would appear therefore, that after subcutaneous inoculation, viral infection of brain cells is a slower process allowing time for other conditions to develop (20,23) which give rise to cellular destruction, loss of function and death.

Although this investigation was primarily concerned with diagnostic applications of the FAT, the histopathological findings have been included and discussed at some length. This is because they assist in demonstrating differences between the two experimental hosts. On this basis a reasonable diagnostic interpretation of results, when using the FAT, can be made. The findings are related to transmission studies with WEE virus, strain 1540-1544 as follows. The FAT is useless as a diagnostic tool when applied to the brains of chicks which die after infection by transmitting mosquitoes. However, if the same or other tissue suspension is passaged to 3-week old mice intracerebrally, the technique becomes the method of choice for identification of the virus.

TABLE 1

STAINING PROTOCOL

Protocol Stage	Tissue (Smear or Section)	Treatment		Purpose for each stage of protocol
		Step 1	Step 2	
1	Infected	PBS	PBS	Autofluorescence control.
2	Infected	PBS	Evans blue in PBS	Suppression of autofluorescence.
3	Infected	Antiserum in PBS	FA conjugate in PBS	Establish extent of total (specific and non-specific) fluorescence.
4	Infected	Antiserum in PBS	FA conjugate in Evans blue	Suppression of portion of non-specific fluorescence.
5	Infected	Antiserum absorbed with normal tissue powder and diluted in PBS.	FA conjugate in Evans blue	Suppression of all components of non-specific fluorescence; remaining fluorescence largely or entirely specific.

TEST PREPARATION

Table 1 (Continued)

Protocol Stage	Tissue (Smear or Section)	Treatment		Purpose for each stage of protocol
		Step 1	Step 2	
6	Infected	Antiserum absorbed with normal tissue powder and diluted in PBS. Further absorbed with 10% infected tissue just before use.	FA conjugate in Evans blue	Suppression of all fluorescence. INHIBITION CONTROL
7	Infected	Normal rabbit serum in PBS	FA conjugate in Evans blue	No fluorescence. NORMAL SERUM CONTROL
8	Normal	Antiserum absorbed with normal tissue powder and diluted in PBS.	FA conjugate in Evans blue	No fluorescence. NORMAL TISSUE CONTROL

TABLE 2

CHICKS SHOWING NEUROLOGIC SIGNS TO WEE INFECTION*

Virus Dose		No. Showing Neurologic Signs*/ No. Inoculated
0.1	Mouse LD_{50}	1/50
0.01	Mouse LD_{50}	5/50
0.001	Mouse LD_{50}	5/50

* All Chicks developed neurologic signs between 96-120 hours after infection.

TABLE 3

COMPARISON OF WEE LD_{50} TITERS IN CHICKS AND MICE

Animal	No. of Titrations	Range Log_{10} LD_{50} Titer	Mean Log_{10} LD_{50} Titer[1]
Chicks	16	8.0-9.4	8.7
Mice	16	5.2-6.3	6.1

[1] Significant at the 0.5% level. $F_{0.005}$ >9.18 at 1 and 30 degrees of freedom.

TABLE 4

DEMONSTRATION OF WEE VIRUS IN CHICKS AND MOUSE BRAIN BY IMMUNOFLUORESCENCE.

EFFECT OF ROUTE OF INOCULATION.

Summary of Results: No. Positive/No. Examined

Route of Inoculation	Chicks		Mice
	Specific Fluorescence in Neurons and Ground Substance	Specific Fluorescence in Meninges and Choroid Plexus	Specific Fluorescence in Neurons and Ground Substance
Subcutaneous	0/24	5/24	15/24
Intracerebral	1/24*	21/24	24/24

* All samples showed a few scattered fluorescent cells, but one only showed a substantial number to be significant for diagnostic purposes.

TABLE 5

INABILITY TO DETECT WEE VIRUS BY IMMUNOFLUORESCENCE IN BRAINS OF CHICKS DYING LATER THAN 24 HOURS AFTER SUBCUTANEOUS INOCULATION

	Time of Death in Hours		
	24 - 48	49 - 96	97 - 144
No. Positive/ No. Examined	0/10	0/10	0/10*

* Virus could not be reisolated from 10% suspensions of the brains of two of these chicks when inoculated intracerebrally in weanling mice. Both chicks died at 138 hours after inoculation.

TABLE 6

IDENTIFICATION OF WEE VIRUS IN HOST TISSUE FROM TRANSMISSION EXPERIMENTS. COMPARISON BETWEEN NEUTRALIZATION TESTS AND IMMUNOFLUORESCENCE

Transmission Experiment and Host Tissue	Neutralization Index	Result of FAT on Mouse Brain
Series I Immune Serum 270/1		
Controls		
Secondary Seed Virus	2.4	Positive
Normal Mouse Brain	-	Negative
Infected Mouse Brain and Normal Serum	-	Negative
Transmission Samples		
Culex tarsalis, Expt. 1 Chick Blood 3	1.7	Positive
Culex tarsalis, Expt. 8 Chick Liver 102	2.2	Positive
Culex tarsalis, Expt. 11 Chick Blood 24, 1st passage	2.9	Positive
Culex tarsalis, Expt. 13 Chick Brain 1	2.8	Positive
Culex tarsalis, Expt. 13 Chick Brain 2	2.3	Positive
Culex tarsalis, Expt. 19 Chick Liver 2	2.7	Positive
Series II Immune Serum 270/2		
Controls		
Secondary Seed Virus	2.7	Positive
Normal Mouse Brain	-	Negative

Table 6 (Continued)

Transmission Experiment and Host Tissue		Neutralization Index	Result of FAT on Mouse Brain
Infected Mouse Brain and Normal Serum		-	Negative
Transmission Samples			
Culex tarsalis, Expt. 17	Chick Liver 119	3.0	Positive
Culex tarsalis, Expt. 17	Chick Liver 122	2.8	Positive
Culex tarsalis, Expt. 17	Chick Liver 129	3.3	Positive
Culex tarsalis, Expt. 17	Chick Liver 149	>2.8	Positive
Aedes dorsalis, Expt. 17	*Gerbil Heart 1	2.5	Positive
Series III Immune Serum 469			
Controls			
Secondary Seed Virus		4.2	Positive
Normal Mouse Brain		-	Negative
Infected Mouse Brain and Normal Serum		-	Negative
Transmission Samples			
Aedes campestris, Expt. 27	Chick Liver 3	<1.0	Negative
A. fitchii, Expt. 34	Chick Liver 3	4.0	Positive
Culex tarsalis, Expt. 17	Chick Liver 154	>3.0	Positive
Culex tarsalis, Expt. 18	Chick Liver 213	4.0	Positive
Culex tarsalis, Expt. 18	Chick Liver 215	4.4	Positive
Culex tarsalis, Expt. 18	Chick Liver 226	>2.2	Positive

Table 6 (Continued)

Transmission Experiment and Host Tissue	Neutralization Index	Result of FAT on Mouse Brain
Culex tarsalis, Expt. 18 Chick Liver 233	3.8	Positive
Culex tarsalis, Expt. 18 Chick Liver 240	3.7	Positive
Culex tarsalis, Expt. 18 Chick Liver 245	3.8	Positive
Culiseta inornata, Expt. 45 *Gerbil Heart 3	>3.0	Positive
Culiseta inornata, Expt. 45 *Gerbil Heart 4	4.7	Positive

* These gerbils acquired infection by the ingestion of infected mosquitoes.

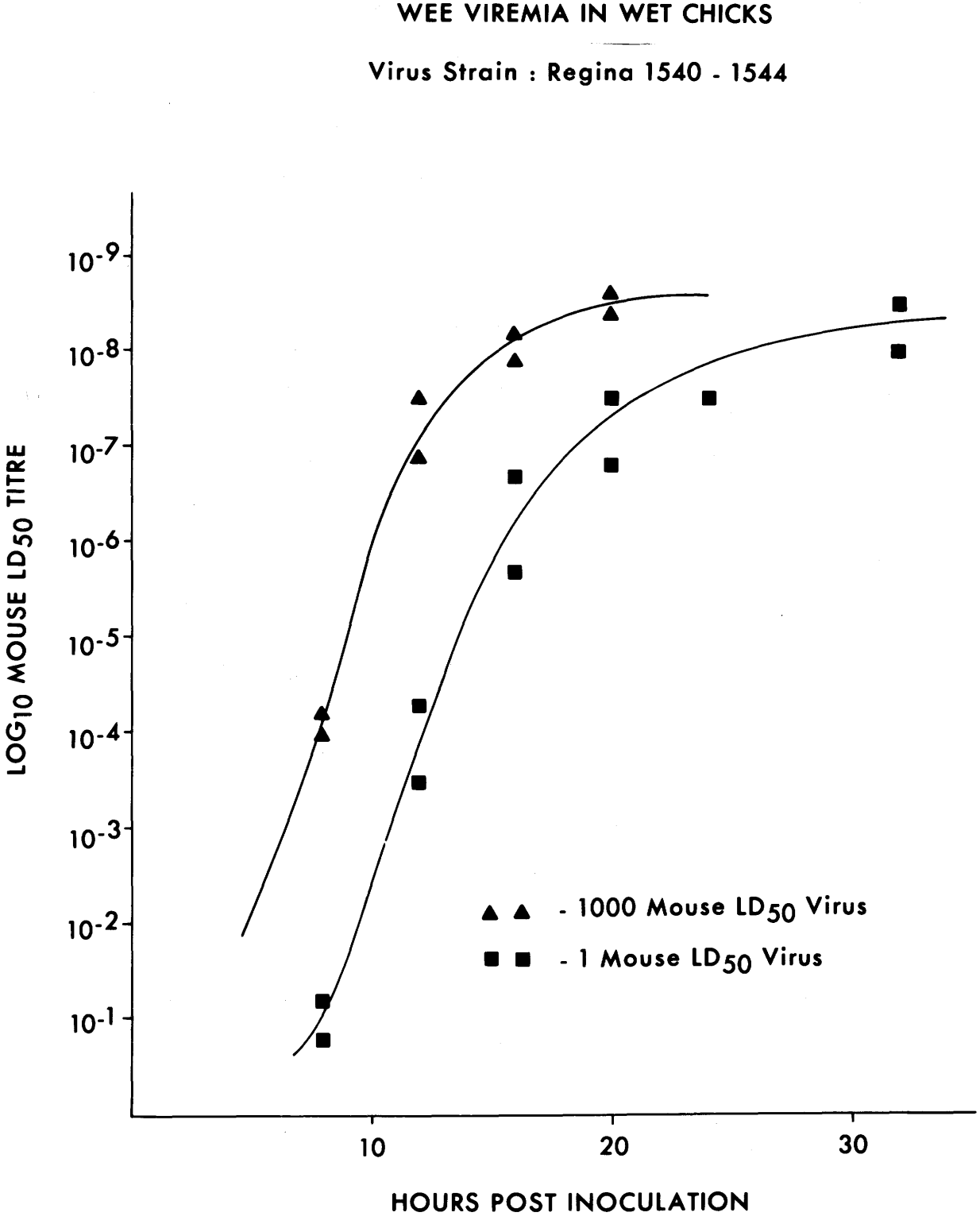

WEE VIREMIA IN WET CHICKS
Virus Strain : Regina 1540 - 1544
LOG10 MOUSE LD50 TITRE
10-9
10-8
10-7
10-6
10-5
10-4
10-3
10-2
10-1
10
20
30
HOURS POST INOCULATION
▲ ▲ - 1000 Mouse LD50 Virus
■ ■ - 1 Mouse LD50 Virus

Figure 1

Figure 2

Figure 2 Indirect fluorescent antibody stained sections of brain from chicks inoculated subcutaneously with WEE virus.

(a) Total non-specific fluorescence in choroid plexus. (Staining protocol, stage 3, Table 1).

(b) Partial suppression of non-specific fluorescence in choroid plexus by counterstain (Protocol 4).

(c) Complete suppression of non-specific fluorescence in choroid plexus by counterstain and tissue absorption of WEE antiserum. (Protocol 5). Shows absence of specific fluorescence.

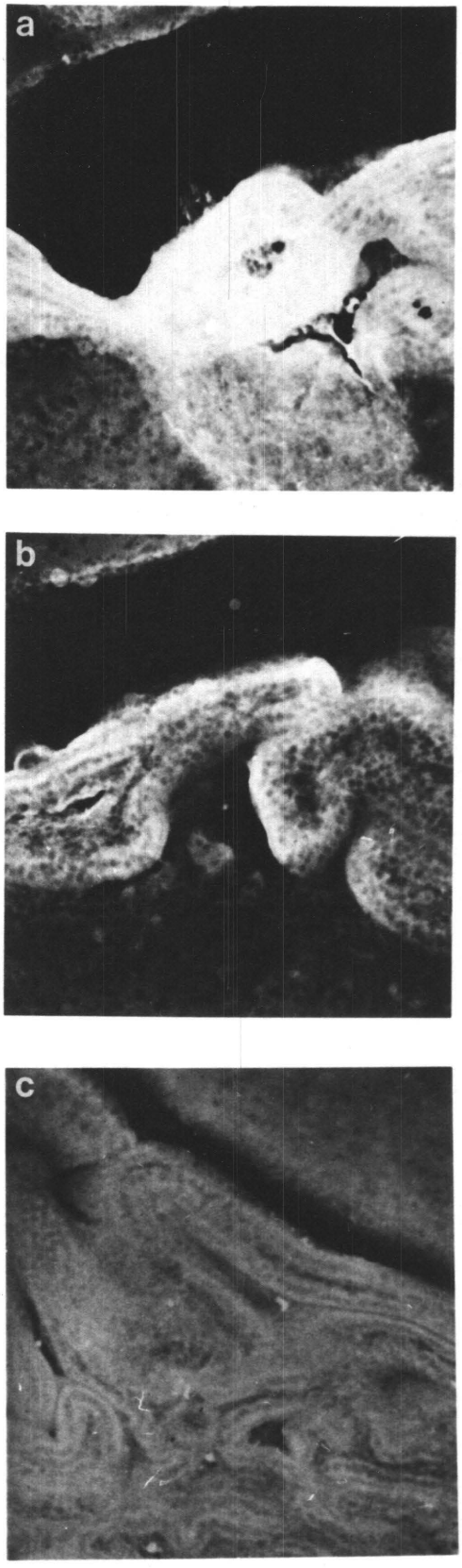


Figure 2

Figure 3

Figure 3 Indirect fluorescent antibody stained sections of brain from chicks inoculated intracerebrally with WEE virus.

(a) and (b) Specific fluorescence (Protocol 5), in choroid plexus.

(c) Specific fluorescence (Protocol 5) in meninges. Note absence of specific fluorescence in neurons and ground substance of cerebellum.

(d) Inhibition control (Protocol 6) in choroid plexus.

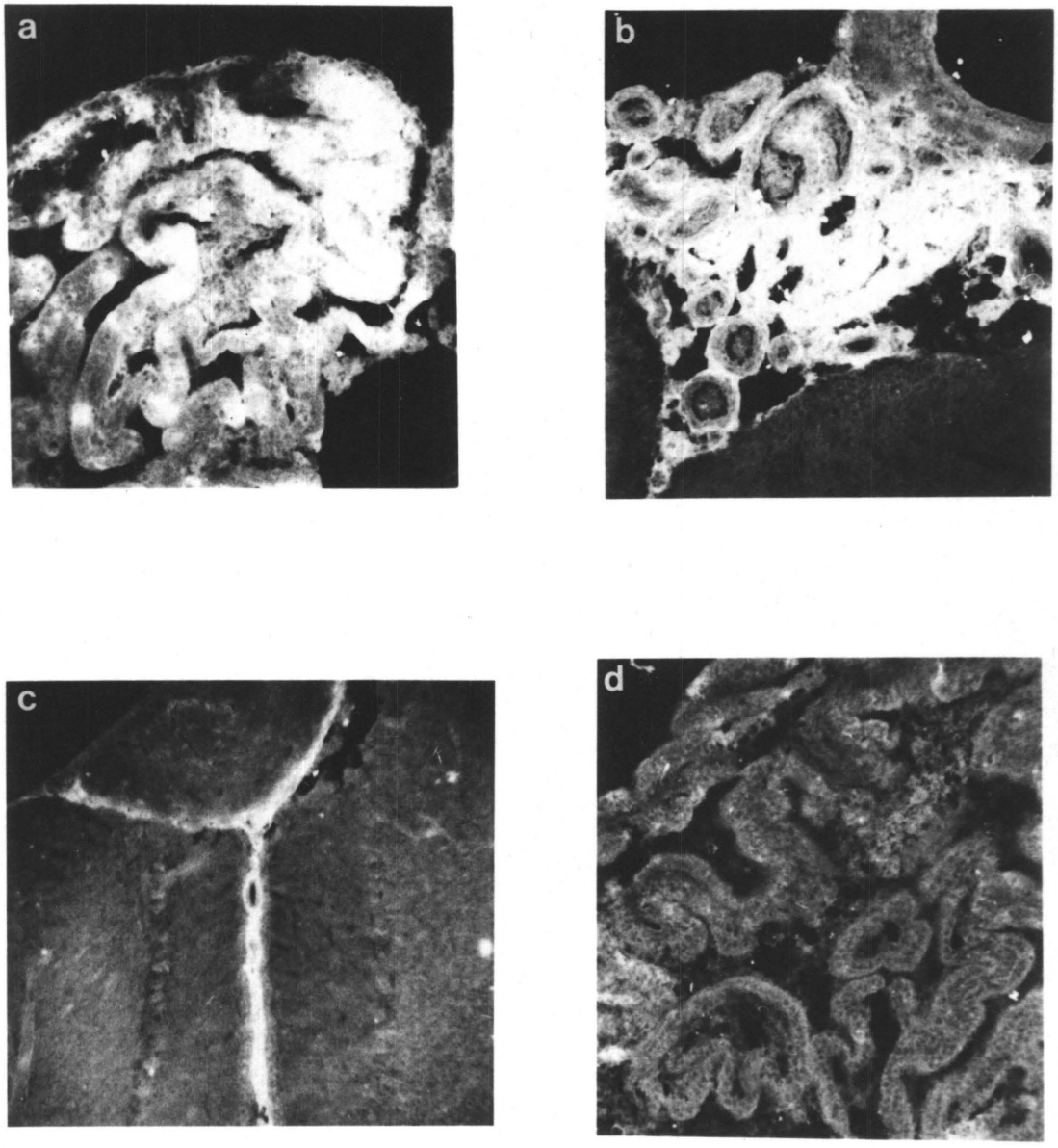


Figure 3

Figure 4

Figure 4 Indirect fluorescent antibody stained sections of mouse brain infected with WEE virus.

(a) Specific fluorescence (Protocol 5) in brain stem of mouse inoculated intracerebrally.

(b) Specific fluorescence (Protocol 5) in brain stem of mouse inoculated subcutaneously.

(c) Inhibition control (Protocol 6) in brain stem of mouse inoculated intracerebrally.

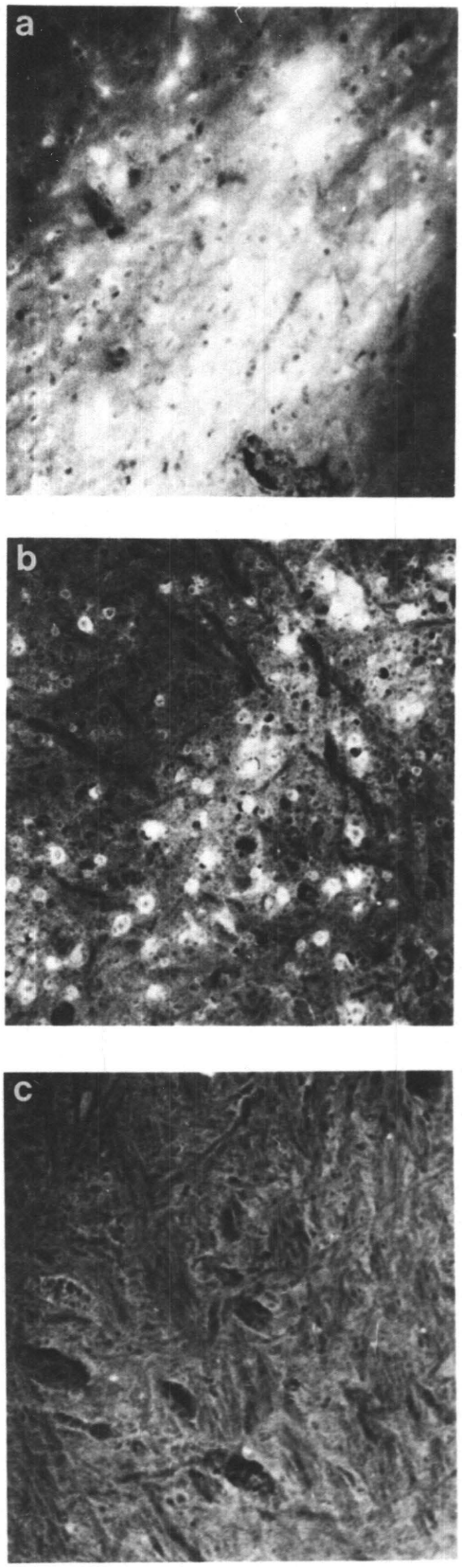


Figure 4

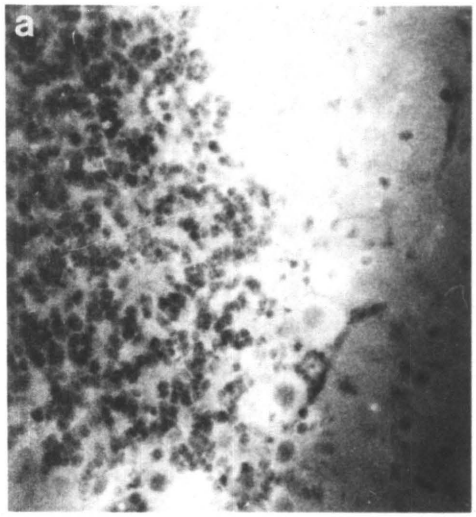
Figure 5

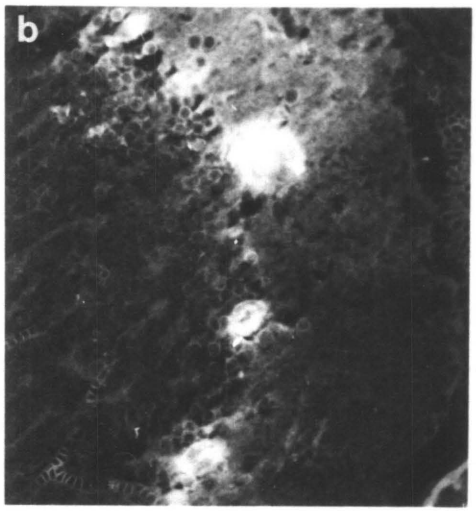
Figure 5 Indirect fluorescent antibody stained sections of mouse brain infected with WEE virus.

(a) Specific fluorescence in cerebellum of mouse inoculated intracerebrally.

(b) Specific fluorescence in cerebellum of mouse inoculated subcutaneously.

(c) Inhibition control in cerebellum of mouse inoculated intracerebrally.





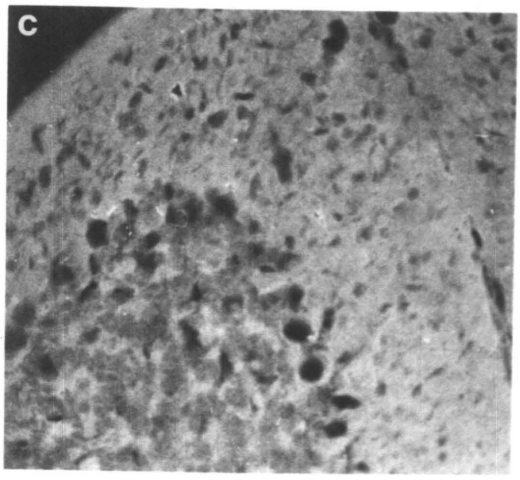


Figure 5

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CHAPTER 4

LABORATORY STUDIES ON THE TRANSMISSION OF WESTERN EQUINE ENCEPHALITIS VIRUS BY SASKATCHEWAN MOSQUITOES

I. *Culex tarsalis*

SUMMARY

A Saskatchewan strain of the mosquito Culex tarsalis, transmitted a local isolate of western equine encephalitis virus from one susceptible vertebrate to another, between four and 44 days after an infective blood meal. At incubation temperatures of 69 and 75°F, 120 transmissions occurred out of a possible 141. All but seven of these were by single infected mosquitoes. Measurements of virus titers in individual mosquitoes showed that viral growth at 75°F was more uniform than at 69°F, but infection rates were comparable at both temperatures. Another effect of differences in temperature was reflected in transmission. After prolonged incubation, C. tarsalis at 69°F transmitted less efficiently than those incubated at the higher temperature. It was deduced from these observations that transmission would not occur where virus levels in whole mosquitoes were below a certain minimum. However, variations in titer above this level had no effect on the mosquitoes' ability to transmit. The minimum concentration of virus required to infect 50% of C. tarsalis was $10^{2.5}$ intracerebral 3-week old mouse LD_{50} per 0.03 ml of donor blood.

Based on the laboratory findings, C. tarsalis is now a confirmed vector of western equine encephalitis virus in Saskatchewan. When ecologic and epidemiologic

findings are considered as well, this mosquito fulfills the requirements for performing a dual role as endemic and epidemic vector of the virus in this province. The laboratory study provides substantial evidence that it could be highly efficient in performing either of these functions.

INTRODUCTION

Western equine encephalitis (WEE) was recognized in Saskatchewan in 1935 (10). The first isolation of the virus from mosquitoes of the province was made in 1962 (20), and since that time, epidemiological studies have provided much information on the local vertebrate and mosquito hosts (2,4,5,22). Nine species of Saskatchewan mosquitoes have been found infected in nature (16, A.N. Burton and J. McLintock unpublished information) and detailed studies on the complex relationships between geographic and seasonal distribution of these hosts, weather conditions, and the occurrence of epidemics, have been undertaken (14-17). Attempts were made to determine whether the known mosquito hosts can transmit the virus from one susceptible vertebrate to another. This is the first report of a series of studies relating to this aspect of the investigations on WEE in Saskatchewan. Apart from attempting to demonstrate the ability of Culex tarsalis to transmit the virus, these studies were also

designed to assess its transmission efficiency. Accordingly, measurements of extrinsic incubation periods, infection thresholds, infection and transmission rates, were included.

MATERIALS AND METHODS

Virus

The WEE virus used in the study was strain 1540-1544 Regina 66. Details of its isolation, passage history and preparation of virus stocks are given elsewhere (11). The material used in transmission experiments was from a stock of 20% chick embryo allantoic fluid. It contained $10^{6.0}$ intracerebral 3-week old mouse LD_{50} per 0.03 ml (ic mouse LD_{50}). Diluent for all virus-containing material consisted of beef infusion broth, pH 7.4, with 0.75% bovine albumin fraction V. It contained 500 units of penicillin and 2.5 mg of streptomycin per ml.

Vertebrate Hosts, Mosquitoes and Environmental Conditions

Chicks 12 to 15 hours old were used as experimental hosts. They were held in covered disposable cardboard cages in a room maintained at 80-85^{o}F.

All but two of the experiments were performed with mosquitoes from a laboratory colony which was established from wild adults caught in Saskatoon in 1969. Mosquitoes

were reared throughout all developmental stages at 75°F, relative humidity 75-80% and a photoperiod of 16 hours provided by incandescent lighting. Larval diet consisted of powdered baker's yeast and finely ground commercial guinea pig feed in Bates' medium S (18). Adults were maintained on 25% sucrose solution. Experiment 1, table 1, and experiment 1, table 3 were performed with mosquitoes from a colony which had been established for several years.

Experimental and Virologic Procedures

For mosquito infection, chicks were inoculated subcutaneously (sc) with 100 to 1000 ic mouse LD_{50} doses of virus. Preliminary investigations had shown that the majority of mosquitoes would feed on chicks between 8:00 and 11:00 p.m. This period of time included an hour when the rearing room lights automatically dimmed to simulate a period of dusk. Each chick was therefore inoculated at about 1:00 a.m. on the day that mosquitoes were to be infected, so that the time of maximum viremia would coincide with that during which the majority of mosquitoes would feed. At about 5:00 p.m. on the same day, 0.1 ml of blood was obtained from the chick by cardiac puncture. It was immediately added to 0.9 ml of diluent containing 20 USP units of heparin per ml, shaken and placed at -70°C for determination of donor titer at a suitable time. The

infected chick was then exposed to a group of mosquitoes which had been deprived of sugar for at least 48 hours. On the following morning, all blood-fed mosquitoes were removed from the cage. This constituted an experimental lot which was provided with sugar and an oviposition dish. Depending on the number of blood-fed mosquitoes, each lot was used for one or more of the experimental procedures for determination of transmission efficiency.

At intervals after infection, groups of mosquitoes were removed from an experimental lot and examined individually for evidence of infection. The methods for preparation of syspensions and determination of infection are given below. The percentages of individual mosquitoes of different groups found to contain virus, constituted the infection rates.

Transmission experiments were performed at intervals, with mosquitoes from an experimental lot. In these experiments, infected mosquitoes were allowed to feed on normal chicks. Where measurements of transmission rates were required, individual mosquitoes were used and in other instances, groups of up to ten per chick were employed. Exposure of the chick was allowed for up to 15 hours (overnight) and following transmission feedings, the mosquitoes were killed by freezing and ground in diluent at a ratio

of 0.5 ml diluent to one mosquito. The suspension was centrifuged and the supernatant used for determination of mosquito infection by sc inoculation of 0.05 ml into normal chicks. Chicks were observed for five days, for evidence of WEE infection. Each suspension was examined for bacterial contamination by inoculating an aliquot into two tubes of thioglycollate broth and incubating these at 37^{o}C for two days.

The chicks upon which infected mosquitoes had fed were held singly. Those which survived for more than five days were regarded as not having been infected and were discarded. Where death occurred within this period of observation, liver or brain was removed aseptically and a 20% suspension prepared. The suspension was used to provide evidence of infection in the chick by the same method used for mosquitoes. Confirmation was achieved by ic passage of material to 3-week old mice from which brain sections served for immunofluorescence diagnosis (11). In this manner, WEE infection was confirmed in all chicks which showed evidence of infection following transmission feedings during the first ten days of each experiment. This procedure was adopted in order to provide firm evidence for extrinsic incubation. Immunofluorescence diagnosis was subsequently used in about 10% of cases as

a constant monitor of chick inoculation for proof of transmission. The percentages of chicks which became infected after they were bitten by single infected mosquitoes of the various groups, constituted the transmission rates.

Mosquito infection thresholds were determined by allowing mosquitoes to feed on chicks circulating virus at different levels. Following ten to 12 days of incubation at 69°F, infection rates were determined. The concentration of virus in donor blood, which was required to infect 1-5% and 50% of mosquitoes (ie infection thresholds) were determined by probability plots of donor viremia titers against infection rates. On the basis that C. tarsalis ingests 0.003 ml of blood, the amounts of virus (ic mouse LD_{50}) required to cause infection at the above threshold levels were calculated. Except for infection thresholds, all experimental procedures related to transmission were performed with mosquitoes incubated at 69 and 75°F.

Virus titrations were performed by intracerebral inoculation of 3-week old mice with 0.03 ml of ten-fold serial dilutions of mosquito or tissue suspensions. Mice were observed for 12 days and titers calculated by the method of Reed and Muench. To calculate the number of ic mouse LD_{50} per mosquito, ie per 0.5 ml, the virus concentration per 0.03 ml as indicated by the titer, was multiplied

by 16.6 which is approximately $10^{1.2}$. Consequently, the lowest level of virus that could be measured in mice was $10^{1.2}$ LD_{50}. Any suspension which failed to kill 50% of mice at the 1 in 10 dilution was subsequently titrated in chicks. Those which failed to kill chicks at the undiluted level were regarded as containing no virus. Those which contained less than $10^{2.5}$ sc chick LD_{50} per 0.03 ml (1 ic mouse $LD_{50} \equiv 10^{2.5}$ sc chick LD_{50}) (11) were regarded as containing less than 10 ic mouse LD_{50} per mosquito and are represented as such in the tables and figures.

RESULTS

Mosquito Infection

Tables 1 and 2 show the amounts of virus recovered from infected mosquitoes at intervals during incubation at 75 and 69°F. Individual mosquitoes were examined in all experiments except experiment 1 (Table 1) where pools of five were employed. Inspection of figure 1 indicates that at 75°F, there was a sharp decline in virus content during the first two to three days of infection, followed by a slight rise, then a slow general decline over the total period of incubation. After the initial decline at 69°F, titers were less uniform than at 75°F (Fig. 2). Differences in temperature had no marked effect on infection rates. They remained at 100% or close to this level throughout.

Transmission

C. tarsalis transmitted on 120 occasions out of 141 possible transmissions. This occurred throughout a period lasting from four to 44 days after infection, and in all but seven instances these were effected by individual mosquitoes.

The extrinsic incubation period which is the interval between mosquito infection and ability to transmit, was four days at both 75 and 69°F. No mosquito would take blood, subsequent to the infective meal, before this time. At this time also, virus levels in individual mosquitoes, had, to a large extent passed the stage of initial decline. It is therefore reasonable to deduce that transmissions which occurred at, and later than, four days were biological rather than mechanical in type.

Table 3 shows transmission rates at weekly intervals during incubation at 75°F. Taking variations in experimental lots into account, there is a two-fold increase in rates from close to 50% in the first week (days four to six) to 100% or close to this level in the third. Comparison with table 4 which shows the rates at 69°F, suggests that at this temperature transmission was less efficient during the later stages of incubation. It should be

observed from the table however, that the mosquitoes fed comparatively poorly at 69°F and only one experimental lot provided a measure of transmission rates.

The variation in virus content of whole mosquitoes throughout the period of incubation (Figs. 1 and 2), suggests at first glance that virus level is unrelated to ability to transmit, once the extrinsic incubation period has passed. Closer inspection, particularly of figure 2, indicates that the lack of such a relationship is true only where variations in titers occur above a minimum level. In other words, some infected mosquitoes do not transmit because the level of infection is too low. When such mosquitoes are examined qualitatively for evidence of infection, the result is positive, provided a highly sensitive detection system such as the chick, is employed. Consequently, the infection rates at 69°F were comparable throughout to those at the higher incubation temperature but transmission rates tended to be lower with continuing incubation. Due to the more rapid stabilization of virus levels at 75°F, fewer mosquitoes exhibited this low level of infection, so that at this temperature, almost all infected mosquitoes eventually transmitted. The situation did not arise where virus could not be recovered from a mosquito which transmitted. This fact

also lends support to the concept of a minimum mosquito titer below which, transmission will not occur.

Infection Threshold

A virus concentration of about $10^{1.0}$ ic mouse LD_{50} per 0.03 ml of blood, was found as the requirement to infect 1-5% of C. tarsalis. This corresponds to 1.0 LD_{50} total virus content of each blood meal. The 50% infection level was $10^{2.5}$ or 31.6 LD_{50}, in the same order as above. Table 5 shows the donor titers and corresponding infection rates in two experiments for determination of infection thresholds.

DISCUSSION

Numerous field and laboratory studies have revealed the role of C. tarsalis as the primary vector of WEE in the western United States (7-9,13). Although there is abundant epidemiological evidence which suggests the same role for this mosquito on the prairies of western Canada (14,16,21), it is important to recognize that the same species of arthropod may effectively function as a vector in one locality and be unimportant in another. This and other complex factors in the epidemiology of the arboviruses have been recognized by the World Health Organization

Study Group on Arboviruses. The group has set out certain criteria which must be fulfilled before an arthropod can be considered as a confirmed vector (1). They may be summarized as follows. The virus must be recovered from wild caught specimens of the arthropod. There must be a significant association of the infected arthropod with the appropriate vertebrate population in which the infection is occurring. Thirdly, the ability of the arthropod to become infected by feeding on a viremic vertebrate host, or on an artificial substitute, and its ability to transmit the virus biologically by bite, must be demonstrated.

On the prairies of western Canada, and particularly in Saskatchewan, WEE virus has been isolated from C. tarsalis on several occasions (14,16,20,21). Accordingly, proof of the ability of this mosquito to transmit the virus, assumes considerable importance. The present report provides this proof. It is pertinent to examine, firstly, the basis on which the third criterion as outlined above, is considered fulfilled by the results of the study reported here. A second consideration concerns the nature and extent of the ecological and epidemiological evidence by which C. tarsalis fulfills the other criteria. By relating field and laboratory

studies in this manner, not only can an assessment be made of this mosquito as a confirmed vector, but its role in the spread of WEE in western Canada can be more fully appreciated.

Several findings in this investigation demonstrate the effectiveness of C. tarsalis of Saskatchewan to transmit a local strain of WEE virus. Among them are the frequency with which single infected mosquitoes transmitted, the low infection thresholds and the short extrinsic incubation period at 69 and 75°F. These temperatures are within the range at which mosquitoes are normally active in Saskatchewan (14,17) and are therefore particularly meaningful in this study. The extrinsic incubation period was as brief as four days. The fact that it coincides roughly with the intervals between blood meals, suggests that during the infective life of a mosquito, several transmissions can take place. The actual number of transmissions in nature would depend, among other things, on the longevity of the mosquito. Although this is not known, it has been demonstrated that under laboratory conditions at least, the survival of C. tarsalis is unaffected by infection with WEE virus (23). Further, the results of the present investigation confirm those of previous workers that C. tarsalis, having become infective, remains so for life (2,7,8,12,23).

The application of quantitative methods to laboratory transmission studies provide additional means for the assessment of vector efficiency. The measurement of virus content of mosquitoes at intervals after the infective blood meal, showed at 75°F the typical phases of mosquito infection; a decreasing, an increasing and a fairly stationary phase (6). The use of whole mosquitoes obscured the relationship between ability to transmit and virus levels in particular tissues. Nevertheless, the relationship between transmission and minimum titer which was observed, probably reflects virus levels in salivary glands. Thomas (23) who performed extensive investigations on the development of WEE virus in C. tarsalis, demonstrated that although there was no relationship between transmission rates and titers in whole mosquitoes, the former varied directly up to a certain level, with the concentration of virus in salivary glands. Further evidence for the efficiency with which C. tarsalis becomes infected and transmits WEE virus derives from examination of infection and transmission rates. After an infective blood meal of high titer, infection rates were maintained at or close to 100% for up to six weeks, which was the longest period of incubation. Very high transmission rates ranging from 82-92% were attained during the second week of incubation. This

occurred at both incubation temperatures and was maintained throughout at 75°F where the rates occasionally reached 100%. Similar rates were obtained by Barnett (2) and Chamberlain and Sudia (7).

Determination of infection threshold has in the past been made to assess the susceptibility of a particular mosquito species to infection. It has been vaguely defined as the minimum amount of virus which must be ingested to infect 1-5% of mosquitoes. It is a very important concept. It can be used to compare the susceptibilities of different species of mosquitoes or different strains of the same species to a particular strain of virus. Similarly, comparisons between different strains of virus can be made. On the basis of these comparisons, along with some knowledge of feeding patterns, the role of vertebrate hosts in infecting mosquitoes in nature can be assessed. Because of its importance, an attempt was made to define and to measure as precisely as possible, the infection threshold of C. tarsalis. It is defined as the minimum concentration of virus which must be ingested to infect 50% of mosquitoes under standard laboratory conditions, that is, incubation at 69°F for a period of ten to 12 days. Measurement is slightly more difficult than usual but it allows for more precise comparisons between species where this is required.

In addition, having determined the 50% infection level, the 1-5% level can also be obtained, so the original concept of Chamberlain et al. (8) remains. A level of $10^{2.5}$ (at 50%) was found for C. tarsalis in this study. This is low and is in accord with earlier findings (7,23). In this connection it may be observed that Thomas (23) also employed the 50% infection level for expression of infection threshold of C. tarsalis to WEE virus. However, his incubation periods varied considerably from one experimental group to another.

Finally, in connection with ecological and epidemiological findings, McLintock et al. (16) reported that between 1962 and 1965, 53% of WEE isolations from Saskatchewan mosquitoes were from C. tarsalis. This establishes the close association of this mosquito with natural sources of infection. Serologic and virus isolation studies have revealed that birds are the principal vertebrate hosts of WEE in the province during summer, but that snakes and frogs and several species of wild mammals are also involved (4,5,15,22). Investigations on the feeding habits of C. tarsalis have indicated that it feeds most readily on birds, is also attracted to man, horses, other mammals and even reptiles (9,13,19,24). On the basis of the above observations and on the results of the laboratory studies

TABLE 1

WEE VIRUS RECOVERY AND INFECTION RATES IN *CULEX TARSALIS* at 75°F

Days Incubation	Mouse intracerebral LD_{50} per Mosquito			Infection Rates			
	Experiment 1	Experiment 2	Experiment 3	Experiment 4		Experiment 5	
				No. Pos./ No. Tested	%	No. Pos./ No. Tested	%
1		<1.0,4.8,4.2	4.7,5.2			15/15	100
2	1.9						
3			2.5,2.2,3.7,1.9				
4		3.2,1.9,1.2		9/9	100		
5	3.0		2.2,2.5	8/8	100		
6		4.2,2.5	2.2,1.9	4/5	80	10/10	100
8-14	3.5	2.7,2.5,0, 2.9,2.6,2.5	2.2,2.5	31/33	94	15/15	100
15-21	3.7,4.0	1.9,1.9,2.2, 2.2	2.4,1.9,3.0,2.2, 0	29/32	91	9/10	90
22-28	3.1	0,2.9,1.9	1.9,<1.0				
29-35	3.5,1.2	1.2,<1.0,1.9	2.5,<1.0	19/20	95	10/10	100
36-42	2.7,2.5	2.4	2.2,2.4,1.9, <1.0	15/15	100	10/11	91

TABLE 2

WEE VIRUS RECOVERY AND INFECTION RATES IN CULEX TARSALIS AT 69°F

Days Incubation	Mouse Intracerebral LD_{50} per Mosquito		Infection Rates			
	Experiment 1	Experiment 2	Experiment 3		Experiment 4	
			No. Positive/ No. Tested	%	No. Positive/ No. Tested	%
1	3.2,3.9,4.9,4.5		14/14	100		
2	<1.0,1.9					
4	3.7,2.2,2.1,2.2				5/5	
5	2.2,1.9				5/5	
6	<1.0		10/10	100		
7	2.2,1.2					
8-14	2.5,3.0,2.8,2.7 2.2,0,3.6,4.7	3.0,3.2,0,4.2 2.2,4.5,3.6,3.2 <1.0,2.2,1.9	9/10	90	17/18	94
15-21	3.6,1.2,1.5,<1.0 4.9,<1.0	2.6,1.9,0,<1.0 3.2,2.2	16/16	100	33/34	97
22-28	2.6,<1.0,<1.0				11/11	100
29-35			13/13	100		
36-42			9/9	100		

TABLE 3

EXPERIMENTAL DESIGN AND RESULTS OF WEE TRANSMISSION TO WET CHICKS BY CULEX TARSALIS AT 75°F

	Experiment 2			Experiment 3		
LD_{50} Titer of Infective Blood Meal, per 0.03 ml.		$>10^{-6.8}$			$>10^{-7.2}$	
		Transmission Rates			Transmission Rates	
Days Incubation	No. Mosquitoes Fed/ No. Attempted Transmissions	No. Chicks Positive/ No. Mosquitoes Positive	% Trans-mission	No. Mosquitoes Fed/ No. Attempted Transmissions	No. Chicks Positive/ No. Mosquitoes Positive	% Trans-mission
3	0/20				Not Done	
4	1/20	0/1		6/58	2/6	Days 4
5		Not Done		6/50	5/6	to 6, 54
6	0/20			1/20	0/1	
8-14	11/40	9/11	82	12/88	11/12	92
15-21	9/20	9/9	100	17/40	14/16	88
22-28		Not Done			Not Done	
29-35	7/20	7/7	100	8/20	8/8	100
36-44		Not Done		8/42	8/8	100

In experiments 2 and 3, mosquitoes were fed to chicks individually. In experiment 1, the results of which are not included in the table, mosquitoes were fed in groups of 5 to each chick. Transmissions occurred on days 10, 15, 20, 25, 29, 34, and 39 post infection. Transmission failed on days 5 and 39. The latter was one of two groups.

TABLE 4

EXPERIMENTAL DESIGN AND RESULTS OF WEE TRANSMISSION TO WET CHICKS BY CULEX TARSALIS AT 69°F

	Experiment 1		Experiment 2		
LD_{50} Titer of Infective Blood Meal, per 0.03 ml.		$>10^{-7.0}$		$10^{-7.2}$	
					Transmission Rates
Days Incubation	No. Mosquitoes Fed/ No. Attempted Transmissions	No. Chicks Positive/ No. Mosquitoes Positive	No. Mosquitoes Fed/ No. Attempted Transmissions	No. Chicks Positive/ No. Mosquitoes Positive	% Transmission
3	0/5				
4	0/5		5/40	1/5	Days 4 to
5	0/10		5/40	5/5	7, 67
6	0/24		1/6	1/1	
7			1/13	1/1	
8-14	5/90	1/5	15/131	12/14	Weeks 2 to
15-21	4/20	3/4	8/78	8/8	3, 91
22-28			5/50	3/5	Weeks 4 to
29-35	0/20		6/26	5/6	5, 73

TABLE 5

WEE INFECTION THRESHOLD OF CULEX TARSALIS

Experiment No.	Infective Blood Meal: Log_{10} LD_{50} Mouse per 0.03 ml.	Infective Blood Meal: LD_{50} Ingested per mosquito[1]	Infection Rates: No. Positive/ No. Tested	Infection Rates: %	Infection Threshold: 1-5%	Infection Threshold: 50%
1	1.2	1.585	4/46	8.9		
	2.8	63	10/12	83.3		
	3.5	316	14/20	70	Mouse LD_{50} titer of $10^{-1.0}$ per 0.03 ml of blood, or Ingestion of 1.0 LD_{50} virus per mosquito.	Mouse LD_{50} titer of $10^{-2.5}$ per 0.03 ml of blood, or Ingestion of 31.6 LD_{50} virus per mosquito.
	8.5	3.16×10^7	23/24	96		
2	1.2	1.585	8/68	11.8		
	2.0	10	6/26	23.1		
	2.2	15.85	44/106	41.5		
	2.6	39.8	37/92	40.0		
	3.5	316	27/30	90		
	5.3	2.0×10^4	17/17	100		
	5.7	5.0×10^4	8/8	100		

[1] Based on ingestion of 0.003 ml of blood.

Figure 1

WEE VIRUS RECOVERY, INFECTION AND TRANSMISSION RATES IN CULEX TARSALIS AT 75°F

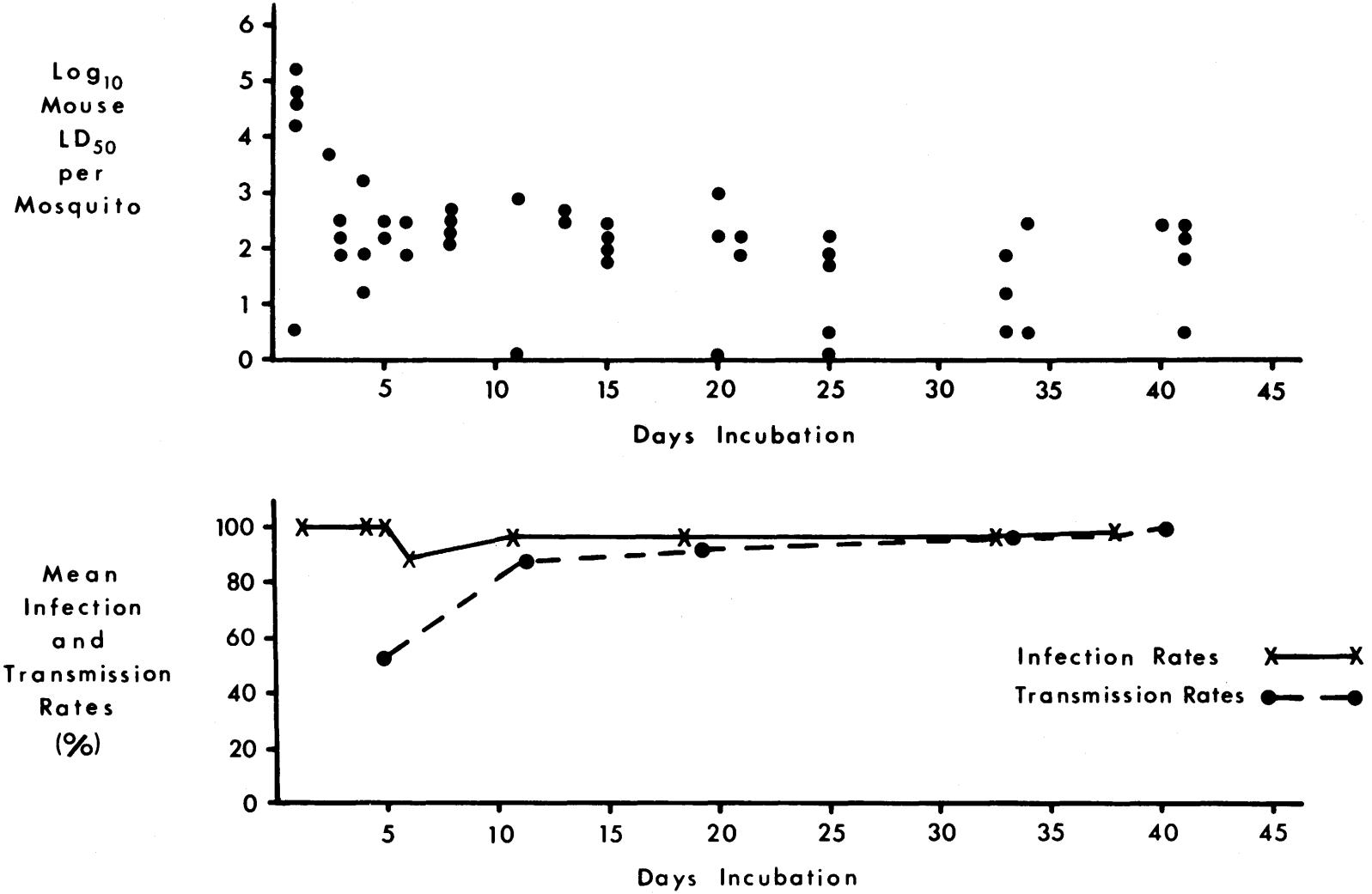
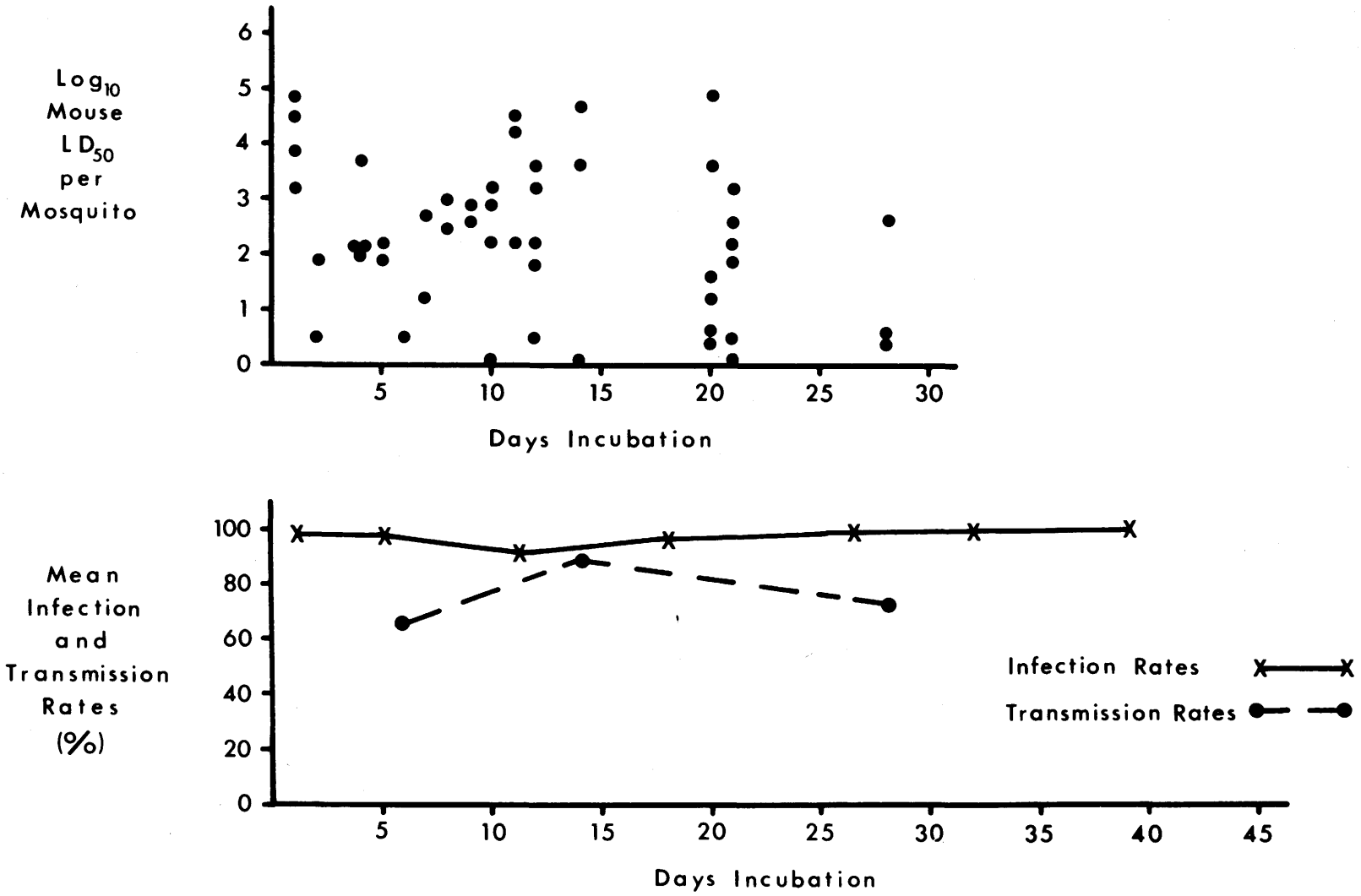


Figure 2

WEE VIRUS RECOVERY, INFECTION AND TRANSMISSION RATES IN CULEX TARSALIS AT 69°F



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CHAPTER 5

LABORATORY STUDIES ON THE TRANSMISSION OF WESTERN EQUINE ENCEPHALITIS VIRUS BY SASKATCHEWAN MOSQUITOES

II. *Culiseta inornata*, *Anopheles earlei*, *Aedes* mosquitoes

SUMMARY

A local isolate of western equine encephalitis virus was used in transmission studies with several species of Saskatchewan mosquitoes. Culiseta inornata supported viral growth for up to five weeks and maintained infection rates between 63 and 88% from the second week of infection onwards. Transmission trials involving close to 3,700 wild and laboratory reared females gave negative results. When the paucity of feedings by infected mosquitoes in the laboratory is considered together with the high infection rates found both in laboratory and field studies, C. inornata remains a highly suspected vector of western equine encephalitis in Canada.

While Aedes vexans and Anopheles earlei failed to support viral growth for 12 or more days after infection, Aedes campestris, A. flavescens and A. spencerii maintained infection for close to two weeks. The failure of these mosquitoes to transmit the infection in the laboratory must be considered in relation to poor adaptation to laboratory conditions which resulted in poor feedings and early deaths. A. fitchii transmitted the virus 9 days after infection. Although this mosquito has not yet been found infected in nature, it becomes a potential vector and may contribute to the spread of the disease in the early spring. It may be particularly important in

northern Saskatchewan where it is abundant, and where Culex tarsalis, a confirmed vector, is rare.

INTRODUCTION

Nine species of mosquitoes have been found infected with the virus of western equine encephalitis (WEE) in Saskatchewan (15, J. McLintock and A.N. Burton, unpublished information). From among these, Culex tarsalis has been shown to be highly efficient in transmitting the virus from one susceptible vertebrate host to another (10). This finding supported epidemiological evidence which suggested that C. tarsalis is the principal vector of WEE in the province. Also on the basis of ecological and epidemiological findings, the other known mosquito hosts were considered to be primarily concerned with maintaining the enzootic status of the disease. In an attempt to determine their role more precisely, laboratory transmission studies were conducted. Included in these studies were Culiseta inornata, Anopheles earlei, Aedes campestris, A. dorsalis, A. fitchii, A. flavescens, A. spencerii and A. vexans. The ability of each species to support the growth of a local isolate of WEE virus and to transmit the infection by bite, were the major areas of concern.

MATERIALS AND METHODS

Virus and Virologic Procedures

Details of virus history, preparation of virus stocks and of virologic procedures are contained in previous reports on laboratory transmission studies (9,10). A 20% suspension of WEE infected chick embryo allantoic fluid was used. It contained $10^{6.0}$ intracerebral 3-week old mouse LD_{50} (ic mouse LD_{50}) per 0.03 ml.

Mosquitoes and Environmental Conditions

Both laboratory reared and wild mosquitoes were used. Laboratory reared Culiseta inornata consisted of first generation adults obtained from colonies established with wild females caught in Saskatchewan in 1969. Colonies from mosquitoes caught in different areas (See table 3) were maintained separately. In addition to these, some adults were obtained from a colony established several years previously. All rearing procedures were carried out at $69^{\circ}F$, relative humidity 70-80% and a photoperiod of 16 hours. The detailed methodology for rearing C. inornata was that which was devised for this species by McLintock (13,17) and which has been successfully employed for several years.

Wild mosquitoes were collected by a permanent light trap which was situated close to Saskatoon and run nightly

throughout the mosquito season. Miscellaneous collections were made primarily in the Saskatoon area with a hand aspirator and with a CDC miniature battery-operated light trap (23). Dry ice was used as an attractant with the latter. After sorting and identification in the laboratory, the mosquitoes were held at 69°F, provided with blood and with oviposition dishes. Where eggs were not required, blood was replaced by a 25% sucrose solution. The eggs collected from wild aedines in 1969 were held at 69°F for three months, after which they were conditioned for hatching in the following spring. Conditioning consisted of exposure to 35°F for a further three months and a post cold temperature of 69°F for 24 hours before they were placed in hatching medium (12). Moist conditions were maintained throughout storage and hatching took place in a medium consisting of nutrient broth (Difco) dissolved in tap water at a concentration of one gram per liter. Subsequent rearing followed the procedure used for C. inornata. Due to limited incubator facilities for rearing several species at the same time, most of the transmission experiments with Aedes mosquitoes were performed with wild females.

Experimental Procedures

Mosquitoes were infected by allowing them to feed

on infected half-day old chicks during the period of maximum viremia which occurred between 20 and 24 hours after infection with 100 to 1000 LD_{50} doses of virus (9). Many refused to feed on the chick. For this reason, some were infected by feeding on a pledget saturated with infected blood which was prepared as follows. Several infected chicks were bled at about 20 hours after infection and the pooled sample added to a mixture of defibrinated bovine blood and 10% sucrose (3:1), at a ratio of one to nine. The bovine blood had been treated with 500 units of penicillin and 2.5 mg of streptomycin per ml and freedom from bacterial contamination ensured beforehand by inoculation of thyoglycollate broth. The infected blood was thoroughly stirred and two samples taken for titration. One was frozen immediately and the other allowed to remain in the mosquito incubator overnight. This was the period over which mosquitoes were allowed to engorge. Whenever possible, both samples were titrated at the same time so that a mean value of the virus content of the blood meal could be obtained. This value was always greater than $10^{6.0}$ ic mouse LD_{50} per 0.03 ml. Mosquitoes were deprived of sugar from 48 hours to several days before attempted blood feedings.

Transmission experiments were performed in the same

manner as those for Culex tarsalis (10), except that in addition to the use of normal chicks, suckling mice, weanling gerbils (Meriones unguiculatus), hamsters and guinea pigs were also used as experimental hosts. This was to encourage as many mosquitoes as possible to take a blood meal. Other forms of encouragement included the use of dry ice as attractant, starvation for different periods, and the inclusion of males in experimental lots. None of these maneuvers succeeded in inducing them to engorge in large numbers. Confirmation of WEE infection in hosts which were bitten by infected mosquitoes, or in those which were exposed to them and died without evidence of having provided a blood meal, was made by neutralization and immunofluorescence tests (9).

Infection thresholds were measured by allowing mosquitoes to feed on pledgets saturated with known dilutions of stock infected blood. For this purpose, infected stock was diluted in normal defibrinated bovine blood. The methods employed for preparation and titration of mosquito and tissue suspension, for calculation of titers and the 1-5% and 50% infection thresholds, are contained in the previous report on transmission studies (10).

RESULTS

Culiseta inornata

Infection. The amounts of virus recovered from infected C. inornata incubated at 75°F and 69°F are presented in tables 1 and 2. In table 2, titers listed for experiment 1 represent those of single mosquitoes which were infected by feeding on a chick. Titers in experiments 2 and 3 are each from a pool of two mosquitoes, all of which were infected from a pledget. In general, titers fell during the three to four days following infection. There was then a period of increase lasting for the next ten to 15 days, followed by a gradual decrease extending to the fifth week of incubation (Fig. 1). This general pattern was reflected in measurements of infection rates (Fig. 1, Table 2). Towards the end of the first week, the infection rate was about 50%. It would appear that in half of the mosquitoes, infection either failed to become established, or having done so, it was rapidly lost. It is also possible that toward the end of the first week, the amounts of virus in these mosquitoes fell to undetectable or barely detectable levels. Then, following viral multiplication over the next few days, virus was again fairly easily detected and the infection rate rose. However, the infection rate

did not reach 100%. It would seem therefore, that all three events suggested above, occur concurrently.

Transmission. Close to 3,700 C. inornata females were employed for transmission trials. This included both laboratory reared and wild individuals. Only 18% of these could be induced to take an infective blood meal, and 3% fed a second time. None of these mosquitoes transmitted WEE virus by bite over a period ranging from four to 20 days after infection. All the mosquitoes involved in transmission feedings were examined for virus, so that, the infection status of each was known. Tables 3 and 4 present the details of these transmission trials and it will be observed that two gerbils which were among those used as transmission hosts, died of WEE infection. It is uncertain whether they were bitten by one or more mosquitoes before the latter were ingested. Since none of the other hosts, including other gerbils, which were known to have been bitten by infected mosquitoes developed WEE infection, it is reasonable to conclude that these animals were infected orally.

Infection Thresholds. Table 5 gives details of the results obtained in measuring infection thresholds in C. inornata. A virus concentration of $10^{1.5}$ ic mouse LD_{50} per 0.03 ml

of blood, infected 1.5%. Based on the ingestion of 0.002 ml of blood from a pledget, this is equivalent to 3.16 LD_{50} virus in the mosquitoes' blood meal. At the 50% infection level, $10^{4.0}$ or 666 LD_{50} were the requirements in the same order as above. Infection rates were measured after ten to 12 days of incubation of 69°F.

Aedes mosquitoes and Anopheles earlei

Infection. A. campestris, A. fitchii, A. flavescens and A. spencerii, when held at 69°F, supported viral growth for ten to 16 days after infection (Tables 6 and 7). Under the same conditions, A. vexans and Anopheles earlei were found to be free of infection at ten and 12 days respectively after their infective blood meals. None of the A. fitchii examined at 20 days were infected (Table 7). A. dorsalis did not survive beyond the first week of infection but at that time there was the typical fall in titers exhibited by members of most of the other species listed in Table 7. Had they survived, it is possible that titers could have increased with continuing incubation.

Due to unwillingness to engorge in sufficiently large number, attempts to measure infection thresholds in Aedes campestris failed.

Transmission. The details of transmission trials are shown in tables 8 and 9. A. fitchii was the only species which transmitted WEE infection by bite. This occurred nine days after an infective blood meal from a chick. Under conditions similar to those occurring with C. inornata, a gerbil died of WEE infection after ingestion of infected A. dorsalis.

DISCUSSION

Except for A. fitchii, all the mosquitoes which were employed in the experiments reported here, are known hosts of WEE virus in western Canada (8,10, J. McLintock and A.N. Burton, unpublished information). The criteria which must be fulfilled and the nature of the investigations which must be undertaken to confirm their roles as vectors of the disease have been outlined (10). Among these investigations, are laboratory transmission studies. In such studies, mosquitoes are expected to adapt to the artificial conditions of the laboratory, but where they fail to do so, certain limitations are automatically imposed on the scope of the experiments. In the present study, severe limitations arose from difficulties experienced in inducing mosquitoes to feed on a live host and in maintaining their survival for long periods of time. The use of laboratory reared mosquitoes and of several vertebrate hosts were attempts to obviate these difficulties. Although the results must

be examined within the context of these limitations, they present several important features.

Culiseta inornata maintained infection for up to five weeks. At 69°F, infection rates settled between 63 and 88% during the second week and this suggests that the establishment of infection in this mosquito is a relatively slow process, compared for example, to Culex tarsalis (10). This deduction must be qualified by stating that the rates were measured in mosquitoes which had been infected from a pledget. The qualification is important because it is known that artificial infection of this nature cannot always be compared to the natural method (18). Nevertheless, numerous studies have shown that its use is justified where natural infection is difficult or impossible and that it is sufficiently reliable where blood-virus, rather than sugar-virus mixtures are employed (5,18). In this connection, C. inornata in experiment 1 (Table 2) were infected naturally from a chick while those of experiments 2 and 3 were artificially infected. Although there was a greater variation in titers in the latter two experiments, it should be observed, that compared to experiment 1, considerably more mosquitoes were examined. Throughout the study, it was noticed that most mosquitoes were reluctant to engorge fully from a pledget and the effect of this can be seen in lower

titers on the first day of infection (Table 2). Dissections revealed that in a few mosquitoes, all or part of the blood meal was diverted to the ventral diverticulum which may differ in susceptibility from the midgut. These two factors may account for the slightly lower titers observed in artificially infected C. inornata and A. campestris (Table 6) compared to those of naturally infected individuals. Perhaps too, higher infection rates could be expected from natural infection. From table 1, a higher temperature resulted in a more regular pattern of viral growth and slightly higher titers in C. inornata.

The standard pattern of viral growth exhibited by A. fitchii, A. flavescens and A. spencerii, indicates that infection in these mosquitoes at ten or more days after the infective blood meal was not simply a case of virus stabilization. To a lesser extent, this also applies to A. dorsalis. The relatively few transmission feedings of the aedines all took place within three to 12 days after infection, and except for A. fitchii, it is possible that no transmissions occurred because the extrinsic incubation period had not been exceeded. As far as is known, Saskatchewan is the only area in North America where A. campestris, A. flavescens, A. spencerii and Anopheles earlei have been found infected with WEE virus in nature.

This may therefore be the first occasion on which transmission attempts have been made with these species. With regard to A. dorsalis and A. vexans, Ferguson (7) reported that variable results were obtained in transmission experiments with these. On the other hand Chamberlain and coworkers (6) considered the latter a good vector of WEE virus.

Aedes fitchii presents a peculiar situation. WEE virus has never been isolated from this mosquito in Saskatchewan, nor as far as can be ascertained, elsewhere in North America. Although only limited conclusions can be drawn from the single transmission effected by this species, it becomes a potential vector. Rempel (19) mentions that it is not abundant in the agricultural area of the province. This is the area where most of the mosquito collections have been made over the past eight years, and McLintock et al. (14,15) indicated that relatively few members of this species were examined for WEE virus between 1962 and 1965. However, it is a common mosquito in the Aspen Grove and Coniferous Forest zones of Saskatchewan where it is on the wing by mid-May (19). If its vector potential is confirmed, this would partially explain transmission of WEE virus in northern Saskatchewan and other northerly areas of Canada where Culex tarsalis,

a confirmed vector, is rare (3,15).

Transmission feedings by C. inornata occurred on 11 occasions from four to 20 days after infection. No virus transmissions occurred. Apart from the paucity of feedings, several factors make it difficult to decide on the significance of this finding. Firstly, the incubation period may have been insufficient. If this is not applicable, and C. inornata of Saskatchewan is indeed unable to transmit WEE virus, then the possible significance of persistent infection at high rates should be examined. In most instances where persistent infection has been associated with inability to transmit, virus is usually present in trace amounts and infection rates are minimal (8,24). An unusual example similar to that found here with C. inornata, was reported by Chamberlain and coworkers (6). They found that although 79% of Anopheles quadrimaculatus contained considerable amounts of eastern equine encephalitis virus at thirteen days after an infective blood meal, none was capable of transmitting. These workers did not attempt to explain the significance of this finding. They did indicate however, the broad infection spectrum of arthropods for arboviruses and this has been adequately substantiated by both in vitro (2) and in vivo (11) studies. In spite of this, the present findings with C. inornata

cannot be dismissed so lightly. This is because it is abundant in Saskatchewan where WEE virus has frequently been isolated from it, and it is the only mosquito so far found infected in northern areas, where clinical cases of the disease have occurred in horses (15,16,19). It is known to have a preference for the blood of larger mammals (19,25) but it also feeds on birds (20). In addition it has been shown to be capable of transmitting WEE virus in the western United States (8). Variations in the two mosquito populations and differences in virus strains could account for differences in ability to transmit. The problems encountered in obtaining transmission of St. Louis encephalitis virus by Culex quinquefasciatus on account of such differences, are worth noting (1). On the basis of these complex but interrelated factors, C. inornata remains a highly suspected vector of WEE virus in western Canada.

Finally, it was observed in this study that laboratory hosts could be infected by ingesting infected mosquitoes, and several known vertebrate hosts of WEE virus can be infected orally (4,22). In this manner, mosquitoes with persistent infection could theoretically serve as a source of virus to these hosts, but the epidemiological significance of this is questionable.

TABLE 1

WEE VIRUS RECOVERY FROM CULISETA INORNATA AT 75°F

Days Incubation	Log_{10} Mouse LD_{50} per Mosquito*
1	4.2,3.9
3	<1.0,<1.0,<1.0,0,0
4	2.5
5	2.5,3.4,3.7
8-14	3.5,2.2,<1.0

* Mosquitoes infected on pledget, 2 mosquitoes per pool.

TABLE 2

WEE VIRUS RECOVERY AND INFECTION RATES IN CULISETA INORNATA AT 69°F

Days Incubation	Log_{10} Mouse LD_{50} per Mosquito			Infection Rates			
	Experiment 1	Experiment 2	Experiment 3	Experiment 4		Experiment 5	
				No. Pos./ No. Tested	%	No. Pos./ No. Tested	%
1	5.6,5.7,4.9	2.9,3.7	3.5,1.5	-		-	
2	-	-	-	5/6		-	
3	3.0	<1.0,<1.0	3.7,3.0	4/4		-	
4	3.9,3.0	-	-	-		-	
5	5.0,<1.0	0,<1.0,2.2 <1.0,0	2.9,0,0	8/17	47	-	
7	-	0,<1.0	0,<1.0	-		-	
8-14	3.0,4.2,<1.0	2.1,2.2,2.2, 3.0,1.5,2.1	1.5,1.5,0 <1.0	19/24	80	29/33	88
15-21	3.3	1.5,0,3.2,3.2	2.7,3.4,2.4,0	15/18	83	25/40	63
22-28	2.1	-	<1.0,<1.0,1.5	14/17	82	-	
29-35	<1.0	<1.0,<1.0	2.2,0	-		4/6	

TABLE 3

EXPERIMENTS ON TRANSMISSION OF WEE VIRUS BY CULISETA INORNATA AT 69°F

Experiment No.		Infective Blood Meal: No. Female Mosquitoes (Mosqs)	Infective Blood Meal: No. Mosqs. Fed	Infective Blood Meal: Source	Transmission: 2nd Blood Meal from Normal Host: No. Mosqs. Fed/No. Positive	Transmission: No. Transmissions
Established Lab Colony	1	120	20	Chick	0	-
1st Lab. Gen. (Weyburn)	2	60	3	Chick	0	-
1st Lab. Gen. (Estevan)	3	50	0	Chick	-	-
Wild	4	150	3	Chick	0	-
Wild and 1st Gen.	5	400	2	Chick	0	-
Wild	6	1500	32	Chick	4/3 chicks Transmissions failed at 4,6,8 and 12 days post infection. Three possible transmissions.	0
Wild	7	530	352	Pledget	1/1 suckling mice At 8 days post infection.	0

Table 3 (Continued)

Experiment No.		No. Female Mosquitoes (Mosqs)	Infective Blood Meal: No. Mosqs. Fed	Infective Blood Meal: Source	Transmission: 2nd Blood Meal from Normal Host: No. Mosqs. Fed/No. Positive	Transmission: No. Transmissions
Wild	8	300	100	Pledget	3/3 (2 chicks, 1 gerbil) Transmissions failed at 14,16 and 20 days post infection.	0*
Wild	9	200	0	Chick	-	-
Wild	10	260	130	Pledget	2/2 chicks At 10 days post infection.	0
			7	Chick	1/0 chick At 10 days post infection.	0
Totals		3570	67 on chick 582 on pledget		11/9	0 Of 9 possible Transmissions

* On the 16th day post infection, 2 four-week old gerbils ate from 8-10 mosqs. after recovery from anaesthesia. Death occurred 5 and 7 days later, and confirmed as due to WEE by fluorescent antibody tests on mouse brain (20% suspension of heart and brain pooled) and by neutralization tests. Neutralization indices, >3.0 and 4.7 logs.

TABLE 4

EXPERIMENTS ON TRANSMISSION OF WEE VIRUS BY CULISETA INORNATA AT 75°F

	Infective Blood Meal	Transmission	
No. Female Mosquitoes	No. Mosqs. Fed	2nd Blood Meal from Normal Host No. Mosqs. Fed/No. Positive	No. Transmissions
120	42	5/5	0
		Transmission failed on days 5,6,7 and 10 post infection.	

TABLE 5

WEE INFECTION THRESHOLD OF CULISETA INORNATA

Infective Blood Meal			Infection Rates No. Positive/No. Tested %				Infection Threshold	
Dilution of Stock[1]	Log_{10} Mouse LD_{50} per 0.03 ml	LD_{50} Ingested per Mosquito[2]	Experiment 1		Experiment 2		1-5%	50%
Undiluted	7.2	1.056×10^{6}	10/12	83.3	8/10	80	Mouse LD_{50} titer of $10^{-1.5}$ per 0.03 ml of blood, or Ingestion of 3.16 LD_{50} virus per mosquito.	Mouse LD_{50} titer of $10^{-4.0}$ per 0.03 ml of blood, or Ingestion of 666 LD_{50} virus per mosquito.
10^{-2}	5.2	1.056×10^{4}	17/20	85	15/19	79		
10^{-4}	3.2	1.056×10^{2}	12/32	37.5	3/15	20		

[1] Stock suspension of infected bovine blood.

[2] Based on ingestion of 0.002 ml of blood.

TABLE 6

RECOVERY OF WEE VIRUS FROM *AEDES CAMPESTRIS* FOLLOWING LABORATORY INFECTION

Days Incubation at 69°F	Log_{10} ic Mouse LD_{50} per Mosquito	
	Experiment 2	Experiment 3
	Infective blood meal from chick. LD_{50} titer $>10^{-6.5}$ per 0.03 ml	Infective blood meal from pledget. LD_{50} titer $>10^{-7.0}$ per 0.03 ml
1	5.0,4.7	
2	-	4.7,<1.0,<1.0,<1.0,<1.0 <1.0,<1.0
4	3.7,4.7,4.2,3.0	4.0,3.8
6		3.5,0
8	2.1,2.1,2.1,2.1	
9		4.9,<1.0,0
11		0,0
12	2.1,2.1	
13		0,<1.0
16	5.2,<1.0,1.5	
	1 mosquito per pool	2 mosquitoes per pool

TABLE 7

RECOVERY OF WEE VIRUS* FROM AEDES SPP. AND ANOPHELES EARLEI

FOLLOWING LABORATORY INFECTION

Days Incubation at 69°F	Aedes dorsalis	Aedes fitchii	Aedes flavescens	Aedes spencerii	Aedes vexans	Anopheles earlei
1	4.2,4.7	3.4,3.2		5.2,5.4,3.4	3.2,3.4	
5	<1.0,<1.0	<1.0,<1.0	<1.0	<1.0,<1.0		
7			2.2,<1.0,1.8	2.2,<1.0		
8		<1.0,1.8				
9			2.4,1.8	3.2,2.2,3.2		
10			<1.0,2.2	3.2,2.4,3.2	0,0	
12		1.8,2.9				0,0
16					0,0	0,0,0
20		0,0				
25					0,0	
30					0	

* Expressed as Log_{10} ic Mouse LD_{50} per mosquito.

TABLE 8

EXPERIMENTS ON TRANSMISSION OF WEE VIRUS BY AEDES CAMPESTRIS AT 69°F

Experiment No.		No. Female Mosquitoes (Mosqs)	Infective Blood Meal: No. Mosqs. Fed	Infective Blood Meal: Source	Transmission: 2nd Blood Meal from Normal Host: No. Mosqs. Fed/ No. Positive	Transmission: No. Transmissions
Laboratory Reared:	1	71	8	Chick	4/4 Chick 2 fed twice in trials with individual mosquitoes. Transmission failed on days 3,7,9, and 12 post infection. Total of 6 possible transmissions.	0
Wild:	2	618	28	Chick	0	-
Wild:	3	330	91	Pledget	3/2 Suckling mice 1 positive from each of 2 lots of 10 mosqs. on day 5 post infection. 2 possible transmissions.	0
Wild:	4	50	7	Pledget	0	-
Wild:	5	41	7	Pledget	0	-
Totals		71 Lab. Reared 1039 Wild	36 on Chick 105 on Pledget		7/6	0 Of 8 possible transmissions

TABLE 9

EXPERIMENTS ON TRANSMISSION OF WEE VIRUS BY *AEDES* SPP. AND *ANOPHELES EARLEI* AT 69°F

Mosquito Species	No. Mosquitoes (Mosqs)	Infective Blood Meal: No. Mosqs. Fed	Infective Blood Meal: Source	Transmission: Second Blood Meal from Normal Host — No. Mosqs. Fed/ No. Positive	Transmission: No. Transmissions
Aedes dorsalis	83	11	Pledget	3/2 Chicks Transmissions failed on days 3 and 4 post infection	0+
Aedes fitchii	196	6	Chick	2/2 Chicks Fed individually 9 days post infection	1*
		8	Pledget	0	-
Aedes flavescens	57	11	Pledget	3/3 Chicks Transmissions failed on days 5,8 and 9 post infection	0
Aedes spencerii	468	86	Pledget	1/1 Gerbil 8 days post infection	0
Aedes vexans	48	13	Pledget	0	-
Anopheles earlei	33	5	Pledget	0	-

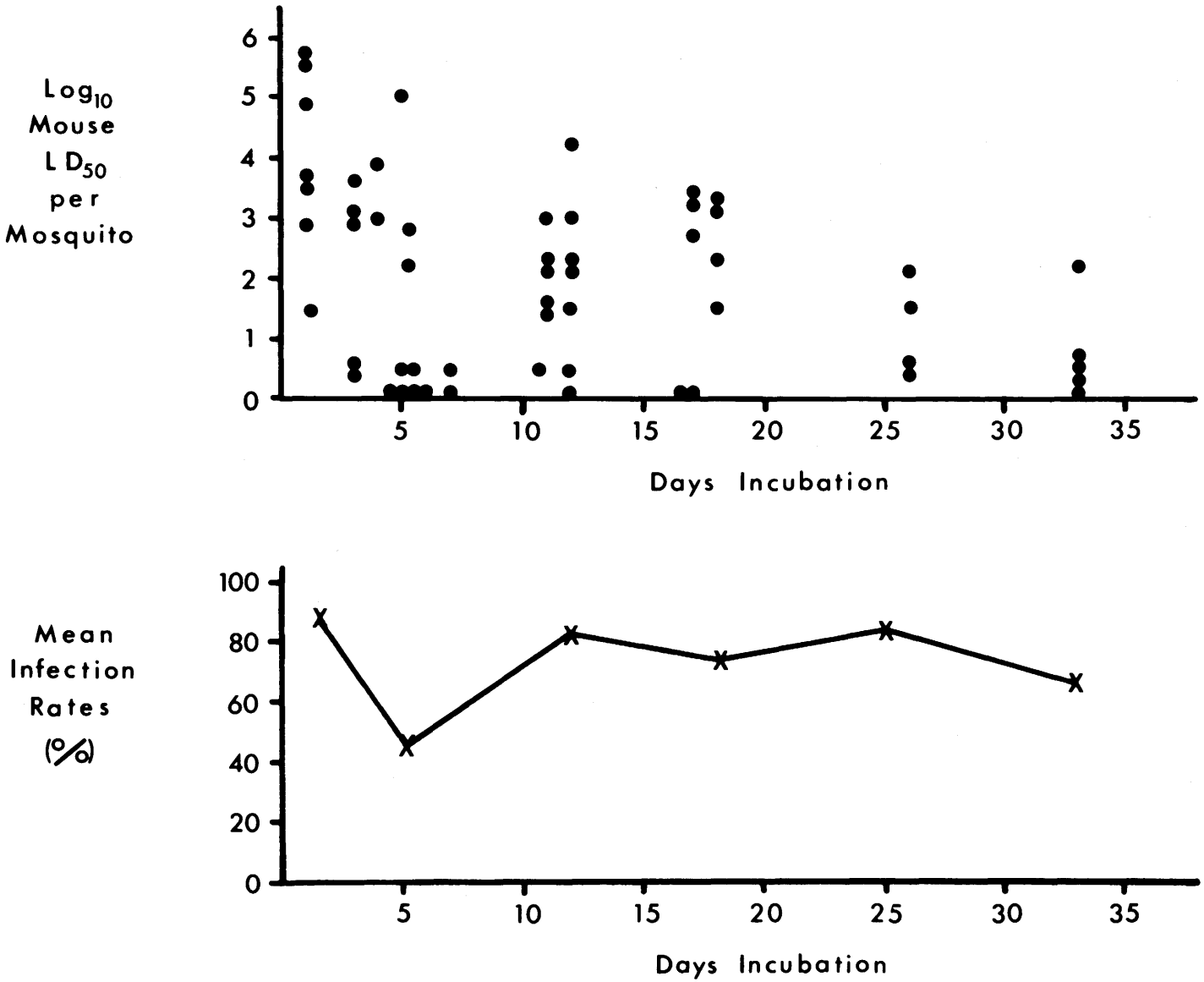
\+ A 4-week old gerbil died of WEE infection 6 days after ingestion of one mosquito which had been infected 4 days previously. WEE infection confirmed by neutralization test (Neutralization Index = Log 3.5) and by fluorescent antibody test.

* WEE infection confirmed by neutralization test (Neutralization Index = Log 4.0) and by fluorescent antibody test.

Figure 1

WEE VIRUS RECOVERY AND INFECTION RATES

IN CULISETA INORNATA AT 69°F



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CHAPTER 6

SUSCEPTIBILITY OF THE MONGOLIAN GERBIL (*MERIONES UNGUICULATUS*) TO WESTERN EQUINE ENCEPHALITIS

SUMMARY

The Mongolian gerbil (Meriones unguiculatus) was highly susceptible to peripheral infection with Western equine encephalitis virus. Small doses of virus ranging from 0.1 to ten intracerebral three-week old mouse LD_{50} which induced no clinical response in three-week old mice when inoculated subcutaneously, caused death in four-week old gerbils. At this age, a standard dose of virus produced uniform viremia patterns between individuals. Peak virus concentration approaching $10^{5.0}$ intracerebral three-week old mouse LD_{50} per 0.03 ml of blood, were attained at 48 hours post inoculation. Resistance to infection increased with age but ten-week old gerbils remained susceptible and exhibited marked clinical signs of encephalitis during the course of infection. Brains from animals which died within ten days of infection, yielded large amounts of virus.

INTRODUCTION

During the course of investigations on mosquito transmission of Western equine encephalitis (WEE) virus, the need arose for a mammalian host which was highly susceptible to peripheral infection. Specific requirements were that at least one aspect of the response, preferably death, should be fairly uniform between

individuals; that this response should occur after infection with small doses of virus, and that recovery of the virus from one or more tissues of dead animals should present no difficulty. In addition, it was important that this host exhibit high levels of viremia, sufficient for example, to infect mosquitoes, and that individuals could be held singly for observation. Although suckling and weanling mice are routinely used as laboratory hosts for WEE virus, neither of these fulfill all the above requirements. For this reason, an investigation was undertaken to determine whether the Mongolian gerbil might be more suitable. This paper describes the response of the gerbil to peripheral infection with the virus.

MATERIALS AND METHODS

WEE virus, strain 1540-1544 Regina 66, which had undergone three suckling mouse brain passages, and was in its sixth chick embryo passage, was used. It was originally isolated from the brain of a human being who died of the disease in Saskatchewan in 1966. The stock material of 20% chick embryo allantoic fluid was stored in sealed containers at $-70^{\circ}C$. It contained $10^{5.5}$ intracerebral three-week old mouse LD_{50} (ic mouse LD_{50}) per 0.03 ml.

Four and ten-week old gerbils and three-week old Swiss mice were inoculated subcutaneously (sc) with

various doses of virus contained in 0.03 ml of the appropriate dilution of stock virus. They were observed for 21 days. Tissues were removed from some of the gerbils which died within this period and 20% suspensions were prepared in beef heart infusion broth. For viremia studies, animals were bled (0.1 ml) by cardiac puncture. Blood was held as a 10% suspension in broth containing 20 USP units of heparin per ml, and along with tissue suspensions were kept at -70°C until virus titration was performed. This was done by intracerebral (ic) inoculation of three week old Swiss mice. Mice were observed for 12 days and LD_{50} titers calculated by the method of Reed and Muench.

The titer is expressed as the end-point dilution of a virus suspension which gave 50% mortality. As such, it is the dilution which contained one infectious unit (ic or sc LD_{50}) per 0.03 ml.

For histopathological diagnosis, brains were fixed in formalin and sections stained with hematoxylin and eosin.

RESULTS

Susceptibility to Infection and Clinical Signs

The mortality patterns in four and ten-week old gerbils are set out in table 1. The results show that for both groups, the duration of infection is dose dependent.

Subcutaneous LD_{50} titers per 0.03 ml of stock virus were $10^{-6.2}$ and $10^{-4.4}$ in four and ten-week olds respectively, indicating that resistance to infection increases with age. The younger gerbils were considerably more susceptible than were three-week old mice (Table 1) in which the sc LD_{50} titer was $10^{-3.1}$, and the older gerbils were only slightly more sensitive than mice. Since the titer of stock virus was $10^{-5.5}$ in three-week old mice inoculated ic, the results further indicate that four-week old gerbils are at least as sensitive to WEE virus by the sc route, as mice are by the ic route of inoculation.

No clinical signs of disease were observed in gerbils receiving 0.01 ic mouse LD_{50} doses of virus, but in all other groups early signs appeared from 24 to 72 hours after infection. Affected animals lay curled up, with hair ruffled, in a corner of the cage, and in contrast to their normal habits, were reluctant or unable to play when encouraged. Absence of their usual curiosity and alert manner was particularly striking. In four-week old animals receiving one or more LD_{50} doses of virus, this phase progressed to anorexia, depression, and varying degrees of hyperexcitability, coarse tremors and incoordination by the third to fifth day. Most remained at this stage until death which was preceded in some instances by

recumbency lasting for three to eight hours. A few of those dying after five days showed posterior paralysis.

In most of the ten-week olds, the early signs of illness lasted for about 24 hours and were followed by apparent recovery. Subsequently, a second phase of clinical illness, with time of onset varying from two to eight days, developed in those which later succumbed to infection (See table 1). Onset of the second phase was related to dose of virus, being generally later in those receiving smaller doses, and the nature of the clinical signs was similar to that already described for the younger age group. The main difference was that in the ten-week olds the illness was more prolonged and gradually became more severe. Muco-purulent ocular discharge was common and many animals showed posterior paralysis, spontaneous convulsions, and recumbency, the last extending for up to 24 hours before death. Others would assume an abnormal posture during which they appeared to be in stupor. In some, violent convulsions lasting for a few seconds could be induced by simple interference, eg by lifting or gently shaking the cage, and on occasion, death followed with the animal in extensor rigidity.

Viremia and Recovery of Virus from Tissues Post Mortem

Viremia was present in four-week old gerbils

between 24 and 96 hours after infection with 100 LD_{50} doses of virus (Table 2). Peak ic mouse LD_{50} titers approaching $10^{-5.0}$ per 0.03 ml of blood occurred mostly at 48 hours. Infection of four-week olds with one ic mouse LD_{50} caused viremia of a less uniform pattern (Table 2) similar to that in ten-week old animals after infection with 100 ic mouse LD_{50} doses (Table 3).

Table 4 shows that large amounts of virus can be recovered from brain post mortem, particularly from young animals dying after a short clinical course of disease. Among the older animals receiving relatively small doses of virus and having prolonged infection, smaller amounts were present in brain and other tissues at death (Table 4).

Histopathological Evidence of Encephalitis

In the limited number of sections examined from animals which died on the eighth day of infection, histological changes were readily apparent. These included perivascular cuffs, focal and diffuse areas of gliosis and neuronal degeneration. Between the animals, lesions were scattered throughout the brain and no attempt was made to assess their relative distribution in particular regions. Meningitis was present with congestion of meningeal vessels, some hemorrhage and infiltrations of mostly mononuclear cells.

DISCUSSION

The Mongolian gerbil is employed in a wide variety of laboratory studies in North America (2,5) but as far as can be ascertained, its use in research on viral diseases has not been investigated. Renoux (4) mentioned that Meriones shawi, a close relative of the Mongolian gerbil, was routinely used in "viral" and other studies in the Pasteur Institute in Tunisia and Lepine et al. (1) reported the high susceptibility of these animals to infection with Coxsackie virus.

The results of the present investigation show that the Mongolian gerbil at four weeks of age is considerably more susceptible to peripheral infection with WEE virus than is the three-week old mouse infected in the same manner. High levels and uniform patterns of viremia are obtained with a standard dose of virus and large amounts of virus can be recovered from some tissues at death. These features should prove useful in laboratory studies related to the epidemiology of the disease. In particular, gerbils could be valuable hosts in transmission experiments, providing of course, that mosquitoes will readily feed on them. Despite their small size, another possible application is the use of these animals as sentinel hosts for monitoring virus activity in nature. Sensitivity to WEE

infection extends for up to ten weeks of age at least, and minimum attention would be required during exposure.

In general, the ease of handling and maintaining the gerbil in the laboratory and its relative freedom from spontaneous infectious diseases (3), suggest that this may be a valuable animal for virological studies.

ACKNOWLEDGEMENTS

I am grateful to Dr. Frank Loew for providing the original gerbils used to establish the laboratory colony from which animals were obtained for this study. The Department of Veterinary Pathology prepared the material for histopathological diagnosis.

TABLE 1

MORTALITY PATTERNS IN MICE AND GERBILS INOCULATED SUBCUTANEOUSLY WITH WEE VIRUS

Approximate Virus Dose*	3-Week Old Mice Mortality**	Gerbil Mortality**	Days to Death										
			4	5	6	7	8	9	10	11	12	13	14
			4-Week Old Gerbils										
0.01	-	0/10											
0.1	-	2/10					2						
1	0/10	10/10	2	1	1	4	2						
10	0/10	6/6		1	3	2							
100	3/10	12/12	4	4	3	1							
1000	9/10	-											
			10-Week Old Gerbils										
1		2/10										1	1
10		4/10					1		1	1			1
100		6/7			1		2	2				1	
1000		5/5			2		2	1					

* Expressed as ic mouse LD_{50}.

** Numerator = number dead. Denominator = number inoculated.

TABLE 2

WEE VIREMIA IN 4-WEEK OLD GERBILS

Virus Dose	Gerbil No.	Viremia* at Hours 24	48	72	96
100 ic	316	4.5	-	2.7	-
mouse LD_{50}	317	-	4.7	-	2.7
	318	3.0	-	4.5	-
	319	-	4.5	-	3.7
	320	-	5.0	-	-
1 ic	321	0	-	2.0	-
mouse LD_{50}	322	-	0	-	5.5
	323	3.0	-	4.0	-
	324	-	4.3	-	-
	325	-	-	-	3.5

* Log_{10} ic mouse LD_{50} per 0.03 ml of blood.

TABLE 3

WEE VIREMIA IN 10-WEEK OLD GERBILS INOCULATED WITH 100 LD_{50} DOSES OF VIRUS

Gerbil No.	Viremia* at Hours							
	24	48	72	96	120	144	168	192
295	2.2	-	0	-	-	Dead		
296	-	3.4	-	0	0	-	-	0
297	<1.0	-	2.0	-	-	0	0	Dead
298	-	1.7	-	<1.0	<1.0	-	0	-
299	-	-	-	-	-	0	-	0

* Log_{10} ic mouse LD_{50} per 0.03 ml of blood.

TABLE 4

RECOVERY OF WEE VIRUS FROM TISSUES OF THE GERBIL AT DEATH

Age	Virus Dose*	Gerbil No.	Days to Death	Virus Recovered** From: Heart Blood	Liver	Heart	Brain
4 weeks	100	305	5	1.0	<1.0	4.2	5.0
		306	5	-	0	4.7	6.0
10 weeks	100	346	8	-	-	<1.0	3.7
		347	9	-	-	0	2.7
10 weeks	1000	300	8	<1.0	0	<3.0	3.2
		301	6	-	0	<3.0	3.6
		315	6	-	0	2.5	2.5

* Expressed as ic mouse LD_{50}

** Log_{10} ic mouse LD_{50} per 0.03 ml.

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CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH

As a background for presenting general conclusions from the study reported in this thesis, and for suggesting areas of continuing research, it is pertinent to refer briefly to the original objectives of the research project. The objectives were, to investigate the behavior of Western equine encephalitis (WEE) virus in nine species of Saskatchewan mosquitoes, to determine the ability of these mosquitoes to transmit the virus from one susceptible vertebrate host to another, and to assess the efficiency of virus transmission wherever appropriate. The project was designed to assist in elucidating the role of these mosquito species, eight of which were known hosts of WEE virus, in the epidemiology of WEE in Saskatchewan. As such, it formed part of a wider interdisciplinary study on the natural history of the disease. With this broad epidemiological approach in mind, the conclusions which may be drawn from the thesis, and some areas of research which may provide important information, can be listed as follows.

1. The results of transmission experiments with Culex tarsalis (Chapter 4), have confirmed that this mosquito is an efficient vector of WEE virus in Saskatchewan. A

definite statement can now be made that where control of the disease is to be effected through reduction of vector population, some emphasis must be placed on C. tarsalis. In this connection, research could be directed towards eradicating this mosquito, or, from a more immediately practical point of view, towards reducing population levels for given periods of time. The latter would depend in part, on the ability to predict an impending outbreak of WEE, and in part, on a knowledge of the biology of this mosquito in Saskatchewan. Information on the age composition of C. tarsalis populations throughout the summer, along with knowledge of the natural host range, incidence of multiple feedings, and autogeny rates, would provide estimates of longevity and of the number of times that an individual female could transmit virus. These estimates, when considered along with the build-up of the adult female C. tarsalis population and WEE infection rates as each summer advances, could provide the means for predicting an outbreak. Finally, information on C. tarsalis larval habitats could be employed to effect larval control at a suitable time.

2. Experiments with mosquitoes other than C. tarsalis (Chapter 5) have indicated that Culiseta inornata and Aedes fitchii may also be important vectors of WEE virus in

Saskatchewan. The results with the other species are less definite, but additional transmission studies could assist in clarifying the role of A. fitchii in northern areas, of the early spring aedines (A. campestris, A. flavescens, and A. spencerii) in the agricultural area, and of C. inornata throughout most of the province. Mass transmission experiments, using a susceptible mammalian host, eg the Mongolian gerbil, rather than an avian host on which these mosquitoes are known to be reluctant to feed, are suggested as a first step (see item 4 below). Positive findings would lead to more detailed studies as outlined for C. tarsalis.

3. On account of numerous technical difficulties, immunofluorescence (FA) methods have, hitherto, found limited application in diagnostic areas of arbovirology. The results of the FA study reported in this thesis (Chapters 2 and 3) have shown that several of these difficulties can be successfully overcome, and for this reason, they assume particular significance. The study has provided a new and simplified approach to the rapid diagnosis of WEE infection, within the framework of transmission experiments. On a broader scale, the results of this aspect of the research can be expected to form the basis for a cheap, reliable and highly specific method for

rapid diagnosis of arboviral infection. This applies equally to suspected clinical and post mortem cases of human and animal (eg equine) patients. In addition, the techniques appear to have a definite place in epidemiological surveys, as a means for rapidly monitoring infection in mosquitoes, wild vertebrate hosts, and sentinel animals. Research in these areas could be directed towards comparing FA methods with neutralization tests for diagnosis of WEE infection. Comparisons between these two methods should include the time taken to arrive at a specific diagnosis, relative costs and ease of interpretation. It is possible that the FA technique may allow for more rapid monitoring of WEE infection in nature, and in this manner, provide data to assist in the early prediction of an impending WEE outbreak. Similarly, early diagnosis in human and equine patients may allow for the institution of measures to protect the patients themselves or other susceptible hosts from irreversible pathological effects of the clinical disease.

4. The study on the susceptibility of the Mongolian gerbil to WEE virus (Chapter 6), has provided a new laboratory host for the investigation of WEE, and perhaps, other viral diseases. This is the only laboratory mammal so far known, to exhibit clinical signs and histopathological

lesions of encephalitis in response to peripheral inoculation of WEE virus, within the range of doses inoculated by transmitting mosquitoes (See "Estimation of Amount of WEE Virus Inoculated by Transmitting Culex tarsalis" p 252. Appendix). These findings could be of benefit to two broad areas of research on WEE. Firstly, the gerbil would probably be a suitable vertebrate host for studies on transmission of the virus by mosquitoes. The chick, because of its high degree of susceptibility, has been extensively used in transmission experiments, but these experiments, have in the past, been hampered by the reluctance of some mosquito species to feed on birds and by the unavailability of highly susceptible mammalian hosts. Such a mammalian host is now available for the first time. In addition to its use in transmission experiments, the gerbil may prove useful as a sentinel host for monitoring virus activity in nature.

The second area of research in which the high degree of susceptibility of Mongolian gerbil may be used to advantage, concerns investigations on pathogenesis of the arboviral encephalitides. For these purposes, the young adult mouse is usually employed as a model for human infection. However, large amounts of virus in great excess of that normally inoculated by transmitting mosquitoes,

are required to reproduce the clinical disease in this animal. Since the young adult gerbil does not suffer from this severe limitation, it is a more suitable laboratory host for pathologic study. In addition, the immunofluorescence method can be combined with other laboratory procedures, and with clinical observation, for this purpose.

5. Finally, it may be mentioned that firm knowledge is lacking on the possible mechanisms, eg formation of the peritrophic membrane, secretion of digestive enzymes, which determine the susceptibility of the mosquito midgut to WEE infection. Similarly, relationships which may exist between virus transmission by mosquitoes and cyclical physiological processes, eg gonadotrophic activity, salivary secretions, (See "Estimation of Amount of WEE Virus Inoculated by Transmitting Culex tarsalis", p 252, Appendix) are largely unknown. Alterations in antigenic and pathologic properties of WEE virus which may occur after passage in a particular mosquito species, could have a profound influence on the epidemiology of the disease, but this aspect of mosquito infection has been largely neglected as well.

Investigations within these areas could employ WEE virus as the model group A agent. C. tarsalis could serve as a base on which to compare two or more mosquito species,

ranging from those which are refractory to infection, through those of intermediate susceptibility. (See "Determination of Infection Thresholds in Culex tarsalis and Culiseta inornata, p 257, Appendix).

APPENDIX

APPENDIX

Preparation of Antiserum to Western Equine Encephalitis (WEE) Virus

Rabbits were selected from a laboratory colony where there was little likelihood of exposure to WEE virus. From each, a preimmune blood sample was obtained and inoculations of WEE virus consisted of three subcutaneous injections of approximately 5,000 intracerebral 3-week old mouse LD_{50} doses of virus, given once weekly. Three weeks after the last injection, a booster dose was given and a test blood sample obtained one week later. Neutralization tests were performed in mice on the sera from both samples. The standard serum - varying virus method was used, directed against the infecting virus. If the test sample showed a satisfactory neutralization index (2.5 logs or more), the rabbit was deprived of food overnight and exsanguinated on the following day. Exsanguination was by cardiac puncture under general (pentobarbitone) anaesthesia.

Antisera were stored at $-20^{o}C$ in two and 20 ml aliquots. Before use, each sample was allowed to thaw and inactivated at $56^{o}C$ for 30 minutes.

Preparation of Mouse and Chick Brain Powders and Absorption of WEE Antisera for Immunofluorescence Staining

Brain was homogenized in phosphate buffered saline (PBS) pH 7.4. It was washed several times to remove traces of blood, and sedimented by centrifugation. After resuspension in four volumes of acetone, the homogenized tissue was allowed to stand at room temperature for 30 minutes with frequent stirring. The precipitate was packed by centrifugation and the acetone treatment repeated. The suspension was then filtered and the precipitate thoroughly washed with acetone and dried at 37°C overnight while still on the filter paper. The powder was stored in sealed vials at 4°C.

For absorption of antisera, 100 mg of powder per ml of antiserum was used. The mixture was stirred occasionally while being incubated at 37°C for ½ to 1 hour after which the powder was packed by centrifugation, the antiserum removed, and the process repeated with fresh powder. A 30-40% loss of antiserum usually resulted.

Significance of Difference in LD_{50} Titers in Mice and Chicks

Results of Titration

Titration Series	Log_{10} LD_{50} Titer per 0.03 ml. in:	
	sc Chick	ic Mice
1	8.8	6.2
	8.8	6.3
	8.8	6.2
	8.9	6.2
2	8.2	5.5
	8.0	5.9
	8.5	5.6
	8.6	6.0
3	8.9	6.3
	9.4	6.2
	8.7	6.1
	8.7	6.0
4	8.7	6.3
	9.0	6.0
	8.6	6.1
	9.2	6.2
Total (T)	139.8	97.1
Mean ($\bar{y}$)	8.74	6.07

In each titration series, the same vial of stock virus material was used in both mice and chicks. Although there are 4 such series, (replications) the interest here is entirely in comparing mice and chicks. The one-way classification of the analysis of variance was therefore employed to test the significance of the difference between them.

Analysis of Variance

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Value
Total	31	64		
Experimental Animals	1	58	58	
Error	30	6	0.2	58/0.2 = 290

Significant at the 0.5% level. $F_{0.005} > 9.18$, 1 and 30 degrees of freedom.

Mosquito Rearing Procedures

Culiseta inornata

Rearing throughout all developmental stages was carried out in a controlled environment at a temperature

of 69°F, relative humidity 70-80% and a lighting schedule which allowed 16 hours of light and 8 hours of darkness. Lighting was provided by incandescent lamps.

Adults were held in cages measuring 12 x 12 x 16 inches. Each cage had a wooden top, base and back, and the sides were of plexiglass which could be removed to allow cleaning. This cage served for feeding and egg laying (Fig. 1B). Adults were fed from a cotton pledget saturated with a mixture of defibrinated bovine blood and 10% sucrose (3:1), and a dish of distilled water was provided for egg laying. The surface area of the water was at least half that of the bottom of the cage.

Egg rafts were gathered on the day following egg laying and were placed in hatching medium. This consisted of 1,250 ml of Bate's medium S with 50 mg bakers yeast and 240 mg ground guinea pig feed, contained in a plastic dish eight inches in diameter and five inches deep.

Bate's Medium S:

Calcium sulphate ($CaSO_4 2H_2O$)	0.5 gm
Sodium chloride	0.5 gm
Magnesium sulphate ($MgSO_4 7H_2O$)	1.0 gm

in one liter distilled water.

The eggs hatched in 60-72 hours and the first larval moult was allowed to take place in the hatching dish. This occurred in two to three days and was evident when the

cast larval skins of the first instar appeared.

At the same time that the hatching dish was prepared, larval trays were set up as follows; 2,500 ml of Bate's medium containing the same quantity of food as above, was placed in each of several enamel trays measuring 15 x 10 x 2½ inches (Fig. 1A). Subsequently, food was added to each tray every second day until pupation began. Air was bubbled continuously through the medium and a sheet of plain window glass placed over each tray prevented excessive evaporation. Six days after the trays were set, 150 second-instar larvae were transferred from the hatching dish to each larval tray by means of a medicine dropper.

Pupation began at about the 21st day after egg laying and from this time onwards pupae were removed daily. They were counted, pooled, washed and transferred to distilled water. The pupal dish, through which air was bubbled continuously, was placed in an emergence cage of the type described above and adults were allowed either distilled water or 25% sucrose from a soaked pledget. They were held in this manner until required for use in transmission studies.

Culex tarsalis

The general procedure was similar to that employed

for Culiseta inornata differing in a few details as follows.

1. Rearing temperature for all stages was 75°F and the lighting schedule provided one hour of "dawn", 15 hours of "daylight", one hour of twilight and seven hours of darkness.
2. Egg rafts were placed in the same dish used for larval rearing and since the number of larvae per dish was not critical, no larval counts were made.
3. Larvae were present on the third day and pupae from the 11th through to the 17th day after egg laying. Larval trays were fed every other day until pupation was complete.
4. Adults were held in wire mesh cages measuring 24 x 24 x 24 inches (Fig. 1B) and normal chicks were used to provide blood meals.

Aedes mosquitoes

Wild caught females were held at 69°F and provided with cotton pledgets saturated with a mixture of defibrinated bovine blood and 10% sucrose (3:1). They were allowed to engorge over a period of several days, after which they were transferred in groups of 50 or less to half-pint ice cream cartons. Each carton was fitted with a lid of wire mesh and on the bottom was a pad of gauze which was

kept moist with distilled water. Eggs which were laid on the pad were held at 69°F under moist conditions for three months. They were then transferred to 35°F at which temperature they were held for a further three months in a humid atmosphere. Humidity was provided by use of a desiccator containing a saturated solution of potassium chloride. Following this period of conditioning, the eggs were held at 69°F for 24 hours just before they were placed in hatching medium. The hatching medium was nutrient broth (Difco) dissolved in tap water at a concentration of one gm per liter. Larvae were transferred to Bate's medium S and subsequently reared by the method used for Culiseta inornata.

Isolation Facilities and Handling of Infected Mosquitoes

Throughout the study all effort was made to contain the virus in defined working areas. Known or potentially infected mosquitoes were held in isolation quarters used entirely for the purpose and far removed from the breeding colonies and other areas of general activity. These quarters were provided with facilities for mosquito infection, incubation and transmission at a given temperature.

Specially adapted cages were used for transmission trials with individual mosquitoes ro with groups from two to ten. Each measured four inches in height and was made

from clear plastic (Fig. 1B, arrows). The latter, together with the small size greatly facilitated observations on blood feeding. On the side of the cage was an aperture one inch in diamter and this was guarded by a double layer of dental rubber dam. Through the first layer of rubber dam was a vertical slit and through the second, a horizontal slit which together permitted easy access of a mechanical aspirator, while preventing at the same time the escape of mosquitoes. The removable lid was made of fine wire mesh through which mosquitoes could feed on sugar solutions from a saturated gauze pad or on one of the larger experimental hosts. Normally, in transmission trials, chicks were held in a thin nylon stocking within the cage. In this manner, excessive restraint was not required and in addition, mechanical damage to mosquitoes was largely avoided. Other small laboratory hosts, i.e. suckling mice and gerbils were also placed directly in the cage. In the latter case, animals were anaesthetized (pentobarbitone sodium, 60 mg per kg intraperitoneally) and the lateral aspect of thorax and abdomen shaved for transmission attempts. Mosquitoes required for grinding were transported in the transmission cages from the isolation quarters to the virus laboratory. Here, they were immediately immobilized by exposure to -20^{o}C and processed as already described.

Estimation of Amount of WEE Virus Inoculated by Transmitting Culex tarsalis

As a first step for estimating the amount of virus inoculated by transmitting mosquitoes, experiments were performed to examine the relationship between virus dose and time of death in half-day old chick.

Relationship Between Virus Dose and Mean Death Time in Chicks

Two experiments were performed. In each, groups of 50 chicks were inoculated subcutaneously with a given dose of virus contained in 0.03 ml of the appropriate dilution of stock virus material. The doses covered the range 0.001 to 1,000 intracerebral 3-week old mouse LD_{50} in ten or 100-fold steps. A record was made of the number of chicks dead in each group at given intervals of time. The cumulative percentage deaths at these times were then plotted on probability paper (Fig. 2). In this manner, the mean time taken for chicks to die (Time taken for 50% deaths to occur, read directly from the probability plots) was obtained for each dose. Only one group of chicks received 10,000 LD_{50} doses. Within 20 hours, 56% had died and the remainder were discarded. The mean death time for this group was calculated as 18 hours.

Figure 3 shows the relationship between mean death time and virus dose. It can be observed that doses above

10,000 LD_{50} are unlikely to reduce the mean death time, and sensitivity of the chick to doses below 0.001 LD_{50} is minimal. In fact, observations from titration studies in chicks had suggested these relationships and they formed the basis for the selection of the doses used in the experiments. A regression curve of mean death time on virus dose was then plotted. Values for both sets of observations were transformed to logarithms in order to demonstrate the linear relationship as shown in the regression curve (Fig. 4). This curve provided the means for estimation of virus dose when time of death was known.

Calculations from Experimental Data

Chicks from routine transmission experiments involving single mosquitoes provided the information required. An advantage was that several observations relating to transmission efficiency could be made simultaneously. However, the time of death in these chicks, subsequent to their infection by transmitting mosquitoes, could not be determined precisely so that a number of assumptions and estimations had to be made. For example, it had been established before transmission studies began that of those mosquitoes which would take a blood meal during the evening, the majority did so between 8:00 p.m. and 11:00 p.m. During this period the incubator

lights simulated a period of dusk. On the following morning, the blood meal would be dark red and this assisted in identifying the feeders of this time period. Such mosquitoes were considered to have fed at 10:00 p.m. and for the purpose of estimating the amount of virus inoculated, chicks infected at this time were marked for further observation. The majority of chicks which were finally included were those which died (of WEE) between 8:00 a.m. and 5:00 p.m. on any day and a few which died between 5:00 p.m. and 1:00 a.m. A reasonable estimate of time of death could not be obtained for those dying at other periods. Accordingly, less than 50 chicks provided a measure of the amount of virus inoculated by transmitting C. tarsalis. The results, obtained from the regression curve (Fig. 4) are presented in table 1.

Among mosquitoes incubated at 69°F, those which transmitted at 20 days inoculated the largest amounts of virus and at other times, varying and usually smaller amounts were inoculated. From the fifth to the tenth day of infection, those held at 75°F transmitted larger amounts than those at 69°F, and it would appear that among the former, minimal amounts were transmitted after prolonged incubation. Apart from these observations, no definite relationship between length or temperature of incubation

and the amount of virus inoculated can be definitely established. However, a number of other associations can be pointed out. The five to ten day period at 75°F was within the interval when virus titers in whole mosquitoes were on the increase and it also included that period when the highest rate of increase in transmissions (as distinct from transmission rates) occurred. (See Chapter 4). It is also known from an unrelated study that the peak of gonadotrophic activity in normal C. tarsalis at 75°F is within this period. The implication here that gonadotrophic activity may be related to transmission efficiency other than by association with frequency of blood feeding, cannot be expanded further. Although infected mosquitoes were routinely provided with oviposition dishes, the transmission experiments were, unfortunately, not designed to measure this parameter as well.

The main observation which emerges from the study is that the majority of mosquitoes (68.2%) inoculated less than 100 ic mouse LD_{50} virus at time of transmission. The relative values are as follows.

Range in LD_{50} Virus Inoculated	No. Mosquitoes	%
<1.0	8	18.2
1.0-9.9	17	38.6
10-99.9	5	11.4
100-999	10	22.7
>1,000	4	9.1
	44	100.0

It is recognized that virus passage in mosquitoes and the secretions inoculated along with virus could cause alterations in virulence or pathogenicity for particular hosts. Consequently, the small amounts of virus inoculated may not be indicative of the ability of C. tarsalis to transmit the disease to man and horses. In a similar study to the one reported here, Chamberlain et al. (1) also found that the amount of eastern equine encephalitis virus inoculated by Aedes aegypti varied considerably, and that most specimens inoculated less than 100 ic mouse LD_{50}. Like these workers, it is stressed that because of the number of assumptions and estimations that had to be made, the results represent fairly crude approximations. For this reason, they are probably of more value in relative, rather than

in absolute terms.

Determination of Infection Thresholds in Culex tarsalis and Culiseta inornata

Infection threshold as defined by Chamberlain et al. (2), is the lowest concentration of virus causing an infection in approximately 1-5% of specimens of a particular mosquito species ingesting it. The value of this concept in comparing transmission efficiency between mosquito species and in evaluating the place of vertebrate hosts has been outlined (Chapter 4). It is suggested that the 50% infection level may offer a better standard for comparisons, and because the 1-5% levels can be determined at the same time, Chamberlain's implied concept of infection threshold as the starting point of infection, is retained. Since temperature and length of incubation are known to affect infection rates, it seemed that these should be standardized as far as possible. A temperature of 69^{o}F was chosen because C. inornata survives well at this temperature. Incubation for ten to 12 days was selected because there were indications that at this time, the decreasing phase of virus titers in whole mosquitoes was complete in both species.

Measurement of Volume of Blood Meals

Infection threshold may be expressed in terms of the concentration of virus at the infective source or in terms of the total virus ingested per blood meal. For the latter, the volume of the blood meal must be known for each species, and to measure this, mosquitoes were starved for several days and those with flat abdomens were selected. Some were allowed to have a blood meal and the weights of empty and blood fed specimens compared by use of a Cahn electro-balance. The results are shown in table 2.

Calculations from Experimental Data

Tables 3 and 4 show the infection rates in Culiseta inornata and Culex tarsalis when fed on blood containing different concentrations of virus. Very few specimens of Aedes campestris fed so that attempts to determine infection threshold in this species failed.

TABLE 1

ESTIMATION OF AMOUNT OF WEE VIRUS (NO. IC MOUSE LD_{50})

INOCULATED BY TRANSMITTING CULEX TARSALIS

Days Post Mosquito Infection	Time of Chick Death		Approx. LD_{50} Virus Inoculated	Days Post Mosquito Infection	Time of Chick Death		Approx. LD_{50} Virus Inoculated
	Hour	Log_{10} Hour			Hour	Log_{10} Hour	
4	48	1.69	0.16	12	50	1.70	0.13
					50	1.70	0.13
5	38	1.58	2.1		50	1.70	0.13
	26	1.42	89				
	62	1.79	0.02	20	20	1.30	1400
	38	1.58	2.1		20	1.30	1400
	*24	1.38	250		20	1.30	1400
	*24	1.38	250		25	1.40	140
					25	1.40	140
6	28	1.45	45		24	1.38	250
					36	1.56	3.6
9	36	1.56	3.6				
	36	1.56	3.6	26	77	1.89	0.001
	36	1.56	3.6		24	1.38	250
	*24	1.38	250		40	1.60	1.4
	*24	1.38	250				
	*24	1.38	250	30	40	1.60	1.4
					20	1.30	1400
10	36	1.56	3.6		40	1.60	1.4
	36	1.56	3.6		40	1.60	1.4
	*24	1.38	250		40	1.60	1.4
	30	1.48	22.4		40	1.60	1.4
	30	1.48	22.4				
				41	*38	1.58	2.1
11	54	1.73	0.06		*35	1.54	5.6
	50	1.70	0.13		*35	1.54	5.6
					*30	1.48	22.4

* Incubated at 75°F. Remainder Incubated at 69°F.

TABLE 2

VOLUME OF BLOOD INGESTED BY SASKATCHEWAN MOSQUITOES

	Culex tarsalis		*Culiseta inornata*		*Aedes campestris*	
	Unfed	Blood-Fed[1]	Unfed	Blood-Fed[2]	Unfed	Blood-Fed[2]
Weights of Whole Individuals in mg	1.882	4.901	4.752	5.378	2.818	6.348
	2.198	5.446	4.916	9.868	4.306	6.908
	1.778	5.034	4.600	7.712	5.084	9.437
	2.444	6.202	6.274	7.208	3.578	7.702
	1.810	5.240	6.054	7.389	4.002	5.784
	1.842	4.929	4.284	9.966	3.482	6.847
	2.097	5.382	6.134	6.550	3.780	7.814
	1.898	5.268	6.094	9.192	4.226	8.502
	2.178	4.988	5.874	5.880	4.104	8.044
	2.482	5.402	5.252	8.642	3.722	6.682
Mean weight	2.0609	5.2792	5.4234	7.7785	3.9102	7.4068
Mean weight of blood meal	3.2183		2.3551		3.4966	
Mean volume of blood meal	0.0030 ml		0.0022 ml		0.0033 ml	

[1] Avian blood, specific gravity = 1.056

[2] Bovine blood, specific gravity = 1.052

TABLE 3

WEE INFECTION THRESHOLD OF CULISETA INORNATA

Infective Blood Meal			Infection Rates No. Positive/No. Tested %				Infection Threshold	
Dilution of Stock[1]	Log_{10} Mouse LD_{50} per 0.03 ml	LD_{50} Ingested per Mosquito[2]	Experiment 1		Experiment 2		1-5%	50%
Undiluted	7.2	1.056×10^{6}	10/12	83.3	8/10	80	Mouse LD_{50} titer of $10^{-1.5}$ per 0.03 ml of blood, or Ingestion of 3.16 LD_{50} virus per mosquito.	Mouse LD_{50} titer of $10^{-4.0}$ per 0.03 ml of blood, or Ingestion of 666 LD_{50} virus per mosquito.
10^{-2}	5.2	1.056×10^{4}	17/20	85	15/19	79		
10^{-4}	3.2	1.056×10^{2}	12/32	37.5	3/15	20		

[1] Stock suspension of infected bovine blood.

[2] Based on ingestion of 0.002 ml of blood.

TABLE 4

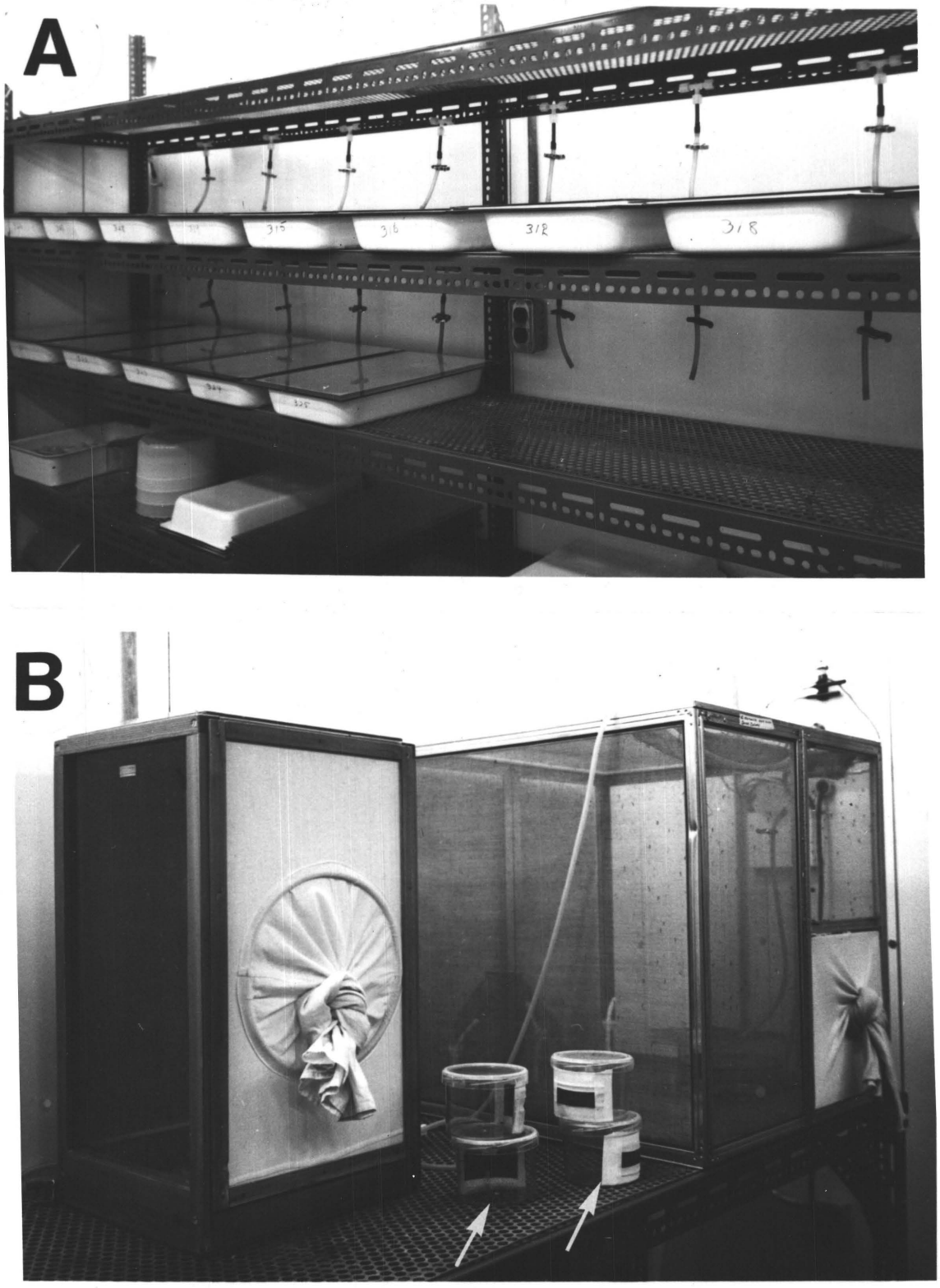
WEE INFECTION THRESHOLD OF CULEX TARSALIS

Experiment No.	Infective Blood Meal: Log_{10} LD_{50} Mouse per 0.03 ml	Infective Blood Meal: LD_{50} Ingested per mosquito[1]	Infection Rates: No. Positive / No. Tested	Infection Rates: %	Infection Threshold: 1-5%	Infection Threshold: 50%
1	1.2	1.585	4/46	8.9		
	2.8	63	10/12	83.3		
	3.5	316	14/20	70	Mouse LD_{50}	Mouse LD_{50}
	8.5	3.16×10^7	23/24	96	titer of $10^{-1.0}$	titer of $10^{-2.5}$
2	1.2	1.585	8/68	11.8	per 0.03 ml of	per 0.03 ml of
	2.0	10	6/26	23.1	blood, or	blood, or
	2.2	15.85	44/106	41.5	Ingestion of	Ingestion of
	2.6	39.8	37/92	40.0	1.0 LD_{50} virus	31.6 LD_{50} virus
	3.5	316	27/30	90	per mosquito.	per mosquito.
	5.3	2.0×10^4	17/17	100		
	5.7	5.0×10^4	8/8	100		

[1] Based on ingestion of 0.003 ml of blood.

Figure 1

VIEW OF MOSQUITO REARING ROOMS



A. Larval trays

B. Holding and transmission (arrows) cages

Figure 2

CALCULATION OF MEAN DEATH TIME IN CHICKS

Virus Dose in Mouse LD_{50}	Cumulative Percentage Deaths at Hours											
	20	22	24	28	30	36	48	60	72	84	96	108
0.001					10				30	42	55	60
0.01					15	20	28		66	72	85	
0.1			18		23	33	58	84		93	99	
10.0			18		40	80	90					
1000.0	50	75	90	97								

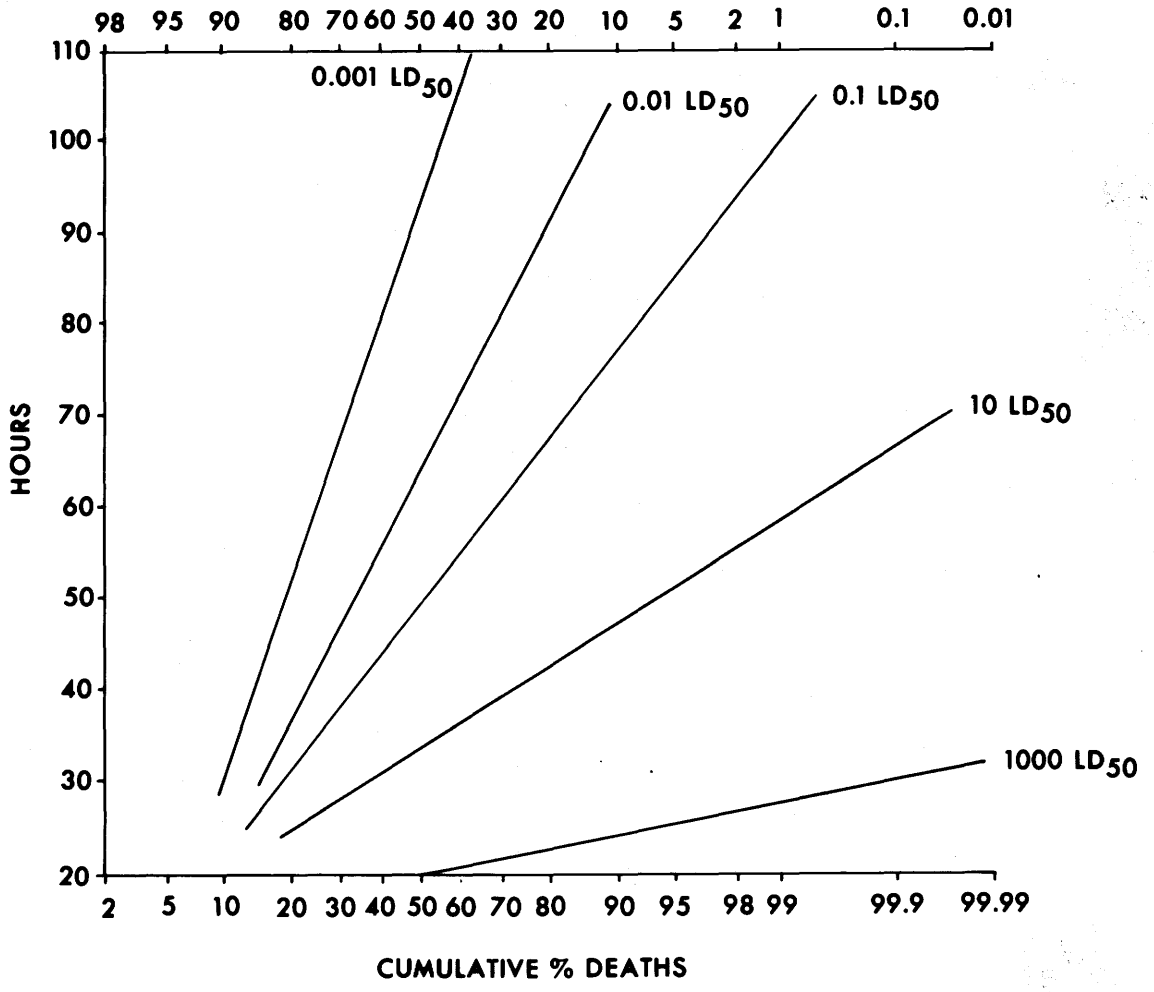


Figure 3

RELATIONSHIP BETWEEN WEE (Strain 1540 - 1544) VIRUS DOSE AND 50% DEATH TIME IN WET CHICKS

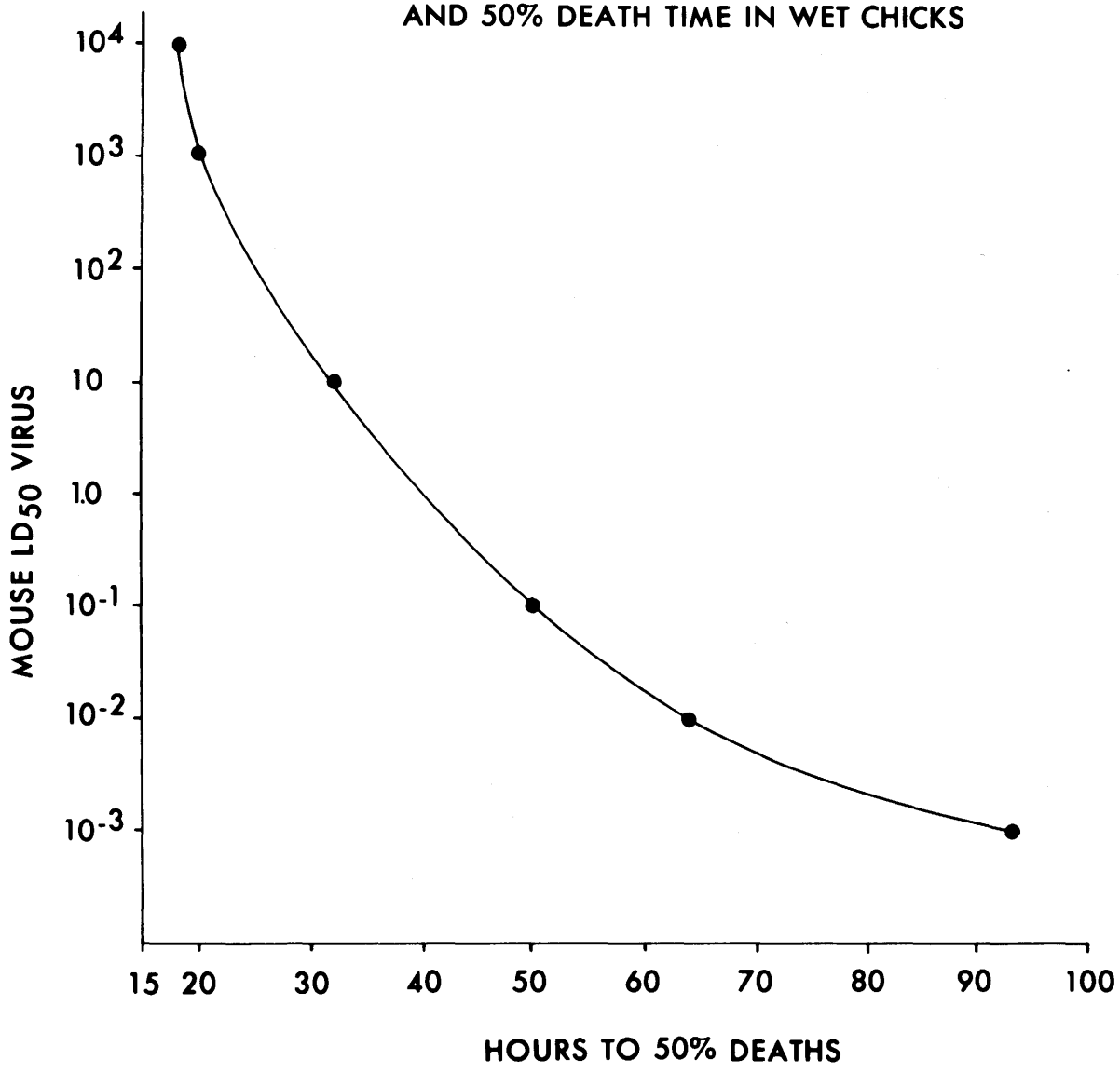


Figure 4

REGRESSION OF MEAN DEATH TIME IN CHICKS ON INTRACEREBRAL MOUSE LD_{50} DOSE OF WEE VIRUS

Mean Death Time (Hr.)	Log_{10} Mean Death Time	Log_{10} LD_{50} Dose
92	1.963	-3
63	1.799	-2
44	1.643	-1
31	1.495	1
20	1.301	3
18	1.255	4

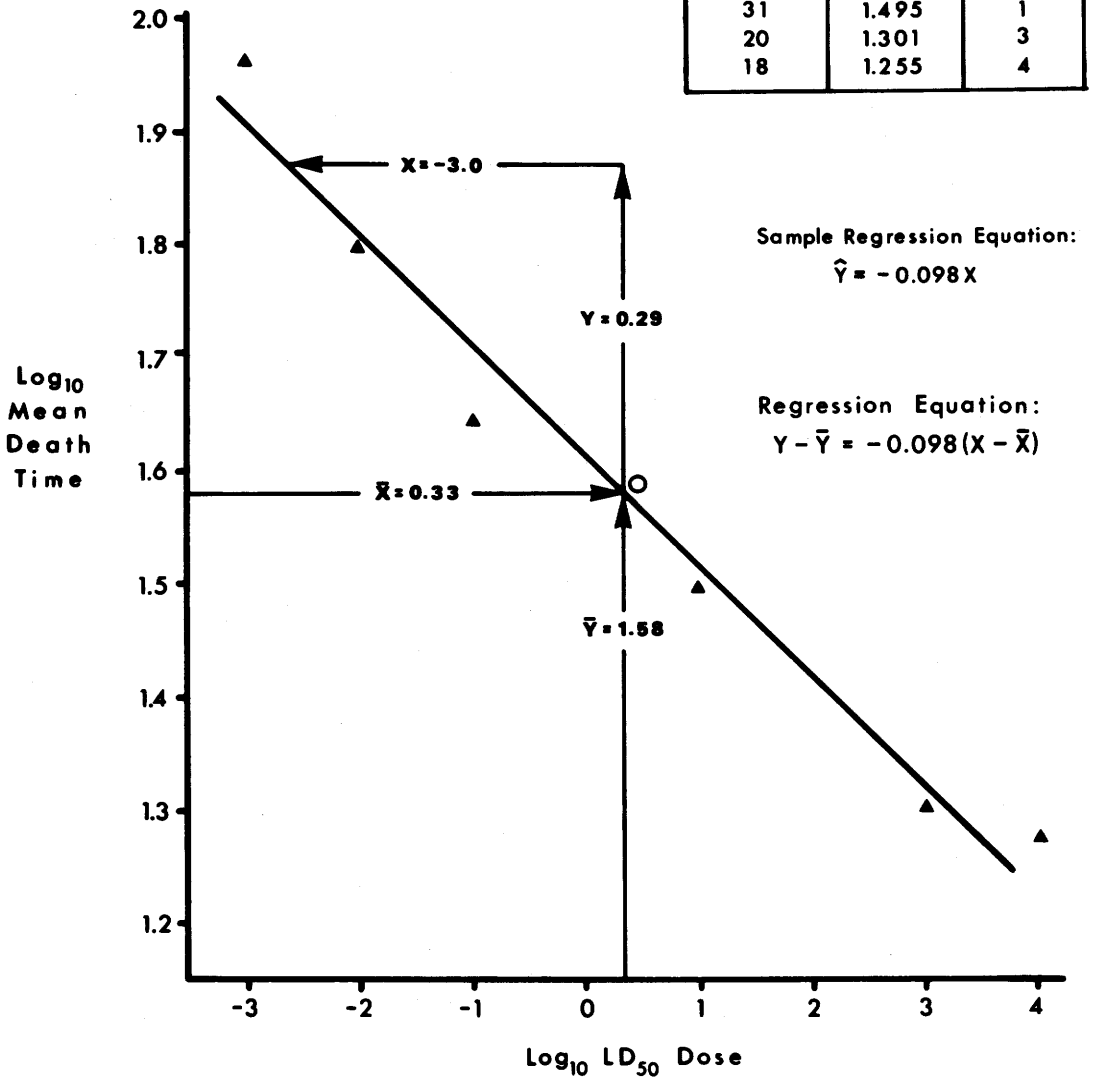
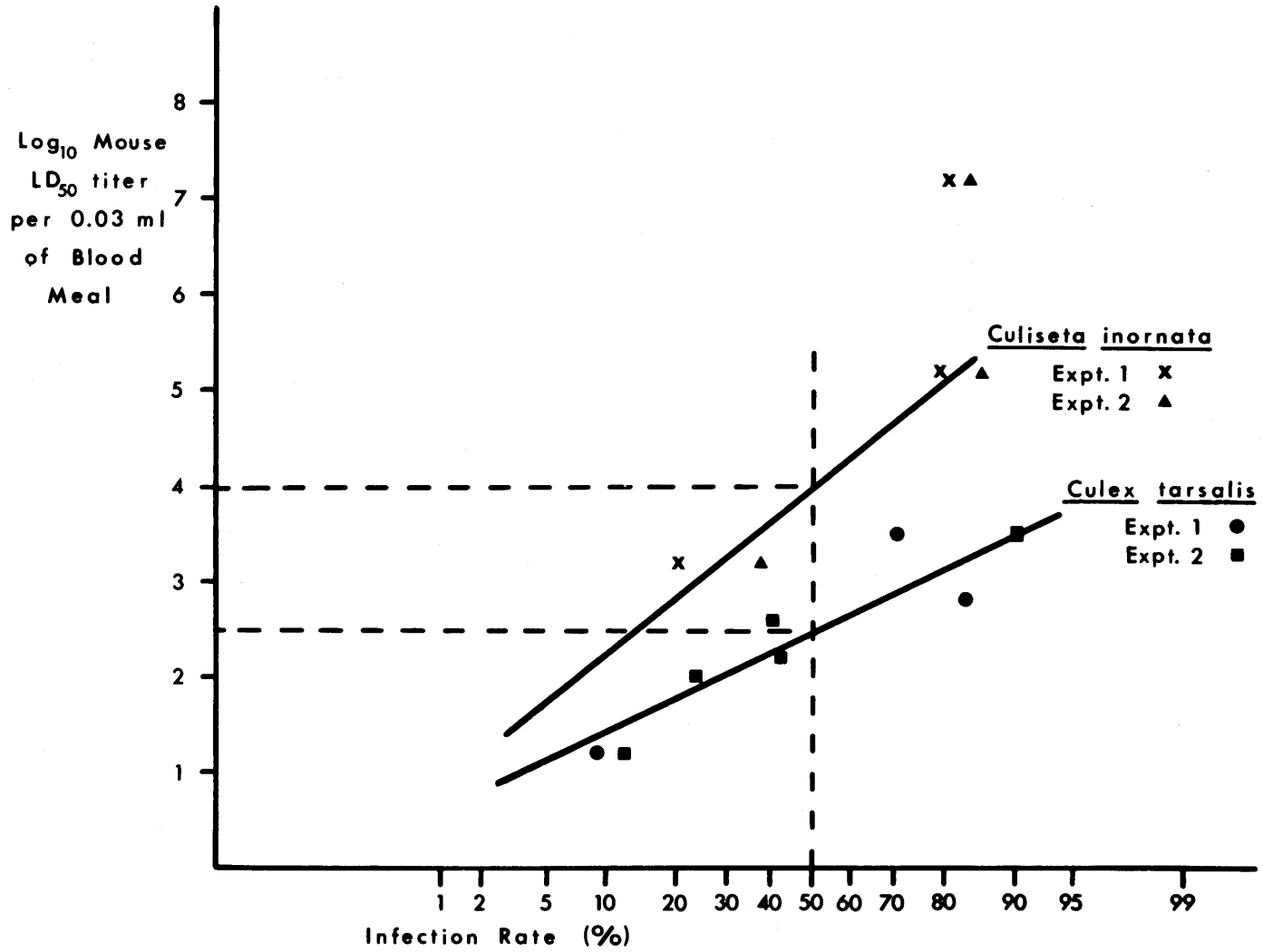


Figure 5

WEE INFECTION THRESHOLDS IN CULISETA INORNATA AND CULEX TARSALIS



References Cited in Appendix

1. Chamberlain, R.W., R.E. Kissling and R.K. Sikes. Studies in the North American arthropod-borne encephalitides. VII. Estimation of amount of eastern equine encephalitis virus inoculated by infected Aedes aegypti. Am. J. Hyg. 60: 286-291. 1954.

2. Chamberlain, R.W., R.K. Sikes, D.B. Nelson and W.D. Sudia. Studies on the North American arthropod-borne encephalitides. VI. Quantitative determinations of virus-vector relationships. Am. J. Hyg. 60: 278-285. 1954.