

THE REGULAR MOSQUITO IRIDESCENT VIRUS (RMIV) IN THE
BLACK SALTMARSH MOSQUITO, Aedes taeniorhynchus
(WIEDEMANN): PRODUCTION, PURIFICATION,
TRANSOVARIAL TRANSMISSION, SITE OF ENTRY,
DEVELOPMENT OF INFECTIONS, AND
POLYPEPTIDE COMPOSITION

By

STEPHEN CARSON HEMBREE

A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF THE
UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA
1974

Dedicated to Jane, my wife

ACKNOWLEDGMENTS

I wish to express gratitude to my supervisory committee: Dr. F. S. Blanton, Dr. R. E. Lowe, Dr. S. G. Zam, Dr. J. L. Nation, and Dr. F. W. Zettler.

Appreciation is also expressed to Dr. D. L. Wiedhaas, Director, and to the staff of the Insects Affecting Man and Animals Research Laboratory, U. S. Department of Agriculture, for their support of this project. I wish especially to thank Mr. D. W. Anthony, for assistance with electron microscopy, and Mrs. A. L. Cameron, Mr. H. R. Ford, and Mr. E. Green for assistance in many ways.

TABLE OF CONTENTS

	<u>Page</u>
ACKNOWLEDGMENTS	iii
LIST OF TABLES	viii
LIST OF FIGURES	ix
ABSTRACT	xi
INTRODUCTION	1
METHODS AND MATERIALS	21
Production of Virus	21
Routine Virus Production Procedure	21
Determination of Optimum Quantity of RMIV Inoculum	25
Determination of the Optimum Larval Age for Exposure to RMIV	26
Purification of RMIV	27
Quantification of RMIV Production	31
Attenuation of Infectivity of RMIV During Purification	32
Transmission of RMIV	33
Per Cent of Larvae Infected with RMIV by Transovarial Transmission	33
Transovarial Transmission Through Isolated Females	34
Sex Specificity of Transovarial Transmission of RMIV	36
Transmission of RMIV by Integument Puncture	37

TABLE OF CONTENTS (Continued)

	<u>Page</u>
Production of Antiserum to RMIV and to <u>A. taeniorhynchus</u> Antigens.	38
Purification of Gamma Globulin	40
Conjugation of Anti-RMIV Antibody with Fluoresceine Isothiocyanate (FITC)	41
Pilot Project on Autoradiography of ³ H-Methyl Thymidine Uptake and Dis- tribution in Larval <u>A. taeniorhynchus</u>	46
Production of ³ H-Methyl Thymidine Labeled RMIV	51
Site of Entry of RMIV into Host Tissue	52
Light Microscope Studies.	52
Autoradiography.	52
Fluorescent antibody	53
Electron Microscope Studies	53
Development of RMIV Infections	55
Autoradiography	55
Fluorescent Antibody.	56
Overt infections	56
Covert infections.	57
Number and Molecular Weights of RMIV Structural Polypeptides.	58
Isolation of RMIV Structural Polypeptides by Hydroxylapatite Chromatography.	60
RESULTS AND DISCUSSION	62
Production of RMIV	62
Determination of Optimum Quantity of RMIV Inoculum.	62

TABLE OF CONTENTS (Continued)

	<u>Page</u>
Determination of Optimum Larval Age for Exposure to RMIV.	65
Quantification of RMIV Production	65
Attenuation of Infectivity of RMIV During Purification.	68
Transmission of RMIV	70
Per Cent of Larvae Infected with RMIV by Transovarial Transmission	70
Transmission of RMIV Through Isolated Females.	72
Sex Specificity of Transovarial Transmission.	73
Total of Overt and Covert Transmission.	75
Transmission of RMIV by Integument Puncture.	78
Pilot Project on Autoradiography of ³ H-Methyl Thymidine Uptake and Distribution in Larval <u>A. taeniorhynchus</u>	79
Site of Entry of RMIV Into Host Tissues.	82
Light Microscope Studies.	82
Autoradiography.	82
Fluorescent antibody	86
Electron Microscope Studies	86
Development of RMIV Infections	97
Autoradiography	97
Fluorescent Antibody.	100
Overt infections	100
Covert infections.	107

TABLE OF CONTENTS (Continued)

	<u>Page</u>
Number and Molecular Weights of RMIV Structural Polypeptides.	110
Separation of RMIV Structural Polypeptides by Hydroxylapatite Chromatography.	114
SUMMARY.	115
BIBLIOGRAPHY	119
BIOGRAPHICAL SKETCH.	125

LIST OF TABLES

<u>Table</u>	<u>Page</u>
1 Net Virus Production Resulting from 24 Hr Exposure of Day Old <u>A. taeniorhynchus</u> Larvae to RMIV.	63
2 RMIV Production Resulting from 24 Hr Exposure of <u>A. taeniorhynchus</u> Larvae of Different Ages to 50 LEQ of Inoculum.	66
3 Attenuation of Infectivity Resulting from purification of RMIV Through the Stage Indicated	69
4 Transovarial Transmission of RMIV by Reciprocal Mating of Exposed and Unex- posed Male and Female <u>A. taeniorhynchus</u> . . .	74
5 Total of Overt and Covert Transmission of RMIV to Larvae Exposed at Different Ages.	77
6 Proteins Used as Markers for the Estimation of the Molecular Weights of RMIV Struc- tural Polypeptides.	111
7 Relative Migration Fractions and Molecular Weights of RMIV Structural Polypeptides . . .	112

LIST OF FIGURES

<u>Figure</u>		<u>Page</u>
1	Per Cent Transmission of RMIV in <u>A. taeniorhynchus</u> Larvae Exposed to Increasing Amounts of Inoculum.	64
2	Per Cent of RMIV Infection Among Progeny of Mosquitoes Exposed to RMIV as Larvae of Different Ages	71
3	Malpighian Tubule Cell with Labeled Nucleus	80
4	Anterior Midgut Region of Larva Showing Residual Deposits of Tritiated Thymidine Peripheral to Caudal Extremity of Foregut Invagination into Midgut.	83
5	RMIV Infected Fatbody Cell in <u>A. taeniorhynchus</u> Larva with Graination Over Cytoplasmic Deposits of Tritiated Thymidine Labeled Viral DNA	85
6	Midgut Epithelium Cell Containing Scattered RMIV Particles.	88
7	Midgut Epithelium Cell with Disarranged Microvilli and Containing Scattered RMIV Particles	89
8	RMIV Particles Pressed Against Peritrophic Membrane in Midgut of <u>A. taeniorhynchus</u> . . .	90
9	RMIV Particles Pressed Against Peritrophic Membrane in Midgut of <u>A. taeniorhynchus</u> . . .	91

LIST OF FIGURES (Continued)

<u>Figure</u>	<u>Page</u>
10	92
RMIV particle Between Peritrophic Membrane and Midgut Epithelium.	
11	93
Virus Like Particle Apparently Within a Microvillus	
12	95
Small Pocket of RMIV in Non-Epithelial Cell in Midgut Wall of <u>A. taeniorhynchus</u> Larva	
13	99
Methyl Green Staining of RMIV Infected Cells	
14	102
Fluorescent Antibody Staining of Epidermal Cells, Imaginal Bud Tissues and Fatbody Tissue Infected with RMIV	
15	103
Fluorescent Antibody Staining of RMIV Infected Tracheal Epithelium Cell	
16	105
Fluorescent Antibody Staining of Fatbody Surrounding Testis.	

Abstract of Dissertation Presented to the Graduate Council
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

THE REGULAR MOSQUITO IRIDESCENT VIRUS (RMIV) IN THE
BLACK SALTMARSH MOSQUITO, Aedes taeniorhynchus
(WIEDEMANN): PRODUCTION, PURIFICATION,
TRANSOVARIAL TRANSMISSION, SITE OF ENTRY,
DEVELOPMENT OF INFECTIONS, AND
POLYPEPTIDE COMPOSITION

By

Stephen Carson Hembree

March, 1974

Chairman: Franklin S. Blanton

Major Department: Entomology and Nematology

A system permitting the weekly production by 1 person of gram quantities of the regular mosquito iridescent virus (RMIV) in the black saltmarsh mosquito, Aedes taeniorhynchus (Wiedemann), was described. The efficiency of the system resulted from exposure of larvae at their most susceptible age to an optimum quantity of RMIV inoculum and from the use of a virus purification procedure designed to minimize waste. Transmission rates of about 10 per cent were routinely achieved. An average of 58 µg of virus was produced per infected larva, constituting 9.35 per cent of the dry weight of the average infected larva. Attenuation of infectivity occurred throughout the virus purification procedure, no major part of it being attributable to a single cause.

Transovarial transmission of the virus increased with the age of larvae at exposure through day 5 of larval life, but decreased when exposure was made just before pupation. All of the larvae produced by 9 isolated, transovarially transmitting females were infected. Transmitting females produced fewer average progeny than non-transmitting females. Males did not transmit virus to previously unexposed females. Total transmission (i.e., transmission resulting in overt disease plus transmission indicated only by infections among progeny) decreased with the age of larvae at exposure and was greatest for larvae in the second instar when exposed.

A probable site of entry of RMIV into host tissue was found by electronmicroscopy in midgut epithelium at the level of the foregut invagination. Infections were found in fatbody, epidermis, imaginal buds and tracheal epithelium by autoradiography of ^3H -methyl thymidine treated larvae 84 hr after the initiation of exposure to the virus. Infections were found in fatbody by fluorescent antibody staining 48 hr after initiation of exposure. Infection had appeared in epidermis and in imaginal buds by 72 hr after initiation of exposure, and in tracheal epithelium by 96 hr after initiation of exposure. Infections resulting in

transovarial transmission could not be detected by fluorescent antibody staining.

Twenty-five polypeptides were found in RMIV by polyacrylamide gel disc electrophoresis of sodium dodecyl sulfate--2-mercaptoethanol disrupted virus. The molecular weights of these were estimated by polyacrylamide gel disc electrophoresis and ranged from 23,500 to 331,000. Fourteen per cent of the genome of the virus could code for its structural polypeptides, assuming no redundancy and that no 5S ribosomal RNA or transfer RNA were coded for.

INTRODUCTION

The relationships between viruses and arthropods are of great interest and importance to man. Viruses are etiologic agents of many diseases of man, of his domestic animals, and of his crops. Arthropods are vectors for many of these and serve also as reservoirs for some diseases of both man and animals (Chamberlain, 1968) and of plants (Maramorosch, 1968). This necessitates an especially intimate relationship between the disease agents and their vectors. Arthropods themselves are susceptible to other viral diseases that are not known to infect either plants or other animals. Most of these have been found affecting insects (Smith, 1967). These diseases are of particular interest in consideration of current efforts to find non-chemical means of reducing some arthropod populations. Although the pathogenicity of the viral diseases of arthropods has long been recognized, the realization that many arboviruses and arthropod-transmitted plant viruses are pathogenic to their vectors is relatively recent (Chamberlain, 1968; Maramorosch, 1968). The relationships between viruses and

arthropods are now known to form a symbiotic spectrum ranging from mutual benefit at one extreme to population decimating lethal parasitism at the other (Maramorosch and Jensen, 1963).

The viruses of insects can be divided into two groups on the basis of whether the virions are enveloped in protein crystals (occluded) or whether they are not (non-occluded) (Smith, 1967, p. 8). Of the non-occluded insect viruses, the iridescent viruses have attracted the attention of virologists for several reasons. They are the largest symmetrical viruses known (Smith, 1967, p. 84); they are produced in great relative quantity, composing up to 25 per cent of the dry weight of their host (Williams and Smith, 1957); they are probably the easiest of all viruses to isolate and purify (Smith, 1967, p. 78); and they are DNA containing viruses which replicate in the cytoplasm of host cells (Wildy, 1971).

The International Committee on Nomenclature of Viruses (ICNV) established at the International Congress for Microbiology in 1966 placed the iridescent viruses in the genus Iridovirus (Wildy, 1971). The members of this genus were defined as large (130 - 180 nm), non-occluded, icosahedral, DNA containing viruses which replicate in

the cytoplasm of the host cell. The Tipula iridescent virus (TIV), isolated from the leatherjacket, Tipula paludosa (Meigen) (Diptera), was designated as the type species. Other members of the genus were Chilo iridescent virus (CIV), from the rice stem borer, Chilo suppressalis Walker (Lepidoptera), and Sericesthis iridescent virus (SIV), isolated from the pruinose scarab, Sericesthis pruinosa (Dalman) (Coleoptera). The literature of these three viruses recently has been well reviewed by Smith (1967) and Bellett (1968). The mosquito iridescent virus (MIV), first described from the black saltmarsh mosquito, Aedes taeniorhynchus (Wiedemann), by Clark et al. (1965), was listed as a probable member of the genus. The uncertainty shown at that time was due to a lack of information of the physical and chemical characteristics of this virus which subsequently became available (Matta, 1970; Matta and Lowe, 1970). At the present time, no species names have been approved for iridoviruses by the ICNV, and an informal nomenclature that identifies the viruses by use of the name of the host has gained acceptance in the current literature and has been used in this dissertation.

The most conspicuous feature of the iridoviruses that have been recognized by the ICNV is their characteristic

iridescence when viewed by reflected light. Visible color is evident both in heavily infected host tissue and when the purified virus is pelleted by ultracentrifugation. This iridescence has been explained as Bragg reflection of visible light from randomly oriented microcrystals of the virus, the formation of which is made possible by the extreme regularity of size and shape of the virions (Williams and Smith, 1957; Smith and Williams, 1958; Klug et al., 1959). This feature of the genus Iridovirus is shared with a number of other insect viruses which could not be included in the genus because too little was known of their characteristics. The extent to which the phenomenon of iridescence is correlated with the physical, biological and biochemical characteristics which define the genus Iridovirus is uncertain. It is conceivable that other viruses might be found that iridesce but differ from the iridoviruses as they have been defined. Several icosahedral cytoplasmic deoxyriboviruses are known which do not form into the paracrystalline arrays necessary for iridescence (Stoltz, 1971). Nevertheless, the iridescent viruses of insects are generally considered to be closely related (Bellett, 1968; Bellett and Inman, 1967; Cunningham and Tinsley, 1968; Glitz et al., 1968; Wildy, 1971). Tinsley and Kelly

(1970) suggested an interim nomenclature system for this group of viruses. Their published list of 12 viruses should now contain two additional ones recently reported from a chaoborid, Corethrella brakelevi, in Louisiana (Chapman et al., 1971) and from a grass grub, Costelytra zealandica, in New Zealand (Kalmakoff et al., 1972):

Iridescent viruses have been reported infecting several species of Aedes mosquitoes and one of Psorophora from widely separated localities:

Aedes taeniorhynchus (Wiedemann)

Florida (Clark et al., 1965; Hall and Lowe, 1971)

Louisiana (Chapman et al., 1966)

Aedes annulipes (Meigen)

Czechoslovakia (Weiser, 1965)

Aedes cantans (Meigen)

Great Britain (Tinsley et al., 1971)

Czechoslovakia (Weiser, 1965)

Aedes fulvus pallens (Ross)

Louisiana (Chapman et al., 1966)

Aedes vexans (Meigen)

Louisiana (Chapman et al., 1966)

Aedes dorsalis (Meigen)

Nevada (Chapman et al., 1966)

Aedes detritus (Haliday)

Great Britain (Service, 1968)

Tunisia (Vago et al., 1969)

France (Hasan et al., 1970)

Aedes stimulans (Walker)
Connecticut (Anderson, 1970)

Psorophora ferox (Humbolt)
Louisiana (Chapman et al., 1966)

The relationship of these isolates is not completely known. Cross infectivity studies were conducted among the known hosts of mosquito iridescent viruses in Louisiana (Woodard and Chapman, 1968), and in all tests the isolates were infective for species other than their own host. However, there was consistently less than 1 per cent infectivity, compared to 15 per cent to 18 per cent infectivity in the original hosts. These data suggest the viruses from different species were not identical. Aedes sollicitans (Walker) is frequently found in natural breeding sites with A. taeniorhynchus, but has never been found infected with RMIV in nature, even in the presence of infected A. taeniorhynchus. However, it can be infected artificially in the laboratory with about the same infectivity rate as the natural host. The iridescent virus from A. stimulans is apparently distinct from other MIV, since it is considerably smaller (135 nm vs. 180 nm) and is lethal to the larvae in the late third rather than the fourth stadium (Anderson, 1970). Isolates of MIV from A. detritus, collected in Tunisia and Southern France, were compared morphologically

and serologically and were considered to be identical (Hasan, et al., 1970). The iridescent virus from A. cantans in Great Britain showed a relationship to that from A. taeniorhynchus by the tube precipitin test but showed no relationship to TIV, SIV, CIV, or to the iridescent virus from Wiseana cervinata (Lepidoptera) in New Zealand (Tinsley et al., 1971). In addition, the iridescent virus from A. taeniorhynchus was unrelated to TIV or SIV when tested by complement fixation (Cunningham and Tinsley, 1968).

Of the iridescent viruses reported from mosquitoes, only the one from A. taeniorhynchus in Florida (Clark et al., 1965) has been well characterized. Two strains of this virus were reported from Louisiana. One exhibited a yellow to green, and sometimes pink, iridescence and the other a blue iridescence (Woodard and Chapman, 1968). The latter has been collected only from Louisiana, and it has been suggested that this isolate is not as prevalent as the former (Hall and Lowe, 1971). The blue isolate was designated the turquoise mosquito iridescent virus (TMIV), and the orange isolate, the regular mosquito iridescent virus (RMIV) (Matta and Lowe, 1970). The infectivity of the 2 isolates was shown to be about the same (Woodard

and Chapman, 1968). Slightly more DNA was present in RMIV than in TMIV (Faust et al., 1968; Wagner et al., 1973) and neither contained RNA (Faust et al., 1968). RMIV and TMIV have been further compared physically, chemically, and serologically (Lowe et al., 1970, Hall and Lowe, 1972). Measurement of the virions of both types, in tissue sections and after negative staining of purified virus, indicated that RMIV was larger than TMIV. Also, RMIV had a greater sedimentation rate in sucrose density gradients and greater density in CsCl gradients. No antigenic differences could be detected between the strains at the level of sensitivity of agar gel diffusion tests. Both strains infected the same host tissues and caused similar cytopathology. Tubular structures of unknown origin, slightly smaller in diameter than the virus they accompanied, were found in tissues infected with both strains (Lowe et al., 1970; Hall and Anthony, 1971). A recent study attributed several biophysical differences between the strains to their relative sizes (Wagner et al., 1973). Additionally, their electrophoretic mobilities were different, but their isoelectric points were similar. Differences were shown in the percentages of protein, DNA, and lipid, but structural differences other than size were not detected.

Several physicochemical characteristics of RMIV have been reported by Matta (1970). Spectrophotometric analysis showed that the ratios of ultraviolet absorption to the concentration of virus suspension were constant at a wavelength of 700 nm, and an extinction coefficient ($E_{700}^{1\%}$) of 10.8 was determined to permit easy quantification of purified virus suspensions. The sedimentation coefficient ($S_{20,w}$) was 4458, and the density was calculated to be 1.354 g/cm^3 . The particle weight, calculated from the diameter (180 nm) and density of the particle, was $4.129 \times 10^{-15} \text{ g}$ or 2.486×10^9 daltons. The DNA content of $15.97 \pm 0.29\%$, determined by diphenylamine with a known quantity of virus, was larger than the 11.7 per cent reported previously by Faust et al. (1968) and accounted for a DNA molecular weight of 397×10^6 daltons. Wagner et al. (1973) reported a content of $16.9 \pm 0.1\%$ DNA, and, using this figure and the particle weight, they calculated the molecular weight of the DNA to be 464×10^6 daltons. Both of these figures for the molecular weight of DNA are more than twice as large as those of any other known non-iridescent virus (Wagner et al., 1973). The amino acid composition was found to be very similar to that of SIV and TIV. Wagner et al. (1973) also found lipid in RMIV,

but the possible structural role of this lipid, like that found in TIV (Glitz et al., 1968), remains unresolved. Some of these data were used to differentiate RMIV from other iridescent viruses (Lowe et al., 1970).

The structure of RMIV has been studied by high resolution electronmicroscopy as one representative of icosahedral cytoplasmic deoxyriboviruses by Stoltz (1971). He reported that the electron dense nucleoid was surrounded by a trilaminar membrane, morphologically resembling a unit membrane, that closely followed the contour of the nucleoid and was thought to be a part of it.

Surrounding the nucleoid and the inner membrane was an outer membrane, closely appressed to the latter in intact virions. The contact line of the 2 membranes was electron dense and twice as thick as a single membrane lamella. In sectioned material there appeared to be a layer of morphological subunits on the surface of the outer membrane, but they could not be resolved by negative staining of intact virions. The rigidity of these external structural subunits was thought to be responsible for the icosahedral shape of the virus. The outer membrane and morphological subunits thereon were defined as the shell of the virus, because they tended to maintain their characteristic angular

configuration in disrupted particles. The inner membrane appeared flexible and could maintain the contour of either the nucleoid or the shell. The inner membrane remained attached to the nucleoid in disrupted particles more frequently than to the shell. In a note appended to his paper, Stoltz (1971) proclaimed that morphological subunits had been detected by negative staining on the surface of collapsed virions and that these were organized into triangular shaped "trisymmetrons" (terminology of Wrigley, 1969, 1970) which were hexagonally arranged. Thus, the structure of the shell of RMIV resembled that of SIV (Wrigley, 1969) and TIV (Wrigley, 1970; Stoltz, 1971).

Clark et al. (1965) demonstrated that per os transmission of MIV was possible, and they also reported that the infectivity of the virus apparently was destroyed by either drying or putrefaction. Woodard and Chapman (1968) routinely transmitted the virus in the laboratory by per os exposures for up to 68 serial passages with a mean transmission rate of 16 per cent. Their studies also indicated that various exposure methods influenced the success of transmission of the virus. Larvae exposed to RMIV for 24 hr during the first through the third, but not the fourth, stadium demonstrated infection before pupation. Optimal transmission appeared to occur when the larvae

were exposed during the second stadium. Transmission increased with the duration of exposure up to 48 hr, but additional exposure caused only a slight increase in per cent transmission. When late first and early second instar larvae were exposed for 24 hr to various numbers of macerated, infected larvae, there was a sharp increase in the transmission rate as the quantity of inoculum increased but also a sharp increase in larval mortality. However, transmission was quantitated on the number of surviving larvae rather than the number of exposed larvae, and the figures given may be deceptive. Also, the net number of infected larvae produced (total number produced minus number of infected larvae used in the inoculum) from the test with the most inoculum was only about 1/3 of that produced from a group exposed to 1/10 of the same amount of inoculum.

Linley and Nielsen (1968a) conducted a series of carefully described experiments on the transmission of RMIV in the laboratory. The percentage of transmission in these experiments was determined on the basis of the number of larvae exposed, and exposure was not done in the condition of extreme crowding that resulted in high larval mortality for Woodard and Chapman (1968). However,

even with the modifications, their results generally substantiated those of Woodard and Chapman (1968).

Matta and Lowe (1970) reported 2 separate infectivity tests with known numbers of first stadium A. taeniorhynchus larvae exposed to known amounts of inoculum and in which the number of survivors was determined. Transmission rates were 7.73 per cent and 15.6 per cent when calculated on the basis of number of survivors, but only 2.67 per cent and 5.95 per cent, respectively, when calculated on the basis of number of larvae exposed, and larval mortality was greater than 60 per cent in both tests. The increase in virus produced (number of infected larvae produced, minus number of infected larvae used as inoculum, divided by number of infected larvae used as inoculum) was 167.5 per cent and 197.5 per cent in the respective experiments. It was stated that the infectivity of the virus was not significantly reduced by treatment with ether, but it was not stated whether the test was performed as previously described by Andrewes and Horstmann (1949).

Experiments relative to the transmission of RMIV in nature were described by Linley and Nielsen (1968b). These experiments demonstrated that infection could be acquired per os by healthy larvae exposed to virus from

either macerated or unmacerated larvae under field conditions. Virus from macerated hosts retained its infectivity in 10 per cent sea water for 3 days in the laboratory, and then slowly attenuated over a period of 30 days. There was less retention of infectivity in artesian water, and the virus was uninfective after 2 days on damp sod.

Transovarial transmission of RMIV was first observed and substantiated by Woodard and Chapman (1968). Infected larvae (never more than 2 per cent) were always found among larvae reared from the eggs of survivors of experiments on the infectivity of RMIV. Sterilization of all containers, instruments and media did not reduce the level of transmission. When late third or early fourth instar larvae were exposed to virus, they showed no gross symptoms of infection before or after becoming adults. However, in 3 experiments the larvae developing from eggs deposited by these adults showed an average infection of almost 30 per cent. Linley and Nielsen (1968a) excluded the possibility of transovum transmission by surface sterilization of the eggs. Additional support for a transovarial mechanism was provided when Hall and Anthony (1971) found the virus in both larval and adult ovaries.

All the progeny of 5 isolated, transmitting females observed by Linley and Nielsen (1968a) and 3 observed by Hall and Anthony (1971) were infected, suggesting an all or none mechanism of transovarial transmission. The 5 transovarially transmitting females produced an average of only 7.4 larvae while non-transmitting females of the same generation produced an average of 26.7 larvae (Linley and Nielsen, 1968a). This indicates the proportion of transovarially transmitting females in a population is not necessarily equal to the proportion of infected larvae produced by that population. Evidence from 19 separate tests indicated that virus infections were not carried covertly from the second to the third generation (Linley and Nielsen, 1968a).

Both Woodard and Chapman (1968) and Linley and Nielsen (1968a) suggested that RMIV could be transmitted to only a susceptible proportion of an exposed population. They made no attempt to test that hypothesis nor did they propose an explanation of why only a proportion of a population would be susceptible. Woodard and Chapman (1968) believed that the susceptible proportion of a population remained fairly constant regardless of when the larvae were exposed. This hypothesis was based on the

untenable assumption that the proportion of covertly infected adults in a population was equal to the proportion of infected larvae they produced.

Linley and Nielsen (1968b) proposed a tentative account of the natural transmission of RMIV in A. taeniorhynchus. They stated,

Transovarial transmission produces infected larvae which die in the fourth instar. These then provide possibly the only, but certainly by far the most important, source of new infection, which is acquired per os by the healthy larvae when they feed on the diseased cadavers prior to pupation. This in turn leads to the presence of infected adults which complete the cycle by depositing infected eggs (p. 24).

Few observations have been made on the gross pathology accompanying the development of infections with RMIV. Chapman et al. (1960) observed that iridescence appeared first in the thorax of late third or early fourth instar larvae and then spread throughout the abdomen during the fourth larval stadium. Most of the patently infected larvae died before pupation. Matta and Lowe (1970) maintained that iridescence first appeared in the lateral portions of one of the first four abdominal segments. Linley and Nielsen (1968a) noted that larvae infected transovarially appeared to move, feed and develop normally

until shortly before death, when they became sluggish and ceased to feed. After death, the abdomen often contracted, and some of the larvae remained suspended from the surface of the water and were often cannibalized.

Clark et al. (1965) first studied the histopathology of RMIV infections and stated that the virus appeared to develop in cell cytoplasm of adipose tissue. Matta and Lowe (1970), using darkfield microscopy and a staining technique previously described by Matta and Lowe (1969), observed the virus in fatbody and imaginal discs and noted that the destruction of these tissues was usually complete in patently infected larvae. In rare instances larvae survived, and in the pupae the fatbody had not been completely destroyed. Using the electron microscope, Hall and Anthony (1971) found the virus in tracheal epithelium and epidermal tissues, in addition to fatbody and imaginal discs, and to a lesser extent in hemocytes, foregut, nerve, muscle, and in both larval and adult ovaries. Disruption of metabolic processes in the larval fatbody was thought to be the cause of death. Virus was never found in midgut or hindgut epithelium or in malpighian tubules.

In attempts to locate the site of entry of RMIV into host tissue Hall and Anthony (1971) found virus particles

in the esophageal region of the foregut epithelium after larvae were exposed to suspensions of the virus. It is unlikely that the virus passed through the cuticular lining in this region of the gut, and it was not stated how long the larvae had been exposed to the virus suspension. Stoltz and Summers (1971) presented the hypothesis that virus entered the epithelium in the extreme anterior region of the midgut. This hypothesis was based, in part, on the observation that intact virus particles were not found more than 100 μ distal to the foregut invagination into the midgut. The peritrophic membrane has been considered an effective barrier to intact virions, but both Hall and Anthony (1971) and Stoltz and Summers (1971) expressed the possibility that subviral components of nucleic acid or nucleoprotein might pass through the membrane.

The present dissertation further elucidates the relationships between RMIV and A. taeniorhynchus. Techniques for mass production and purification of large amounts of RMIV in minimal amounts of time were essential to several of these efforts. Tinsley and Harrap (1972) stated at the Second International Congress for Virology that one of the main difficulties in research with MIV was its low virulence and the resulting lack of sufficient quantities

of the virus obtained from laboratory hosts. Therefore, priority was placed on the development of methods to produce large (gram) quantities of purified RMIV. Standardized management of the host was regarded as essential to the mass production of the virus. Several host management methods involving different feeding regimens and the use of different rearing containers were attempted. Only the most successful of these has been described. Experiments were conducted to determine the optimum quantity of inoculum necessary and the optimum age at which to expose larvae to assure maximum net virus production. The production of RMIV and the effect of infection with the virus on the weight of the host were quantitated. The attenuation of infectivity of RMIV resulting from purification was studied to determine if it could be attributed predominantly to any particular one of the procedures in purification. Several experiments were conducted to elucidate the quantitative aspects of vertical transmission within a host population and to determine if it was possible for RMIV to be transmitted by males to previously unexposed females. Attempts were made to transmit RMIV by integument puncture, as described by Clark (1966), to determine if resistance to entry of virus into host tissues was responsible for

failures to achieve high rates of transmission by per os exposures. The site of entry of RMIV into host tissue was sought by fluorescent antibody and autoradiographic observations with the light microscope and by examination of ultrathin sections of exposed first instar larvae with the electron microscope. The development of overt infections with RMIV in larvae exposed in the second larval stadium was studied by fluorescent antibody staining and by autoradiography. The development of covert infections with RMIV in larvae, pupae and adults, exposed as late instar larvae, was studied by fluorescent antibody staining. The number of structural polypeptides composing RMIV was determined and their molecular weights estimated by polyacrylamide gel disc electrophoresis. Attempts were made to isolate these polypeptides by hydroxylapatite chromatography.

METHODS AND MATERIALS

Production of Virus

Routine Virus Production Procedures

The initial source of RMIV used in these studies was live, infected, fourth stadium larvae of A. taeniorhynchus mosquitoes collected from a small saltmarsh on the southwest side of Atsena Otie Key, an island near the town of Cedar Key, Florida. All mosquito eggs used to produce larvae for routine virus production were acquired from the mosquito colony of the U. S. Department of Agriculture Insects Affecting Man and Animals Research Laboratory, at Gainesville, Florida. The colony procedures used for mosquito production are being described for publication (Ford and Green, in prep.).

Eggs were obtained from the colony on damp sphagnum moss in pans covered with aluminum foil. They could be stored at room temperature for up to 3 months without unacceptable loss of vigor. Periodic examinations of larvae produced in the colony provided assurance that there were no latent

virus infections present. Tap water was used to rinse the eggs from the moss into a round enamel pan, using a screen to collect the moss and debris. The water was swirled rapidly to accumulate the eggs in the center of the pan, and they were then pipetted into graduated conical bottom centrifuge tubes, 1 cm³ per tube. Each tube of eggs was poured into an enamel hatching pan containing 2 liters of 0.05 M NaCl made with distilled water. The eggs were again accumulated by swirling, and an infusion of 200 mg of live brewer's yeast was added to each pan. The eggs were allowed to hatch for 1 hr after which the unhatched eggs were removed. One cm³ of eggs provided about 20,000 larvae in each pan. These were allowed to develop for 24 hr in the yeast infusion.

The progression of mosquito life was measured by days, or 24 hr periods, beginning at hatching, regardless of the time of day this occurred. No effort was made to coordinate any treatment with moulting or ecdysis, but it is pertinent that passage through each of the first 3 stadia required about 24 hr with adequate food and space (Nayar, 1967). All water temperatures were maintained at 27°C throughout rearing.

Twenty-four-hr-old larvae were poured onto an organdy

cloth screen, washed with tap water, and placed in a small volume of 0.05 M saline. Eight oz waxed-paper cups were used as containers to expose the larvae to virus. Twenty-five hundred larvae were counted into 1 cup containing 200 ml of 0.05 M saline, and this cup was then used as a visual standard to pipette an estimated 2500 larvae into each exposure container to be used. Inoculum was prepared by triturating fresh, RMIV infected fourth stadium larvae in a Ten Broeck type tissue grinder until their head capsules were completely destroyed. It was quantitated as larval equivalents (LEQ), 1 LEQ being the virus from 1 patently infected larva, whether freshly triturated or at some stage in virus purification. The inoculum was filtered through an organdy screen before being pipetted into the exposure containers. Routine exposures were for 24 hr without additional food.

Rearing containers, prepared several hours before needed, were white plastic trays,¹ 56.7 x 44.7 x 8.0 cm, containing 7.5 liters of 0.05 M saline made with table

¹panel Control Corp., Detroit, Michigan.

salt and tap water. After exposure, the larvae and inocula were poured into the rearing trays. One gram of high protein, low fat content hog food supplement¹ (ground fine enough to pass through a screen of 50 meshes per inch) was added as a thoroughly wetted slurry to each tray. The larvae were fed an additional 2 grams of food 48 and 72 hr after being placed in the rearing trays. With this schedule, no aeration was necessary, and no fouling of the water occurred. Occasionally, a few pupae appeared late in the fifth day of larval life, but pupation usually began late in the sixth day after the eggs hatched.

The collection of infected larvae began when the first pupae were observed in the trays. The contents of the trays were poured through a screen of 30 meshes per inch to retain the larvae. The trays were washed immediately with cold tap water and a scrub cloth to remove all old infusion and detritus. Soap was never used, and no attempt was made to sterilize the trays, although they were allowed to dry thoroughly before being used again. The larvae

¹Southeast Hog Supplement (40% protein), Purina, St. Louis, Missouri.

were placed in a black photographic tray containing ice water to immobilize them. The infected larvae, which were conspicuously iridescent against the black background, were removed with a bulbed pipette and held in cold 0.05 M Tris-HCl buffer, pH 7.0.

Determination of Optimum
Quantity of RMIV Inoculum

Eggs were hatched and larvae allowed to develop for 24 hr as described above. Six experimental groups of larvae, each with replicates of 2500 larvae, were exposed for 24 hr to 5, 10, 25, 50, 75, and 100 LEQ of inoculum per group, respectively. Controls were exposed to an equal number of triturated uninfected larvae. Groups exposed to an inoculum of only 5 LEQ were given a small amount of food. At the end of the exposure period, all groups were examined for larval mortality, and the larvae were transferred to rearing trays and maintained as described above. Feeding of groups that experienced mortality during exposure was reduced to avoid contaminating the rearing containers with excessive food. Infected larvae from these treatments were harvested and counted late in the fourth stadium. Only larvae showing visible signs of RMIV infection were considered infected. Net production of virus,

quantitated as LEQ, was determined. A qualitative judgment of mortality was made, since only a low level of mortality could be tolerated in any virus production system to be used routinely. The experiment was repeated 3 times in successive weeks to be certain the system was replicable.

Determination of the Optimum
Larval Age for Exposure to RMIV

Six experimental groups, composed of 2 replicates of 2500 larvae each, were exposed for 24 hr to 50 LEQ of RMIV inoculum during days 1 through 6 of larval life, respectively. Exposure of group 1 was made immediately after hatching, and exposure of group 2 was made during the second day of larval life as described for routine virus production. Larvae of groups 3 through 6 were placed in rearing trays when 24 hr old, fed and held by routine methods until the respective groups were old enough to expose. After exposure, all larvae were carefully rinsed with tap water to remove external inoculum and returned to rearing trays. Groups that had been exposed were screened and infected larvae collected and counted at 12 hr intervals beginning early in the fourth day of larval life and continuing through day 7, when pupation was almost

complete. Net production of virus, quantitated as LEQ, was determined from 2 experiments conducted at an interval of 1 month.

Purification of RMIV

All virus purification procedures were performed at 0° - 4°C in 0.05 M Tris - HCl buffer, pH 7.0. Virus infected larvae were triturated in a Ten Broeck type tissue grinder until their head capsules were completely destroyed. The triturant was washed through organdy cloth into a beaker, and the volume was adjusted to 50 ml per 1000 larvae. The beaker was covered and the contents stirred with a magnetic stirrer for 12 - 24 hr to further fragment the tissues. The triturant was then placed in a separatory funnel and brought to a 20 per cent (v/v) mixture with cold ethyl ether. The mixture was shaken vigorously for 5 min, placed in an explosion proof refrigerator, and allowed to separate for 6 hr. The buffer layer was removed and re-extracted with ether. The first ether layer was washed with the original volume of buffer and allowed to separate an additional 6 hr. These extractions were effective in removing much of the debris from the virus suspension. The final buffer layers were combined in a

beaker and placed in an ice bath. The residual ether was removed by stirring the mixture gently (to avoid foaming) for 12 - 24 hr with a magnetic stirrer under a ventilated hood, while passing a gentle stream of air over the top of the virus suspension. Following ether extraction, the virus was pelleted by centrifugation in 50 ml round bottom centrifuge tubes at 10,000 g for 40 min, in an International Equipment Company (IEC) PR-6 refrigerated centrifuge equipped with a high speed accessory attachment. The supernatant buffer was discarded, 10 ml of new buffer were added to each tube, and the pellets were allowed to soften overnight before being resuspended by vortexing. After resuspension, the volume was adjusted to 80 ml per 1000 LEQ being processed, and the virus was subjected to 4 cycles of differential centrifugation. The low speed cycle was 15 min at 1000 g, and the high speed cycle was 40 min at 10,000 g. The low speed pellets of the first 3 cycles were scavenged by resuspending them to 25 per cent of their original volume, combining them, and recentrifuging them for 20 min at 1500 g. The virus in the supernatant buffer was pelleted by a high speed run. All high speed pellets were allowed to soften in buffer for at least 6 hr before being resuspended by vortexing.

Virus in the high speed pellets from the scavenging runs was returned to the main bulk of virus before the succeeding low speed run. The final low speed pellet was discarded.

The concentration of resuspended virus from the final high speed run was estimated spectrophotometrically and adjusted to approximately 20 mg/ml with a Beckman DU-2 spectrophotometer, and the absorption curve at 700 nm (Matta, 1970). Sucrose density gradients were prepared by successively layering 2.5 ml aliquots of 55 per cent (w/v), 44 per cent, 30 per cent, and 15 per cent sucrose in 13 ml, 1.4 x 9.7 cm centrifuge tubes. The gradients were allowed to form naturally in cold for at least 12 hr before use, and gradients not used within 48 hr of preparation were discarded. The virus suspension was diluted 1:1 with 10 per cent sucrose, and 2 ml of this material were layered on the gradients and centrifuged at 20,000 g for 20 min in an IEC B-60 ultracentrifuge equipped with a type SB-283 head. After centrifugation, the virus was seen as a distinctive band about two-thirds of the way down the tube, with a diffuse layer of top component slightly above it. Initially, a small pellet of iridescent material was seen on the bottom of the tubes after sucrose gradient

centrifugation, but later results showed that proper care in disrupting and resuspending the final high speed pellet from differential centrifugation prevented its formation. The virus band, but not the top component, was removed with a needle and syringe. The virus suspension was diluted 1:1 with distilled water and pelleted in a Beckman Model L preparative ultracentrifuge equipped with an SW-39 head. The pellets were resuspended and washed twice with distilled water to remove the residual sucrose. The final pellets were resuspended in a minimum volume of distilled water, quantitated spectrophotometrically, adjusted to a concentration of 15 mg/ml, and lyophilized with a Virtis lyophilizer. The lyophilized virus (15 mg/tube) was stored at -30°C .

Virus purified by this procedure reacted vigorously with its homologous antiserum formed in rabbits, but not with normal rabbit serum, in tube precipitin tests. It did not react with antiserum against acetone extracted A. taeniorhynchus tissue in tube precipitin tests, by agar gel diffusion tests, or by immunoelectrophoresis in agarose. It appeared homogeneous in the electron microscope. Matta (1970) found that virus purified by a similar but less rigorous method produced a single sedimentation boundary in an analytical ultracentrifuge equipped with

Schlieren optics.

Quantification of RMIV
Production

Fifteen groups of 2500 larvae each were exposed to an inoculum of 50 LEQ per group and reared to the advanced fourth stadium as described for routine virus production. Infected larvae were collected and counted, and the total number was divided into 2 equal groups. A third group of larvae, showing no overt symptoms of RMIV infection and equal in number to 1/2 the total number of infected larvae, was also collected. The virus from one group of the infected larvae was purified by methods described above and quantitated spectrophotometrically. The other group of infected larvae and the group of apparently uninfected larvae were lyophilized with a Virtis lyophilizer until weight loss stopped. The weight of both groups was determined on a Mettler H-5 balance. From these data the following results were determined: (1) the per cent of transmission acquired by routine virus production procedures, (2) the quantity of purified virus produced per infected larva, (3) the weights of infected and uninfected larvae from the same rearing set, and (4) the per cent of larval dry weight attributed to the virus.

Attenuation of Infectivity
of RMIV During Purification

Transmission studies with purified, lyophilized virus indicated that there was lower transmission with this material than when other preparation were used. Experiments were conducted to determine if the loss of infectivity occurred at any particular stage of the virus purification procedure.

Six experimental classes were established, each composed of 5 groups of 200 larvae. Twenty-four-hr-old larvae were exposed for 24 hr to virus which had been purified through various stages of purification as follows: (1) fresh, triturated infected larvae; (2) buffer extracted virus; (3) ether extracted virus; (4) virus subjected to 4 cycles of differential centrifugation; (5) virus which had been subjected to sucrose density gradient centrifugation; and (6) virus which had been purified and lyophilized.

Larvae in classes 1 and 2 were exposed to 5 LEQ of inoculum per group. Larvae in classes 3 and 4 were exposed to 10 LEQ per group. Larvae in classes 5 and 6 were exposed to 20 LEQ per group. The use of increasing amounts of inoculum was necessary to assure infections in all classes, because preliminary tests showed that exposure of

classes 1 and 2 to the quantities of inoculum necessary to produce infections in classes 5 and 6 resulted in high mortality during exposure. A small amount of food was provided larvae in classes 3 through 6 during exposure, and for each group 200 larvae were reared in rectangular, white enamel pans containing 1 liter of 0.05 M NaCl. The larvae were examined for RMIV infection in the late fourth stadium, and per cent transmission was determined for each class. The experiment was replicated each week for 4 weeks.

Transmission of RMIV

Per Cent of Larvae Infected with RMIV by Transovarial Transmission

Experiments were conducted to determine the per cent of infected F₁ generation larvae produced by adults that had survived exposure to RMIV at different times during their larval life. The surviving adults from the 6 experimental groups in the experiment "Determination of the Optimum Age for Exposure to RMIV" were maintained in an adult rearing room at 80°C and 70 per cent relative humidity. Cotton pads soaked in 5 per cent sucrose were continually available as a food source, and shaved guinea pigs provided

blood meals on consecutive days. Five days after emergence, 16 oz cups, containing loosely packed, damp sphagnum moss were offered as an oviposition medium. The moss was left in the cages for 5 days, then covered with aluminum foil, and the collected eggs were allowed to develop an additional 10 days. The eggs then were hatched and reared for 24 hr as described for routine virus production. Two trays of 2500 larvae from each group were reared to the advanced fourth stadium. They were examined for RMIV infection, and the per cent transmission in each group was determined. The experiment was conducted twice in successive months.

Transovarial Transmission Through Isolated Females

Larvae were reared to 4 days of age by procedures described above, at which time groups of 2500 were exposed to 50 LEQ of inoculum for 24 hr. The larvae were rinsed thoroughly after exposure to remove inoculum, placed in a rearing tray and checked periodically for evidence of overtly infected larvae until pupation was completed. The pupae were placed in a cage to emerge, and the adults were fed as described above. Five days after emergence was completed, 100 blooded females were taken from the

group and isolated in 35 ml glass vials containing damp sphagnum moss as an oviposition medium. Boiled raisins were placed on the screened tops of the vials for food and as a source of liquid. The females were left in the vials for 1 week, then removed. The vial tops were taped to prevent desiccation of the eggs, and the eggs were allowed to develop an additional 2 weeks. The moss from each vial was then placed on a screen and washed. Egg batches from vials in which oviposition occurred were placed individually in rectangular, white enamel pans with 1 liter of 0.05 M NaCl. Larvae hatching in the pans were fed and allowed to develop to the advanced fourth stadium, counted and examined for RMIV infection. Determined from these data were: (1) the per cent of isolated blooded females that oviposited, (2) the per cent of the egg batches produced which hatched, (3) the per cent of the egg batches which hatched that contained infected larvae, (4) the per cent of infected larvae from each egg batch producing infected larvae, and (5) the number of larvae produced by transmitting and by non-transmitting females.

Sex Specificity of Transovarial
Transmission of RMIV

Four rearing trays of 2500 A. taeniorhynchus larvae each were allowed to develop to 5 days of age. The larvae in 2 of the trays were exposed for 24 hr in 16 oz cups to 100 LEQ of fresh inoculum per cup of 2500 larvae. After exposure, the larvae were rinsed thoroughly and returned to rearing trays. The 2 untreated trays were maintained on a regular rearing schedule. Pupae from all 4 trays were collected and put into cages for emergence. At 6 hr intervals during a 48 hr emergence period, the adults were immobilized in a cold room and separated both according to sex, and whether or not they had been exposed to virus. When emergence was complete the adults in each cage were again immobilized and divided into 2 equal groups and recombined to result in the following experimental classes:

Class 1: Unexposed Males X Unexposed Females

Class 2: Unexposed Males X Exposed Females

Class 3: Exposed Males X Unexposed Females

Class 4: Exposed Males X Exposed Females

The adults were fed as previously described. Eggs were again collected by the standard method and allowed

to develop for 10 days after oviposition. They were then hatched, and 2 groups of 2500 larvae from each class were allowed to develop to advanced fourth stadium, when they were examined for evidence of RMIV infection. The percent of transovarial transmission of RMIV in each class was determined.

Transmission of RMIV by
Integument Puncture

An attempt was made to transmit RMIV by integument puncture to determine if rates of transmission higher than those acquired per os could be achieved. Late third instar larvae (early in their fourth day of larval life) were rinsed thoroughly with tap water and placed in a suspension of 1 mg/ml of purified RMIV in a petri dish placed in a tray of shaved ice. The depth of the virus suspension was sufficient to cover the larvae, and when they were immobilized, they were pinched with a pair of fine tipped forceps, prepared as described by Clark (1966), and transferred to a covered tray of 0.15 M NaCl. After 24 hr, the survivors were transferred to a tray of clear 0.05 M NaCl. To extend their development time, they were maintained at 24°C rather than 27°C, as in routine rearing, and only a small amount of food was provided. Beginning

48 hr after inoculation, the larvae were checked at 12 hr intervals for infection with RMIV.

Production of Antiserum to RMIV and
to A. taeniorhynchus Antigens

Antibodies to both RMIV and A. taeniorhynchus antigens were produced in rabbits. Baseline normal sera were acquired by cardiac puncture of nonimmunized rabbits. Formation of antibody to RMIV was stimulated by 4 weekly injections of virus into each rabbit used. Five mg of purified RMIV was suspended in 1 ml of normal saline, emulsified with 1 mg of Freund's complete adjuvant,¹ and 1 ml of this emulsion was injected intramuscularly into each hip. Weekly bleedings (35-40 ml) by cardiac puncture began 1 wk after the fourth injection. Injection of antigen without adjuvant was continued after the fourth week, with the injections being given immediately after blood was withdrawn. The blood was allowed to clot in 50 ml centrifuge tubes for 1 hr at room temperature. The clots were then ringed with a sterile applicator stick and allowed to retract for 24 hr at a temperature of 4°C. The

¹Difco Laboratories, Detroit, Michigan.

tubes were centrifuged at 5,000 g for 20 min, and the serum was collected with sterile pipettes and stored without glycerine at -30°C . The presence of antibody to RMIV was determined by the tube precipitin test.

Powdered tissue of A. taeniorhynchus was prepared by triturating healthy fourth stadium larvae in a Ten Broeck type tissue grinder, containing acetone, in a dry ice-acetone bath. The tissue was extracted with acetone and the powdered tissue collected by filtration at -30°C . The powdered tissue was air dried, and water soluble proteins to be used as antigens were solubilized with phosphate buffered saline, pH 7.0, by stirring overnight at 4°C . Insoluble matter was removed by centrifugation at 15,000 g for 30 min and discarded. The antigen was sterilized by passing it through an 0.45 μ Millipore filter. The concentration of protein was determined by the Lowry method (Lowry et al., 1951), using bovine serum albumin fraction V¹ as a standard. Antibody to this antigen was produced in a rabbit by injecting 10 mg of antigen suspended in Freund's complete adjuvant weekly for 4 weeks. Only 1 collection of 35 ml of blood

¹Sigma Chemical Company, St. Louis, Missouri.

was made by cardiac puncture before the rabbit expired. The antiserum was prepared and stored as described above. Presence of antibody was determined by the tube precipitin test.

Purification of Gamma Globulin

Methods used to purify gamma globulin from anti-RMIV antiserum were partially those of Goldman (1968) and partially those of Kawamura (1969). All procedures were performed at 0° - 4°C. Antiserum was diluted 1:1 with sodium phosphate buffered saline, pH 7.0. Saturated enzyme grade ammonium sulfate,¹ (NH₄)₂SO₄, was adjusted to pH 7.0 with 0.1 N NaOH. The globulins were precipitated twice with half-saturated (NH₄)₂SO₄ and the euglobulins were precipitated twice with 1/3 saturated (NH₄)₂SO₄. The precipitates were resolubilized in distilled water to the original undiluted serum volume, except for the final precipitates, which were dissolved in 1/2 the original serum volume. The residual (NH₄)₂SO₄ was removed by dialysis against 0.15 M NaCl for 24 hr with stirring and frequent changes of saline. The globulins were then dialyzed against 0.005 M phosphate buffer, pH 8.0. The

¹Sigma Chemical Company, St. Louis, Missouri.

protein concentration was determined by the Lowry method (Lowry et al., 1951) and adjusted to 40 mg/ml by pressure filtration in an Amicon pressure filtration device equipped with a PM-10 membrane.¹ A DEAE cellulose² column was prepared with a dry weight of DEAE cellulose equal to 20 times the actual quantity of proteins to be separated. A column with a height : diameter ratio of more than 10:1 was used. The column was equilibrated and eluted with 0.005 M phosphate buffer, pH 8.0, in which both alpha and beta globulins, but not gamma globulin, adsorbed to the column. The column was eluted with a flow rate of 10 ml/hr. The gamma globulin from the column was quantitated spectrophotometrically, using an extinction coefficient of 1.24, and the concentration was adjusted to 20 mg/ml. The gamma globulin was stored at 4°C for a short time before further processing.

Conjugation of Anti-RMIV Antibody with
Fluoresceine Isothiocyanate (FITC)

Conjugation was performed by the method of Clark and Shepard (1963) as described by Goldman (1968). The

¹Amicon Corporation, Lexington, Massachusetts.

²Sigma Chemical Company, St. Louis, Missouri. 0.89 meg/g.

concentration of the antibody solution was adjusted to 20 mg/ml and the pH was adjusted to 9.5 with 0.5 M carbonate-bicarbonate buffer, pH 9.5, containing 1 mg FITC¹ per ml of immune globulin G (IgG) solution.

A G-25 Sphadex² desalting column was prepared and equilibrated with 0.0175 M phosphate buffer, pH 6.3. The solution of conjugated antibody and free dye was added to the column, and the column was washed with the equilibrating buffer. The conjugated antibody was eluted after the void volume, while the free dye was retained on the column. The volume of the labeled antibody was adjusted to the pre-labeling IgG volume with an Amicon pressure filtration device equipped with a PM-10 membrane. The labeled IgG was then dialyzed overnight against a large volume of 0.0175 M phosphate buffer, pH 6.3.

At this point the antibody solution consisted of molecules of unlabeled antibody and antibody molecules to which were conjugated 1 or more FITC molecules.

¹Baltimore Biological Laboratory, Baltimore, Maryland.

²Sigma Chemical Company, St. Louis, Missouri.

Kawamura (1969) stated that the unlabeled antibody should be removed, because it would compete with labeled antibody for binding sites. Also, antibody molecules with more than 2 FITC molecules attached should be removed, because they had a high net negative charge and tended to combine non-specifically with tissue. To this end, the labeled antibody solution was subjected to chromatography on a DEAE cellulose column equilibrated with 0.0175 M phosphate buffer, pH 6.3. In this buffer unlabeled antibody would not absorb to the column, and it could be eluted from the column with the equilibrating buffer. The labeled antibody was eluted from the column, according to the number of FITC molecules attached, by eluting the column with a flow rate of 10 ml/hr of 0.0175 M phosphate buffer, pH 6.3, containing successively 0.1, 0.2, and 0.3 M NaCl.

The fluoresceine : protein (F : P) ratio of the conjugate was determined by the method of Holborow and Johnson (1967), using the formula:

Concentration of FITC (ug/ml) =

$$\frac{\text{Absorption}_{492} - \frac{\text{Absorption}_{320}}{2} \times \text{Dilution}}{0.2}$$

Protein concentration was then determined by the Lowry method (Lowry et al., 1951), and the F : P ratio was determined by the formula:

$$F : P = \frac{0.41 \times \mu\text{g FITC/ml}}{\text{mg/ml protein}}$$

Fractions with F : P ratios of 0.36 to 1.79 were pooled, with a resulting composite F : P ratio of 0.76. The labeled antibody was dialyzed exhaustively against phosphate buffered saline, pH 7.2. The concentration was adjusted to 8.33 mg/ml, and the conjugate was lyophilized in lots of 10 mg and 15 mg/tube and stored at -30°C .

For evaluation, the labeled antibody was reconstituted to its pre-lyophilization volume with distilled water, diluted with phosphate buffered saline, pH 7.2, to a concentration of 3 mg/ml, and clarified by centrifugation at 15,000 g for 10 min. Dilutions of 2 mg/ml, 1 mg/ml, 0.5 mg/ml and 0.25 mg/ml were prepared to determine the optimum concentration to use for staining. Unexposed and known infected larvae as test specimens were embedded together in Paraplast,¹ the blocks were sectioned at a

¹Scientific products, Evanston, Illinois.

thickness of 8 μ , and the sections were deparaffinized in tertiary butanol at 40°C. The specimens were hydrated through an ethanol series to distilled water and washed in phosphate buffered saline before being incubated in a humidified chamber with dilutions of the conjugated antibody. Following exposure to the antibody, they were given 4 - 5 min washes in phosphate buffered saline and coverslips were mounted over the specimens with a media of 1 per cent Tris \square Tris (hydroxymethyl) amino-methane \square in 9 parts glycerine and 1 part water, with the pH adjusted to 9.9 with 0.1 N NaOH (Mrenova and Albrecht, 1966). The specimens were examined and photographed with a Zeiss Ultraphot II microscope, using dark field illumination. The light source was an Osram HBO-200 Mercury vapor lamp. The primary filter used was the Zeiss UG-1/3mm, and the secondary filter admitted all wave lengths of light greater than 410 nm. Dilutions of the labeled antibody as low as 0.5 mg/ml were found to stain foci of virus infections, and subsequent studies utilized a concentration of 1 mg/ml. The specificity of the stain was indicated by its failure to stain sections of uninfected larvae mounted alternately with sections of infected larvae, on the same slide. The staining

reaction of the labeled antibody was inhibited by treating sections of infected larvae with unlabeled anti-RMIV anti-serum prior to treatment with the labeled antibody. Treating sections of infected larvae with antiserum to A. taeniorhynchus did not inhibit the staining reaction.

Pilot Project on Autoradiography of ^3H -Methyl
Thymidine Uptake and Distribution in
Larval A. taeniorhynchus

No literature was available on certain parameters essential to an autoradiographic study of the development of RMIV infection in A. taeniorhynchus larvae. A pilot project was conducted with uninfected larvae to determine: (1) the level of radioactivity necessary in an aqueous medium to assure uptake of ^3H -methyl thymidine into larvae in quantities detectable autoradiographically, (2) the length of time larvae could be exposed to this activity without destroying the resolution of the technique by translocation of the tritiated methyl group by metabolism, and (3) the optimum length of time to expose a nuclear track emulsion to sections containing the tritiated thymidine, before developing them.

Dilutions of ^3H -methyl thymidine,¹ with a specific

¹International Chemical and Nuclear Corporation, Irvine, California.

activity of 17.4 Curies/millimole (Ci/mM) and a concentration of 1 millicurie/mM (mCi/mM), were made with 0.05 M NaCl to provide solutions of 2.5 microcuries/ml (μ Ci/ml), 5 μ Ci/ml, 10 μ Ci/ml, and 25 μ Ci/ml. Ten ml of each concentration were placed in each of 4 aluminum weighing cups and a small amount of brewer's yeast infusion added. Ten late third stadium larvae of A. taeniorhynchus were placed in each cup. At intervals of 30 min, 1 hr, 2 hr, and 4 hr after initiation of exposure, the larvae in 1 of the 4 cups of each concentration were removed, rinsed thoroughly, and placed for 30 min in 3 oz cups containing a yeast infusion. The post-exposure feeding was intended to flush unabsorbed label from the gut to minimize the nonspecific distribution of the label by the microtome knife during sectioning of the specimens. Following exposure to label, the larvae were washed thoroughly in a dilute solution of Tween-80, rinsed in distilled water, fixed in Carnoy's fixative for 4 hr at room temperature, dehydrated through absolute ethanol, cleared in tertiary butanol and embedded, two larvae per block, in Paraplast. Serial sections of the larvae from the 16 experimental groups were cut at a thickness of 8 μ on a Leitz microtome and placed on gelatin cooled slides. The slides were divided into 5

sets, each set containing the serial sections of two larvae from each of the experimental groups. The slides were deparaffinized in xylene, hydrated through descending concentrations of ethanol to distilled water and allowed to dry. All subsequent dipping, packaging and developing operations were performed in total darkness. The slides were emulsion coated by dipping them in Kodak NTB-2 nuclear track emulsion¹ that had been melted and held in a constant temperature water bath at 45°C. Control blank slides, to determine background graination, and unlabeled sections, to check for possible nonspecific tissue activity, were also coated, and all slides were allowed to dry for 2 hr in a near vertical position. After drying, the slides were sealed in slide boxes containing a drying agent, wrapped in several layers of aluminum foil, and placed in a refrigerator at 4°C. At intervals of 2, 3, 5, 7 and 10 weeks after dipping, 1 set of the slides was developed in Kodak Dektol for 1.5 min at 18°C, dipped for 30 sec into Kodak Indicator Stopbath, fixed for 10 min in Kodak Rapid Fix (diluted 1:1), and washed in running water for 15 min (Rogers, 1967). After rinsing in distilled water,

¹Eastman Kodak Company, Rochester, New York.

the slides were stained in methyl green-pyronine (Pease, 1953) as recommended by Thurston and Jofte (1963). After staining, the slides were dehydrated in tertiary butanol, cleared in toluene and coverslips were mounted with a synthetic resinous mounting medium.

When all sets of the autoradiograms had been processed, they were examined at 500 x (oil) magnification with an AO-Spencer microscope. Background was rated on the control slides and also on each experimental slide, by examining areas of the slide distant from sections, as light (5 grains/100 μ^2), moderate (5-10 grains/100 μ^2) or heavy (10 grains/100 μ^2). The quality of the stain reaction was noted, and the control sections which contained no radioisotope were examined for evidence of nonspecific activity. The following tissues were examined for evidence of Label uptake: epidermis, fatbody, foregut epithelium, midgut epithelium, gastric caecae, imaginal discs, hindgut epithelium, malpighian tubules, tracheal epithelium and gonads. Casual observation was made of incorporation into nerve and muscle tissue. Labeling over the midgut contents was observed to see if the gut had been swept clean by post exposure feeding. Incorporation of label into the cells of each tissue, as indicated by supranuclear

graination, was rated semiquantitatively by code as follows:

Frequency of labeled cells (nuclei)

- 1 = $< 1/10$ of cells labeled
- 2 = $1/10 - 1/2$ of cells labeled
- 3 = $> 1/2$ of cells labeled

Intensity of activity in labeled cells (nuclei)

- 1 = < 25 grains per nucleus
- 2 = 25-50 grains per nucleus
- 3 = 50-100 grains per nucleus
- 4 = > 100 grains per nucleus

Only the effect of the various exposure concentrations and durations on the labeling of the midgut epithelium cells was considered in deciding the concentration of label to use in subsequent experiments. These cells were observed as standards, because (a) they were uniformly large, (b) the nuclei occupied less than $1/2$ the area of the cells in cross-sections, making it easy to discern successful localization of the isotope within the nucleus, and (c) because they had good staining properties.

Production of ^3H -Methyl Thymidine
Labeled RMIV

A. taeniorhynchus larvae were hatched and reared for 24 hr, and 2000 of them were exposed to RMIV as described for routine virus production. The larvae were reared to advanced third stadium and then transferred to a pan containing a liter of 0.05 M NaCl, 10 mCi of ^3H -methyl thymidine (specific activity of 66 Ci/mM), and a small amount of food. The larvae were exposed to the tritiated thymidine for 24 hr. At the end of exposure 128 conspicuously infected fourth stadium larvae were harvested, as were 10 larvae which displayed no evidence of infection. The uninfected larvae and four of the infected larvae were prepared for autoradiography as described above. The virus in the remaining 124 infected larvae was purified by methods previously described and quantitated spectrophotometrically. Five mg of labeled virus were recovered and diluted to 50 mg. An 0.2 ml sample gave 1200 disintegrations per min (dpm) above background when counted in a Packard Tri-carb Liquid Scintillation Counter, indicating a total radioactivity of 0.14 μCi for the purified virus. The virus was used immediately in autoradiographic study of the site of penetration of RMIV into host tissue.

Site of Entry of RMIV into
Host Tissue

Light Microscope Studies

Autoradiography.--Eggs of A. taeniorhynchus were hatched and the larvae allowed to develop for 24 hr by standard procedures. Two thousand larvae were exposed to 5 mg of RVIV, labeled with ^3H -methyl thymidine as described before, with a total radioactivity of 0.14 μCi . At intervals of 1/4, 1/2, 1, 2, 4, 8, and 12 hr, 125 of the larvae were withdrawn from the exposure medium, rinsed thoroughly with tap water, and fixed in Carnoy's fixative for 4 hr at room temperature. The specimens were processed histologically, dipped in Kodak NTB-2 nuclear track emulsion, developed after 2 weeks' exposure and stained as previously described. They were examined at magnifications of 500x (oil) and 1000x (oil) in an attempt to detect a site of entry of the labeled virus through the gut wall.

Exposed specimens that were not processed for autoradiography were reared to the advanced fourth stadium, when they were examined to determine the per cent infection with RMIV.

Fluorescent antibody.--Eggs of A. taeniorhynchus were hatched, the larvae were allowed to develop for 24 hr, and 2500 larvae were exposed to an inoculum of 50 LEQ of RMIV as described for routine virus production. At intervals of 1/4, 1/2, 1, 2, 4, 8, 12, and 24 hr after the initiation of exposure to virus, samples of 125 larvae were removed from the exposure container. They were rinsed thoroughly, fixed in Carnoy's fixative for 4 hr at room temperature, dehydrated through absolute ethanol, cleared in tertiary butanol, and embedded in Paraplast. Sections, processed as before, were incubated for 45 min with 1 mg/ml conjugated anti-RMIV antibody and examined as for other studies for evidence of a specific site of entry of RMIV into host tissue.

Larvae not collected for processing were allowed to develop to the advanced fourth stadium when they were examined to determine per cent infection with RMIV.

Electron Microscope Studies

Two hundred freshly hatched A. taeniorhynchus larvae were placed in a 10 cm diameter petri dish of distilled water containing an inoculum of 10 freshly triturated RMIV infected fourth stadium larvae. Samples of exposed larvae were removed from the exposure medium at intervals

of 5, 10, 15, 20, and 30 min, and also at 1 1/2, 2, 3, 4, and 5 hr after the initiation of exposure. Head capsules and air tubes were excised in glutaraldehyde, and the larvae were fixed for 4 hr at room temperature in 4 per cent glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. They were washed in cacodylate buffer over night, post fixed for 1 hr in 1 per cent osmium tetroxide in cacodylate buffer, dehydrated through ascending concentrations of ethanol, cleared in propylene oxide, and embedded in epon-araldite (Mollenhauer, 1964). Silver to light gold sections, approximately 60 nm thick, were cut on a Sorvall Porter-Blum MT-2 ultramicrotome with glass knives, and the sections were taken up on carbon or Formvar coated grids. The sections were stained with saturated uranyl acetate and lead citrate (Venable and Coggeshall, 1965) and examined and photographed with a Hitachi 125 E electron microscope using accelerating voltages of 50 or 75 KV. Thick sections were also cut and examined by phase contract microscopy as an aid to orientation.

Development of RMIV InfectionsAutoradiography

Eggs of A. taeniorhynchus were hatched, and the larvae were allowed to develop for 48 hr in the hatching container, by which time they had reached the second larval stadium. Two groups, each with 2500 larvae, were exposed for 24 hr to 100 LEQ of RMIV inoculum and then transferred to rearing containers. Twelve hours after the termination of virus exposure, and at 24 hr intervals thereafter, 125 of the exposed larvae were removed from the rearing container and placed for 2 hr into 125 ml of 0.05 M NaCl containing 20 $\mu\text{Ci/ml}$ of ^3H -methyl thymidine with a specific activity of 13 Ci/mM. A small amount of food was provided to encourage the intake and passage of material through the gut. At the termination of exposure to tritiated thymidine, the larvae were thoroughly rinsed and placed in a suspension of food for 30 min. The larvae were processed histologically, and autoradiography and staining were performed as has been described. Exposure of this emulsion was for a period of 10 days. The activity of the exposure medium was increased to 20 $\mu\text{Ci/ml}$, despite the results of the pilot project, and the emulsion exposure time was decreased in an attempt

to shorten the experiment. Autoradiograms were examined at magnifications' 500x (oil) and 1000x (oil) to determine the tissues in which infection could be detected by this technique and to determine how soon after the exposure infections in these tissues could be detected.

Larvae not collected for study were reared to the advanced fourth stadium and examined to determine the per cent infection with RMIV.

Fluorescent Antibody

Overt infections.--Eggs of A. taeniorhynchus were hatched, the larvae were reared for 24 hr, and 2500 of the larvae were exposed to 50 LEQ of RMIV inoculum as described for routine virus production. At the termination of exposure, the larvae were transferred to a rearing container. At that time and at 24 hr intervals thereafter, samples of 125 larvae were collected, processed histologically, and stained with fluorescent antibody, 1 mg/ml, as described above.

The exposed specimens were examined to determine in which tissues infection could be detected and at what time interval after initiation of exposure infections could be detected by this method.

Larvae not collected for this study were reared to the advanced fourth stadium and examined to determine the per cent of infection with RMIV.

Covert infections.--Specimens for this study were collected from the exposed mosquitoes in the experiment, "Sex Specificity of Transovarial Transmission of RMIV." Larvae were exposed to 100 LEQ of RMIV inoculum per group of 2500 larvae during the fifth day of larval life. Specimens for this study were collected as larvae at the end of the sixth day of larval life (48 hr after exposure was initiated), as pupae at the end of the eighth day of life, just prior to eclosion (72 hr after exposure was initiated), and as adult females prior to bleeding (120 hr after exposure was initiated). Known transovarially transmitting females, surviving the experiment "Transmission of RMIV through Isolated Females," were also examined. The specimens were processed histologically, stained with fluorescent antibody to RMIV (1 mg protein/ml) and examined as described above to determine which tissues were infected and at what time interval after the initiation of exposure infection could be detected, with special attention paid to reproductive tissues. The amount of transovarial transmission occurring through the experimental group, exclusive

of the known infected females, was reflected in the transmission through class 4 of the experiment "Sex Specificity of Transovarial Transmission of RMIV."

Number and Molecular Weights of RMIV
Structural Polypeptides

Analytical sodium dodecyl sulfate (SDS)--polyacrylamide gel disc electrophoresis was performed on SDS--2-mercaptoethanol disrupted samples of highly purified RMIV by the methods of Maizel (1971). Purified RMIV was subjected to sucrose gradient centrifugation 1 or 2 additional times, washed 3 times with distilled water to remove residual sucrose, and quantitated spectrophotometrically (Matta, 1970). The concentration was adjusted to 10 mg (protein)/ml in distilled water. The virus was structurally disrupted and the proteins denatured to polypeptides by heating the virus to 100°C for 1 min in 1 per cent SDS¹ and 0.1 per cent 2-mercaptoethanol.² Samples of 100 ug, 200 ug, 300 ug and 400 ug of protein in 250 ul volume of 12 per cent sucrose were overlaid onto 10 x 0.6 cm resolving

¹Fisher Scientific Company, Fairlawn, New Jersey.

²Matheson, Coleman and Bell, Cincinnati, Ohio.

gels of 10 per cent or 13 per cent acrylamide¹ with 2.5 x 0.6 cm stacking gels of 3 per cent acrylamide. Both resolving gels and stacking gels contained 0.1 per cent SDS. Bromphenol blue was added to the samples as a tracking dye. Simultaneously, 50 ug samples of proteins of known molecular weight,² also treated with SDS and 2-mercaptoethanol, were run in identical gels. A current of 2mA per tube was applied until the tracking dye had migrated 0.5 cm into the resolving gel, and then 4mA per tube were applied until the tracking dye approached the bottom of the resolving gels. The gels were cut at the dye front when removed from their tubes. They were fixed and stained in 0.2 per cent Coomassie Brilliant Blue R 250³ in 50 per cent methanol and 7 per cent acetic acid for 12 hr and destained in repeated changes of 5 per cent methanol in 7 per cent acetic acid. The gels were stored in stoppered test tubes containing 7 per cent acetic acid. The stained bands of viral polypeptides were counted, and the relative migration of both

¹Sigma Chemical Company, St. Louis, Missouri.

²Schwarz/Mann, Orangeburg, New York.

³Sigma Chemical Company, St. Louis, Missouri.

viral polypeptides and polypeptides of known molecular weight was determined in relationship to the dye front. Curves were drawn, relating to relative migration of the standard polypeptides in both 10 per cent and 13 per cent resolving gels, to the Log_{10} of their molecular weights. The molecular weights of the viral polypeptides were then estimated from these curves.

Isolation of RMIV Structural Polypeptides
by Hydroxylapatite Chromatography

Hydroxylapatite chromatography techniques described by Moss and Roseblum (1972) were utilized with RMIV. Hydroxylapatite (Bio-Gel HT)¹ was washed repeatedly with 0.01 M sodium phosphate, pH 6.4, 0.1 per cent (W/V) SDS, and 1.0 mM dithiothreitol² (DTT). Columns, 2.5 x 10 CM, were poured over a layer of fine grade Sephadex G-50² or over filter paper discs. Twenty to 40 mg samples of RMIV, purified as described above, were made to 5 ml in 1 per cent SDS, 0.1 per cent 2-mercaptoethanol and placed in a boiling water bath for 2 min. The denatured samples

¹Bio-Rod Laboratories, Richmond, California.

²Sigma Chemical Company, St. Louis, Missouri.

were then diluted to a concentration of 1 mg/ml with 0.01 M sodium phosphate, pH 6.4, 0.1 per cent SDS and placed on the columns. They were washed into the columns with 2 column volumes of the 0.01 M phosphate-SDS-DTT solution and eluted with a linear gradient formed by a 2-chambered gradient former, containing 200 ml of 0.1 M sodium phosphate, pH 6.4, 0.1 per cent SDS, 1 mM DTT in the starting chamber and 200 ml of 0.7M sodium phosphate, pH 6.4, 0.1 per cent SDS, 1 mM DTT in the trailing chamber. A flow rate of about 5 ml/hr was maintained by gravity. Three ml fractions were collected in a refrigerated Buchler fraction collector. The $O.D_{280}$ of the fractions was determined in a Beckman DU-2 spectrophotometer, and curves were drawn relating fraction number to $O.D_{280}$

RESULTS AND DISCUSSION

Production of RMIV

Determination of Optimum Quantity of RMIV Inoculum

For purposes of this dissertation, the optimum quantity of RMIV inoculum for use in the routine production of RMIV was defined as that quantity which resulted in the maximum net production of virus without causing significant larval mortality during exposure. The combined results of 3 experiments designed to determine that quantity are shown in Table 1. An inoculum of 50 LEQ per exposure cup of 2500 larvae was found to be the optimum inoculum for use in the virus production system described and was used in routine virus production.

A graph of these data (Figure 1) indicates that the per cent transmission of RMIV increased in proportion to the quantity of inoculum used until inocula in excess of 50 LEQ/2500 larvae were used. Above this point, high larval mortality during exposure obscured the relationship between per cent transmission and quantity of inoculum.

Table 1

Net Virus Production Resulting from 24 Hr Exposure
of Day Old A. taeniorhynchus
Larvae to RMIV

	Inoculum (LEQ) Per 2500 Larvae					
	5	10	25	50	75	100
Gross Number of Infected Larvae Per 15,000 Exposed	234	317	758	1174	863	131
Per Cent Transmission	1.56	2.1	5.1	7.2	5.7	0.8
Net Virus Production (LEQ) Per 15,000 Exposed	219	287	863	927	638	0
Mortality During Exposure	Low	Low	Low	Low	High	Very High

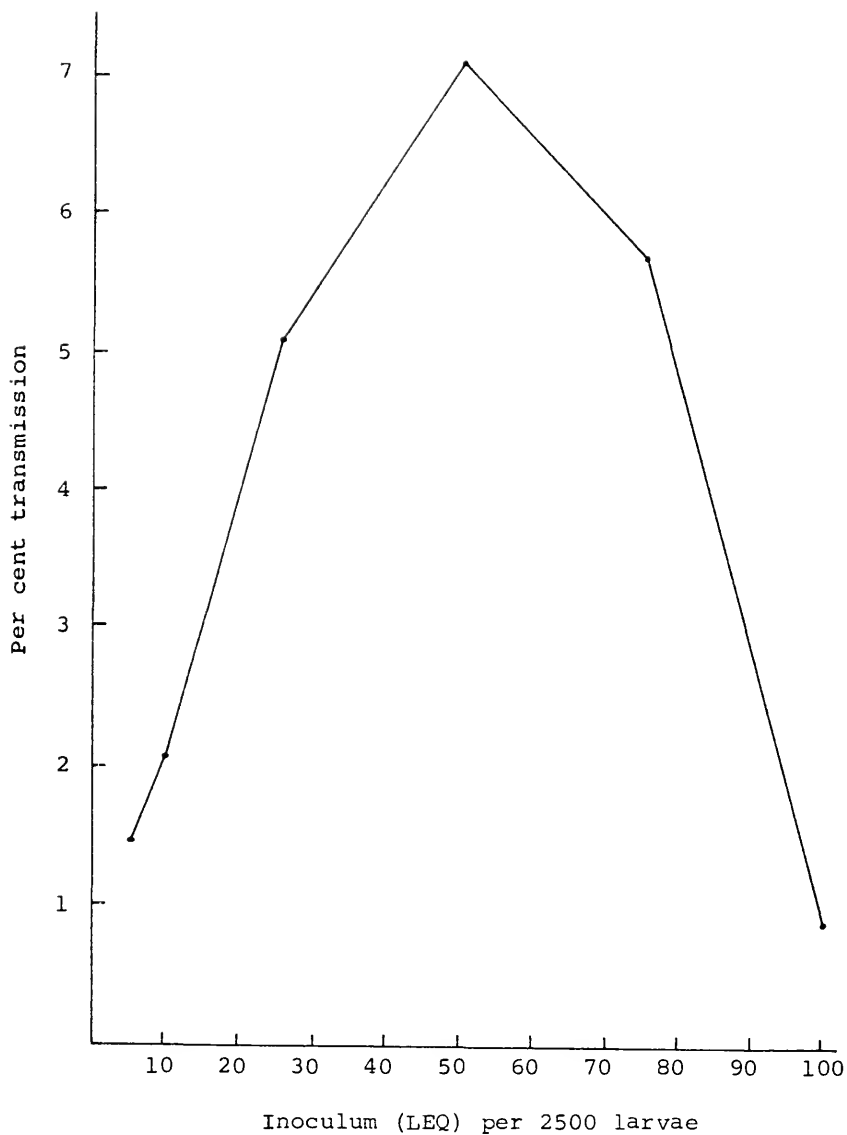


Fig. 1. Per Cent Transmission of RMIV in A. taeniorhynchus Larvae Exposed to Increasing Amounts of Inoculum.

Mortality during exposure in the control groups paralleled that in the experimental groups, indicating that the high mortality in experimental groups exposed to 75 and 100 LEQ of inoculum was not due to a toxic effect of the virus, as experienced by Bellett and Mercer (1964) in studies on the multiplication of SIV in cell cultures.

No infection was observed among the control larvae.

Determination of Optimum
Larval Age for Exposure
to RMIV

The combined results of two experiments designed to determine the effect of the age of A. taeniorhynchus larvae at exposure to RMIV on the net production of RMIV are shown in Table 2. Maximum net production of RMIV resulted from exposures initiated when the larvae were 24 hr old. These results corroborate those of Woodard and Chapman (1968).

Quantification of RMIV
Production

Thirty-eight hundred infected, advanced fourth stadium larvae were collected from 37,500 larvae exposed to RMIV and maintained as described for routine virus production.

Table 2

RMIV Production Resulting from 24 Hr Exposure
of A. taeniorhynchus Larvae of Different
Ages to 50 LEQ of Inoculum

	Day of Larval Life Exposure Was Effected					
	1	2	3	4	5	6
Gross Number of Infected Larvae per 10,000 Exposed	1033	1084	702	329	0	0
Per Cent Transmission	10.33	10.84	7.02	3.20	0	0
Net Virus Production (LEQ) per 10,000 Larvae Exposed	833	884	502	129	0	0

This represented a transmission rate of 10.13 per cent. Nineteen hundred infected larvae and an equal number of uninfected larvae from the same experiment had dry weights of 1.19698 grams and 1.39290 grams, respectively. This represented a mean dry weight of 630 μ g and 733 μ g for infected and uninfected larvae, respectively. From the additional 1900 infected larvae harvested, 112 mg of RMIV were purified. This was a recovery of 58.9 μ g of purified virus per infected larva and showed that 9.35 per cent of the dry weight of an average infected, advanced fourth stadium larva was composed of virus.

The host production system and the virus purification procedures described here resulted in a maximum production by 1 person of 940 mg of purified virus in 1 week and have approached that quantity several other times. Therefore, it is possible for 1 person, with an adequate mosquito colony, setting 80 trays (200,000) of exposed larvae per week, to conveniently produce and purify 1 net gram of virus per week. Virus produced in 1 week can be purified the subsequent week while a new generation of infected hosts are produced. Although this quantity of virus may not be adequate for extensive biological control field testing, it certainly is adequate for numerous laboratory studies.

Attenuation of Infectivity
of RMIV During Purification

An attenuation of the infectivity of RMIV during purification is apparent from the data shown in Table 3. Almost 70 times as many infected larvae per LEQ of inoculum were produced by freshly triturated inoculum as by an inoculum of purified virus that had been lyophilized. This attenuation may have resulted from both quantitative loss of virus during purification and from some damage to virus particles during processing that reduced the ability to enter a host cell or the ability to establish active infections. The proportion of attenuation attributable to any one of these causes cannot be determined from the data. The greatest attenuation appeared to occur following ether extraction and sucrose gradient centrifugation. This was surprising, since RMIV was shown to be insensitive to ether by Matta and Lowe (1970) and because sucrose density gradient centrifugation is commonly used in virus purification because it is a relatively mild technique. The greater relative loss of infectivity during these stages of purification was possibly a reflection either of the use of increased quantities of inoculum in those experimental groups or a reflection of a non-linear relationship between transmission and the quantity of inoculum used. Despite the

Table 3
 Attenuation of Infectivity Resulting
 from Purification of RMIV Through
 the Stage Indicated

Progressive Stage Of Purification	Quantity of Inoculum (LEQ) Per 4,000 Larvae	Infected Larvae Produced Per 4,000 Exposed	Per Cent Trans- mission	Infected Larvae Produced Per LEQ of Inoculum
Trituration	100	337	8.4	3.37
Buffer Extraction	100	189	4.7	1.89
Ether Extraction	200	109	2.7	0.55
Differential Centrifugation	200	69	1.7	0.35
Sucrose Gradient Centrifugation	400	39	1.0	0.10
Lyophilization	400	22	0.5	0.05

ambiguity of the data, it was apparent that the attenuation detected was not caused by any single step in purification, but occurred throughout the whole procedure.

Transmission of RMIV

Per Cent of Larvae Infected with RMIV by Transovarial Transmission

A relationship was found between the age of the parent generation when exposed to RMIV and the frequency of infections transmitted transovarially. As shown in Figure 2, little or no transovarial transmission into progeny occurred when the parent generation was exposed to virus during early larval life. The amount of transovarial transmission increased as the age of the parent generation at exposure increased, up to a point, and then decreased. These data suggest that, when sufficient time for development of infections in larvae is available, overt infections with RMIV result. No definite explanation can be given for the decrease in transovarial transmission through parents exposed during the sixth day of larval life. It is possible that some host physiological change associated with pupation interrupts some infections before reproductive tissues become infected. This possibility will be discussed further in later sections.

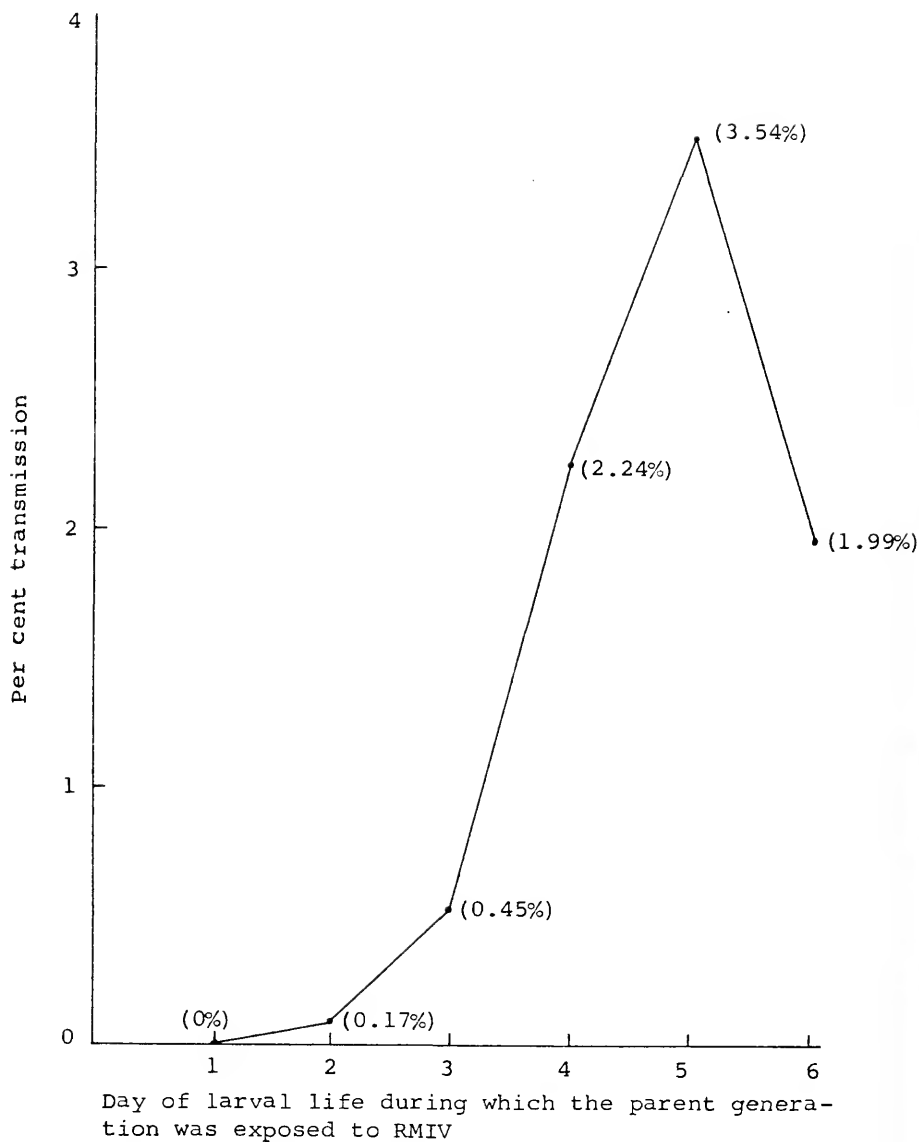


Fig. 2. Per Cent of RMIV Infection Among Progeny of Mosquitoes Exposed to RMIV as Larvae of Different Ages.

Data from 2 experiments were combined, and a total of 10,000 larvae were in each experimental group.

Transmission of RMIV
Through Isolated Females

Eighty-three of 100 isolated, blooded females, exposed to RMIV during the fifth day of larval life, oviposited. Of these, eggs of 67 of the females (81%) hatched, and 9 groups (13.4%) contained infected larvae. Initially, some of the larvae in 4 of the infected groups displayed no gross infection early in the fourth stadium. This suggested that there were exceptions to the apparent all-or-none mechanism of transovarial transmission observed by Woodard and Chapman (1968) and by Hall and Anthony (1971). However, the uninfected larvae were isolated, and without exception they developed overt infections and died before pupating. The 9 groups of infected larvae consisted of from 13 to 104 larvae, with a mean of 63.3 per group, compared to the uninfected groups, which ranged from 1 to 156 larvae with a mean of 94.2 per group. From the total of 6,053 larval progeny observed from the isolated females, only 591 (9.8%) were infected, although 13.4 per cent (9 of 67) of the females were known to have been infected. These data suggested that the per cent of infection among larvae produced in transovarial transmission experiments was not an accurate reflection of the per cent of infection

occurring among the females that produced them. In the experiment described, the per cent of known infected females among the isolated females exceeded the per cent of overt infections developing among the progeny of the experimental group by a factor of 1.37 ($13.4\% \div 9.8\% = 1.37$). Although this factor was acquired from limited data, it will be used in subsequent discussions to estimate the per cent of infected (transmitting) females in transovarial transmission experiments from the per cent of infection among the progeny of the experimental group.

Sex Specificity of Transovarial Transmission

Males of A. taeniorhynchus apparently play no part in the transovarial transmission of RMIV (Table 4). When males were exposed to RMIV as 4 day-old larvae and mated to unexposed females, none of 5,000 progeny were infected. Also, per cent of infection among progeny of exposed females was not diminished by mating them to unexposed males rather than to exposed males.

As will be discussed in later sections, overt infections appeared as often among male larvae as among female larvae when mixed groups were exposed in early instars. Since RMIV is a cytoplasmic virus, the apparent inability

Table 4

Transovarial Transmission of RMIV by Reciprocal
Mating of Exposed and Unexposed Male and
Female A. taeniorhynchus

Mating	Per Cent Infection Among Progeny (5,000 Larvae Per Class)
Unexposed Males x Unexposed Females	0
Unexposed Males x Exposed Females	4.5
Exposed Males x Unexposed Females	0
Exposed Males x Exposed Females	4.8

of males to transmit the virus may be related to the very small quantity of cytoplasm occurring in sperm.

Total of Overt and
Covert Transmission

The total amount of RMIV infection acquired by a group of exposed larvae was defined as the sum of overt infections (identified by the characteristic iridescence of infected larvae and pupae) and covert infections. Overt infection with RMIV were easy to quantitate, and since they are fatal almost without exception, they play no part in transovarial transmission. Little is known of covert infections with RMIV, since at present the only evidence for their existence is the transovarial transmission resulting from a portion of them. The proportion of covert infections which do not result in transmission is unknown. Also, it is not known whether covert infections can result in transovarial transmission into only 1 of numerous ovipositions by a single female. Nevertheless, an attempt was made to determine the total transmission of RMIV to groups of larvae exposed to the virus at different ages. This was done to test the hypothesis (Woodard and Chapman, 1968) that about the same per cent of infections occurred whether the larvae were exposed when young (resulting in

overt infections) or when older (resulting in transovarial transmission).

The following assumptions were necessary: (1) males play no significant part in vertical transmission of the virus, although they become infected with the same frequency as females; (2) the per cent of covertly infected females is about 1.37 times the per cent of infected larvae among the progeny of the exposed generation; and (3) the proportion of covertly infected females which transmit the virus through their first egg batch is not influenced by the age of the larvae when infection is acquired. Support for the first 2 assumptions has been offered. The latter is not unreasonable, considering that overt infections are manifested 3 or 4 days after initiation of exposures in larvae, and that adults are usually a week to 10 days old before they oviposit. Therefore, infections should have had time to develop regardless of when the larvae acquired infection. Overt infections occurring in the experiment "Determination of Optimum Larval Age for Exposure to RMIV" were related to covert infections, reflected by transovarial transmission, in the experiment "Per cent of Larvae Infected with RMIV by Transovarial Transmission," because the ova for the latter experiment

Table 5
 Total of Overt and Covert Transmission
 of RMIV to Larvae Exposed at
 Different Ages

Day During Which Larvae Were Exposed	Per Cent Overt Transmission	Per Cent Covert Transmission*	Total Trans- mission**
1	10.33	0	10.33
2	10.84	0.23	11.07
3	7.02	0.62	7.64
4	3.29	3.07	6.36
5	0	4.85	4.85
6	0	2.73	2.73

*Covert transmission was calculated as 1.37 times per cent of infection among progeny of the exposed generation.

**These data represent observation of 10,000 larvae per class.

were produced by the survivors of the former. Both experiments were conducted twice at an interval of 1 month, and combined data are given in Table 5. These data suggest that older larvae are less susceptible to infection with RMIV than younger larvae and that larvae are more susceptible to RMIV during the second day of larval life than at any other time.

Transmission of RMIV
by Integument Puncture

Attempts to transmit RMIV to A. taeniorhynchus larvae by integument puncture, as described by Clark (1966), were unsuccessful. Clark reported up to 70 per cent transmission of various microbial mosquito pathogens by this method. One hundred and seventy-five of 269 third instar larvae survived the exposure and were fourth instar larvae or pupae 5 days after exposure. Pupation was delayed by a poor diet and by maintaining the larvae at 24°C rather than at 27°C. These conditions attempted to prolong development and may have interfered with the development of infection. Also, there is no assurance that virus ever entered the host, so the data are inconclusive.

Pilot Project on Autoradiography of ^3H -Methyl
Thymidine Uptake and Distribution in
Larval A. taeniorhynchus

Autoradiography detected uptake of ^3H -methyl thymidine into the nuclei of cells of third instar A. taeniorhynchus larvae in the following tissues: epidermis, fatbody, foregut epithelium, midgut epithelium, gastric caecae, imaginal buds, hindgut epithelium, malpighian tubules, tracheal epithelium and the gonads. Uptake was greatest in the malpighian tubules, hindgut epithelium, and gastric caecae, and only slightly less in midgut epithelium, imaginal buds, and fatbody. Less uptake was detected in epidermis, foregut epithelium, tracheal epithelium and gonadal tissue. Uptake into nerve and muscle cells was rarely detected. Exposure to 2.5 $\mu\text{Ci/ml}$, for up to 4 hr, resulted in labeling too sparse to be useful, but exposure to 25 $\mu\text{Ci/ml}$ for even 1/2 hr overlabeled many tissues. A 2 hr treatment of 10 $\mu\text{Ci/ml}$ labeled the cells of most tissues consistently and adequately. On the basis of the response of midgut epithelium (used as a representative tissue) this treatment was selected as ideal for a study of the development of RMIV infections in host tissue. In Figure 3 is shown the sharply circumscribed location of



Fig. 3. Malpighian Tubule Cell with Labeled Nucleus
(1000x).
(N - Nucleus)

graination over a labeled nucleus. The specific activity of the labeled thymidine used in this pilot study was 17 Ci/mM and exposures of 2 weeks were adequate for specimens exposed to the accepted treatment of 10 μ Ci/ml for 2 hr. Grains over the cells of active tissues increased in number as duration of exposure of the emulsion increased, but background graination also increased rapidly and reduced the resolution of the technique.

Examination of control materials indicated that background graination intrinsic to the emulsion was low enough not to reduce the resolution of the technique. A small amount of very fine graination occurred over the anterior thoracic fatbody of all specimens, whether exposed to label or not, and could not be explained. The staining reaction of methyl green-pryone was very good in uninfected tissue. DNA in the nuclei stained a pale blue, and the graination was easy to detect. RNA stained a pale pink in the cytoplasm and a bright pink in the nucleoles, which was very helpful in detecting the nuclei of small cells.

Feeding larvae to flush unabsorbed tritiated thymidine from the gut lumen after exposure was very helpful. In the few specimens that did not feed during the flushing

period, graination was very heavy over the gut contents. It was observed that some of the radioactive thymidine was dispersed from the gut into surrounding areas by the microtome blade during sectioning. An interesting phenomenon was observed in larvae that had fed actively during the flushing period. As shown in Figure 4, a pocket of tritiated thymidine remained in the otherwise cleanly flushed gut. The residual pocket was ring-shaped and located in the anterior part of the midgut at the level of the caudal extremity of the foregut invagination into the midgut. This possibly represented an "eddy" in the flow of material through the gut, and its possible significance is discussed later with studies on the site of entry of RMIV into host tissue.

Site of Entry of RMIV Into Host Tissue

Light Microscope Studies

Autoradiography.--The site of entry of RMIV into host tissue could not be detected on autoradiograms of serial sections of 700 early instar larvae of A. taeniorhynchus exposed to purified RMIV that had been labeled with ^3H -methyl thymidine. Three and one-tenth per cent (30/973)

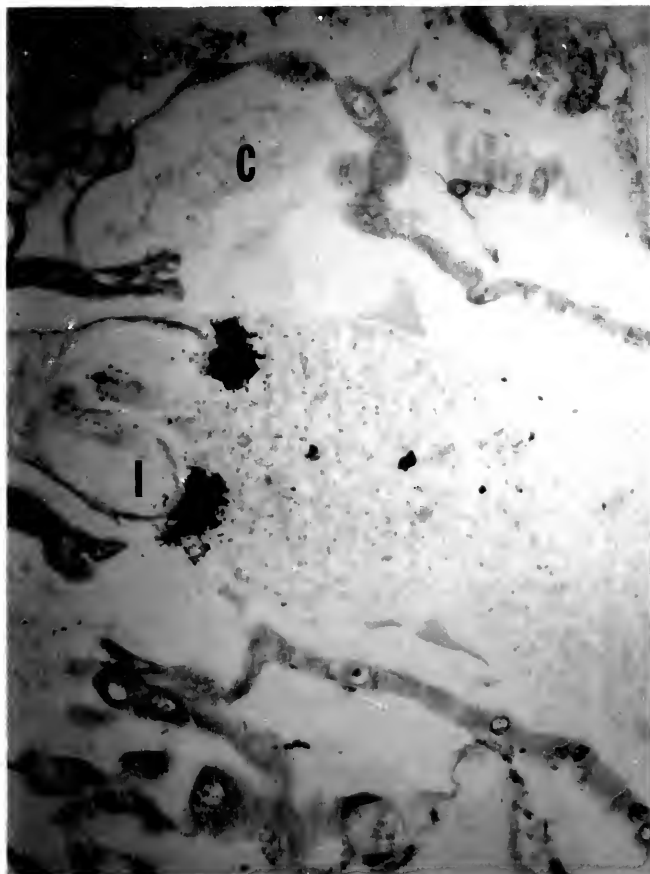


Fig. 4. Anterior Midgut Region of Larva Showing Residual Deposits of Tritiated Thymidine Peripheral to Caudal Extremity of Foregut Invagination into Midgut (100x).
(I - Foregut invagination; C - Gastric caecae)

of the survivors of exposure to the purified, labeled virus developed infections with RMIV, when they were reared to the fourth stadium. That was about the same per cent transmission as achieved with purified, unlabeled virus in the study "Attenuation of Infectivity of RMIV During Purification," suggesting that labeling did not reduce the infectivity of the virus. Small foci of grains were seen scattered in the emulsion over the midgut contents of larvae that had been exposed to the labeled virus, indicating that labeled virus was consumed and that it could be detected autoradiographically. However, these foci of grains were rare. Only 0.14 uCi of activity were detectable in the entire virus inoculum (5 mg) by liquid scintillation counting. Autoradiograms of sections of 4 of the infected larvae in which the inoculum was produced were examined. These revealed that label incorporation into foci of virus, identified in the cytoplasm of infected cells by their staining reaction with methyl green, was much less than into the nuclei of the infected cells (Figure 5). The evidence suggests that the virus inoculum was not heavily labeled. This could have occurred if most of the viral DNA had been produced before the infected larvae were exposed to tritiated thymidine.

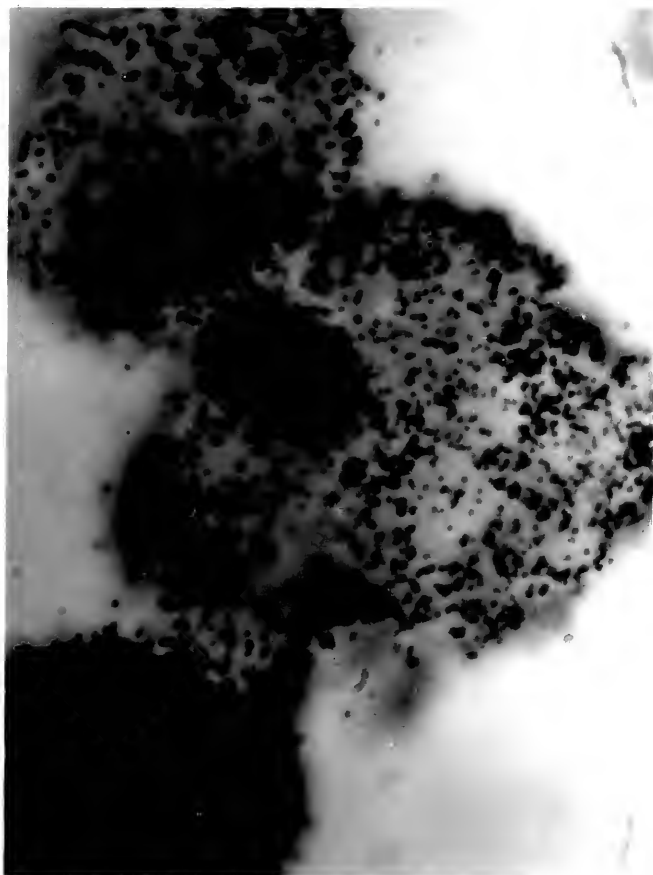


Fig. 5. RMIV Infected Fatbody Cell in *A. taeniorhynchus* Larva with Graination Over Cytoplasmic Deposits of Tritiated Thymidine Labeled Viral DNA (1000x).

If this is true, it might account for failure to observe exit of the virus from the gut lumen into host tissue by autoradiography.

Fluorescent antibody.--The site of entry of RMIV into host tissue was not detected by examination of fluorescent antibody stained serial sections from 800 early instar larvae exposed to an inoculum of triturated, infected hosts. Infection with RMIV developed in 16.8 per cent (191/1131) of the survivors of the exposure, when they were reared to the fourth stadium. Studies to be discussed below suggested that RMIV entered host tissue as isolated virions which did not become aggregated in cells of the midgut epithelium. Neither these virions nor the fluorescence from them by attached labeled antibody could have been detected with the light microscope because their size is below the resolution of the equipment used.

Electron Microscope Studies

A probable site of entry of RMIV into host tissue was found by examination of tissues with the electron microscope. Numerous virus particles with the size and appearance of RMIV were found in midgut epithelium of first instar A. taeniorhynchus larvae killed and fixed 5 hr after

initiation of exposure to the virus. The infected epithelial cell shown in Figures 6 and 7 is at the level of the foregut invagination into the midgut. This was determined by the relative positions of the infected cell, the peritrophic membrane and the cuticular lining of the foregut invagination. The larvae examined were from a colony known to be free of RMIV infection prior to exposure.

A possible mechanism of entry of RMIV into host tissue is as follows. Some of the virus particles entering the midgut are swept into the eddys forming a ring around the caudal extremity of the foregut invagination. (See Figure 4 and related text.) Some of these virus particles are pressed against the peritrophic membrane (Figures 8 and 9), emerging between the foregut invagination and the midgut wall (Figures 6 and 7). Where perforations occur in the peritrophic membrane due to faulty construction or to trauma, virus particles invade the space between the peritrophic membrane and the midgut epithelium. Virus particles and other objects of equal or larger size were seen between the peritrophic membrane and the midgut wall (Figures 8, 10 and 11). Virus particles enter the midgut cells, perhaps passing into and down the microvilli (Figures 10 and 11), or perhaps crossing the cell membrane in an area where the microvilli are disarrayed or damaged

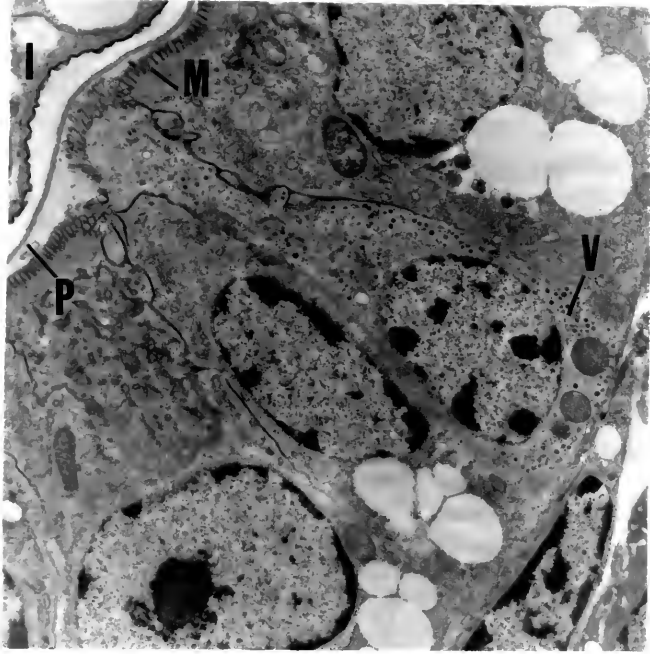


Fig. 6. Midgut Epithelium Cell Containing Scattered RMIV Particles (7500x).
(V - Virus; I - Foregut invagination; M - Microvilli; P - Peritrophic membrane)

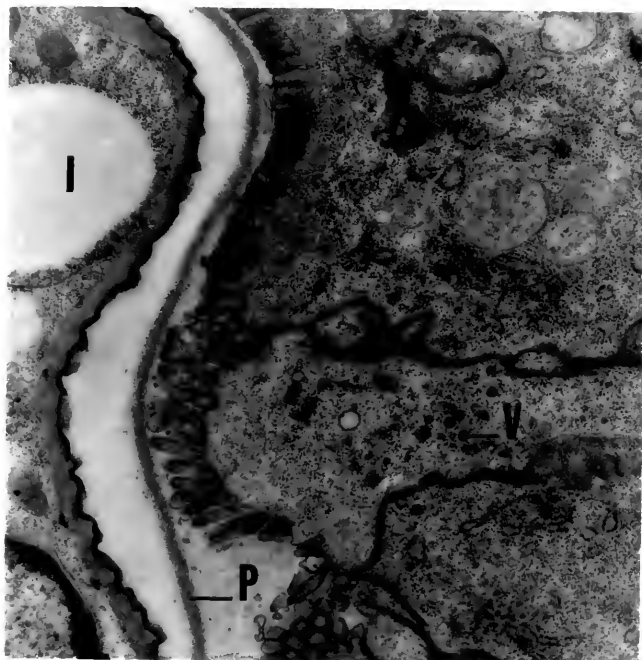


Fig. 7. Midgut Epithelium Cell with Disarranged Microvilli and Containing Scattered RMIV Particles (15,000x). (V - Virus; M - Microvilli; P - peritrophic membrane; I - Foregut invagination)

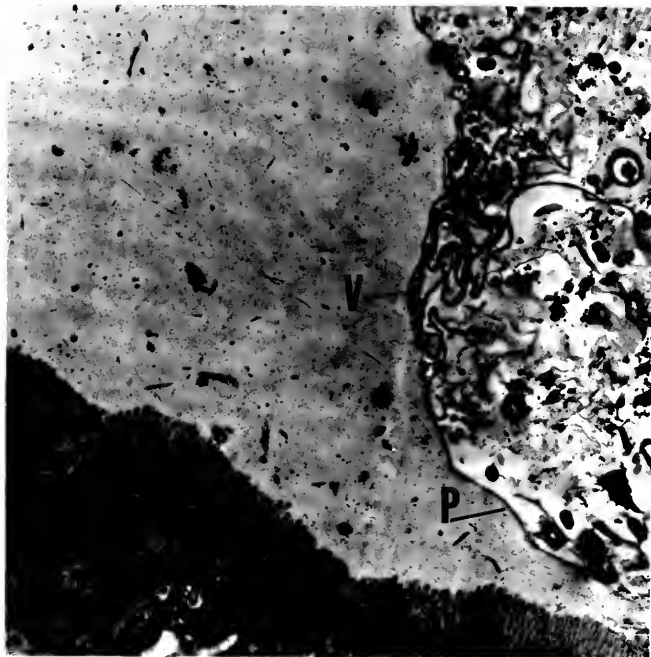


Fig. 8. RMIV Particles Pressed Against Peritrophic Membrane in Midgut of *A. taeniorhynchus* (9000x). (V - Virus; P - Peritrophic membrane; M - Microvilli)

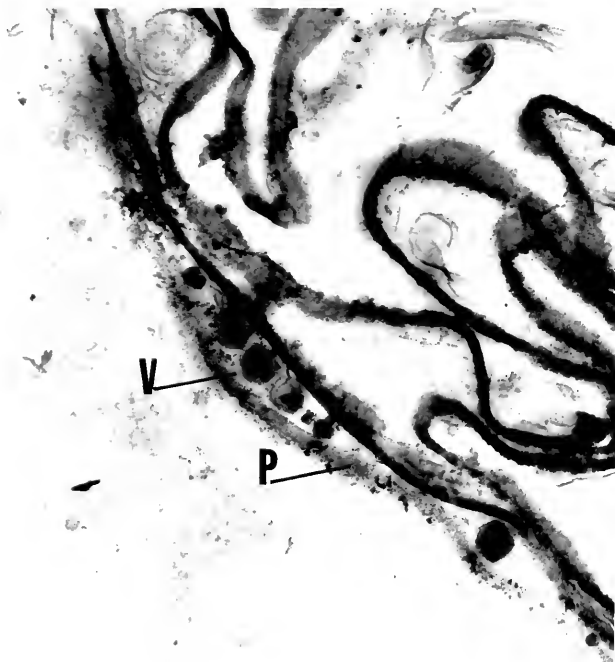


Fig. 9. RMIV particles pressed against peritrophic membrane in midgut of *A. taeniorhynchus* (33,000x).
(V - Virus; P - Peritrophic membrane)

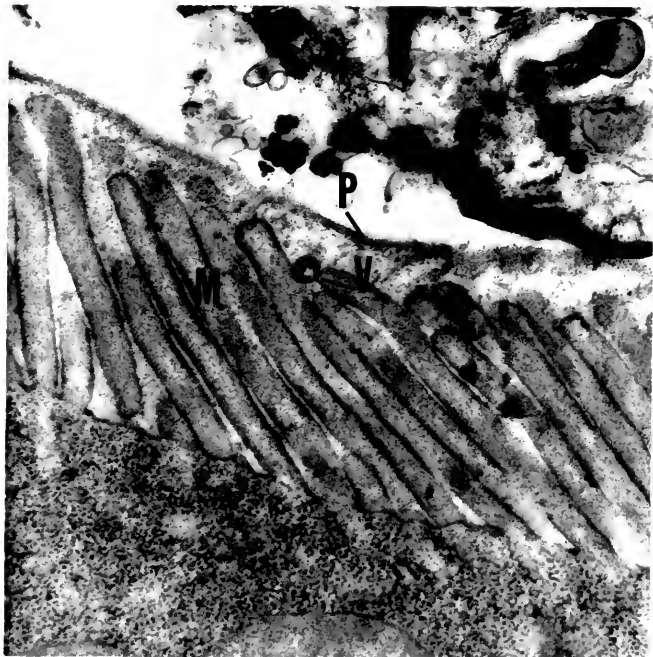


Fig. 10. RMIV Particle Between Peritrophic Membrane and Midgut Epithelium (30,000x).
(V - Virus; P - Peritrophic membrane; M - Microvilli)



Fig. 11. Virus Like Particle Apparently Within a Microvillus (30,000x).
(VLP - Virus like particle; M - Microvilli)

(Figure 7). The large numbers of virions in the infected cell in Figure 6 suggests the possibility that the virus may replicate in some midgut cells. It is also possible that the virus passes to adjacent cells (Figure 12) before entering and being distributed by the hemolymph. Only selected epithelial cells may be receptive, and the availability of receptive cells would constitute a barrier to entry in addition to the peritrophic membrane. The section of an infected midgut cell seen in Figure 6 contains over 100 virus particles, while no virus particles can be seen in any of the adjacent epithelial cells. Also, the microvilli of the cell are disarranged (Figure 7), and the cell is not vacuolated, as the adjacent cells are. It is possible these observations reflect only the level at which the cells were sectioned. However, disarrangement of the microvilli may reflect damage to the cell surface permitting easier entry by the virus. The absence of vacuolation in the infected cell may indicate either that it is different in age than the other cells or that metabolic changes resulting from invasion by the virus have occurred. It is a possibility that the age of a cell may influence its susceptibility.

Some characteristics of the transmission of RMIV in the laboratory were enigmatic. Per cent transmission

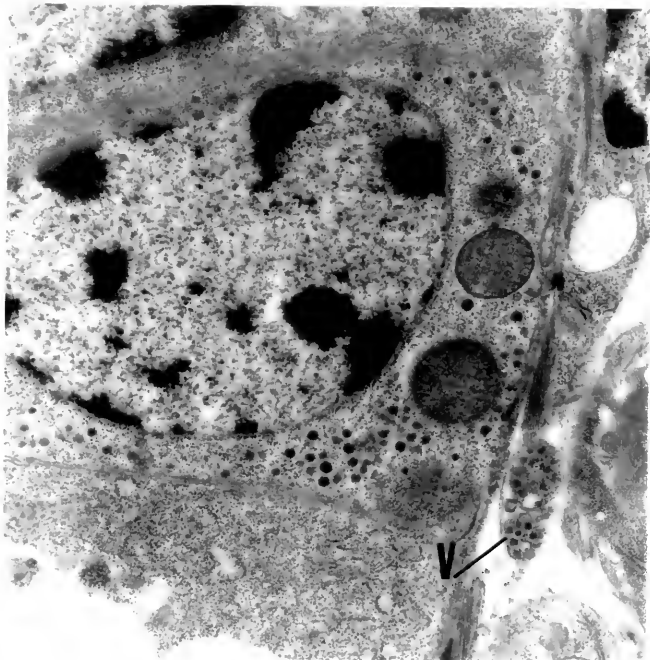


Fig. 12. Small Pocket of RMIV in Non-Epithelial Cell in Midgut Wall of A. taeniorhynchus Larva (14,000x). (V - Virus)

appeared to be linearly related to quantity of inoculum, as shown by the rising side of the curve in Figure 1. However, this was true only to a point, beyond which quantitation of the relationship was impossible, since larval mortality during exposure, probably due to anoxia, became almost total in extremely heavy inocula. Although it has not been established that there is a finite limit to per cent transmission in any given population, no one has achieved high levels of transmission, regardless of the quantity of inoculum used or the conditions of exposure.

Two of the possible mechanisms that may limit transmission of RMIV are the barriers to entry mentioned above and metabolic inhibition of replication by virus which gains entry. The biochemistry of the process by which RMIV is replicated is not known, although the recent finding of RNA polymerase activity in SIV and in CIV (Kelly and Tinsley, 1973) represents a beginning. Therefore, it is impossible to speculate on mechanisms by which transmission is controlled by host reactions limiting replication. If it were possible to achieve high levels of transmission by injection of the virus into larvae, this would suggest that control of transmission was effected

by limiting entry of the virus into the host. However, it has not been possible to do this. The failure of the injection experiment is of no significance because the procedures used may not have assured entry of the virus into the host. Also, it is possible that the virus must pass through the gut epithelium as a prerequisite to the establishment of infection. Why high levels of transmission of RMIV have not been possible remains unresolved.

Development of RMIV Infections

Autoradiography

Infections with RMIV were detected by autoradiography in 16 of 100 late fourth stadium larvae that were killed and fixed 108 hr after initiation of exposure to the virus. Infections were also detected in 20 of 100 early fourth stadium larvae that were killed and fixed 84 hr after initiation of exposure to the virus. It was possible to visually detect infections in 348 of 2000 (17.4%) late fourth stadium larvae exposed with the above experimental larvae. Infections with RMIV were not detected autoradiographically in any of 2 groups of 100 larvae each, killed and fixed as second and third instar larvae 36 and 60 hr after initiation of exposure to the virus. Infected cells

were easily recognized by the presence of heavy graination in the emulsion over the cytoplasm, signalling the incorporation of ^3H -methyl thymidine into viral DNA (Figure 5). Graination over the cytoplasm of uninfected cells was only slightly higher than background graination (Figure 3). Infected cells were also recognized by the histochemical reaction between viral DNA in the cytoplasm and the methyl green stain used. Stained foci of viral DNA appeared as conspicuous green to greenish purple bodies in the cytoplasm of infected cells (Figure 13). Infected cells were found among the following tissues: fatbody, imaginal buds, and epidermis. Fatbody cells, which appeared to be the preferred host of the virus, were conspicuously enlarged when infected.

An attempt to shorten the time of exposure of the emulsion, by exposing larvae to greater activities of ^3H -methyl thymidine than those indicated as optimal by the pilot project on autoradiography, resulted in over-labeling of the tissue and reduced the resolution of the method. Infections in cells with small cytoplasm volume were difficult to detect. The stained foci of virus in epidermal cells were about the same size as the nuclei of these cells, and they could not always be distinguished by

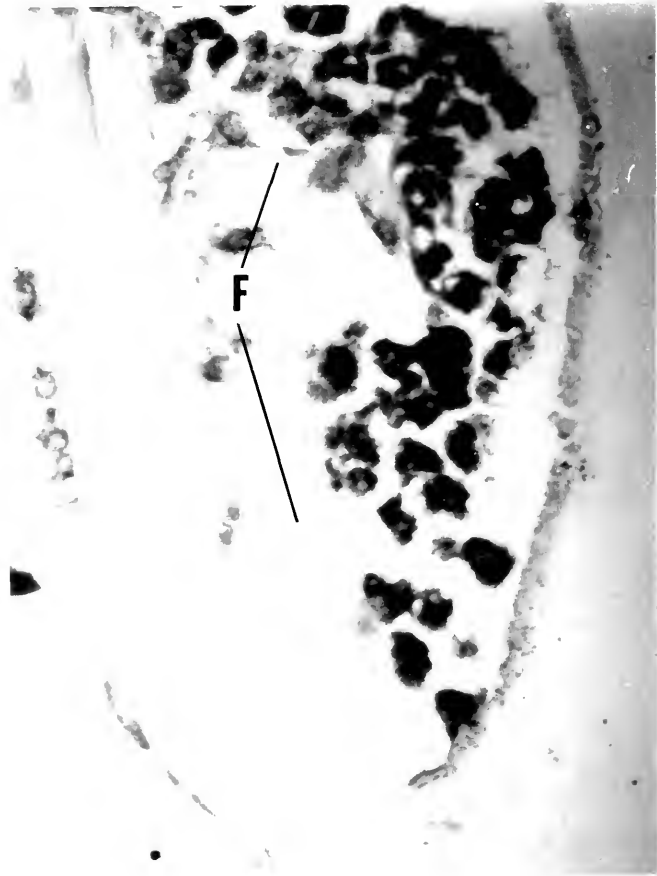


Fig. 13. Methyl Green Staining of RMIV Infected Cells
(100x).
(F - Fatbody)

the presence of the pink staining nucleolus. Use of a lighter stain specific for the nucleus only, such as nuclear fast red, would have enhanced the resolution of the technique.

Infected tissues were distributed throughout the bodies of infected larvae by the time infection could be detected either autoradiographically or histochemically. No point source of origin of the infection was evident. A remarkable progression of disease apparently occurred late in the third stadium or early in the fourth stadium. Within a period of 24 hr, the infection progressed from being inapparent by the methods used to being conspicuously present and distributed through the diseased larvae.

Fluorescent Antibody

Overt infections.--Infections with RMIV were detected by fluorescent antibody staining of viral antigens in 12 of 100 fourth stadium larvae, killed and fixed 96 hr after initiation of exposure to the virus. Infections were also detected in 14 of 100 third stadium larvae, and 1 of 100 second stadium larvae, that had been killed and fixed 72 and 48 hr after the initiation of exposure, respectively. No evidence of infection was found in 100 second instar

larvae, killed and fixed immediately after exposure. Overt infections were observed in 253 of 2000 (12.7%) advanced fourth stadium larvae examined visually 120 hr after initiation of their exposure with the experimental larvae.

Infected cells were recognized by green fluorescence emanating from labeled antibody combined with foci of viral antigen in the cytoplasm. No other fluorescence was present in any location or configuration that could have been confused with the stained antigen. However, a green natural fluorescence was observed lining the entire enteric track, in the oviduct and surrounding the ova of gravid females, and in the cytoplasm of cells forming the malpighian tubules. The latter was caused by small, discrete spherules that were consistent in size, while stained foci of viral antigen were larger and very irregular in shape.

Infection was detected in cells of fatbody, imaginal buds, epidermis, and tracheal epithelium tissues (Figures 14 and 15). Infection was found only in fatbody cells in the infected second instar larva. These occurred singly or in pairs on the medial surface of the fatbody in almost every abdominal segment, but were scattered and infrequent in the thorax. The infected cells were about double normal

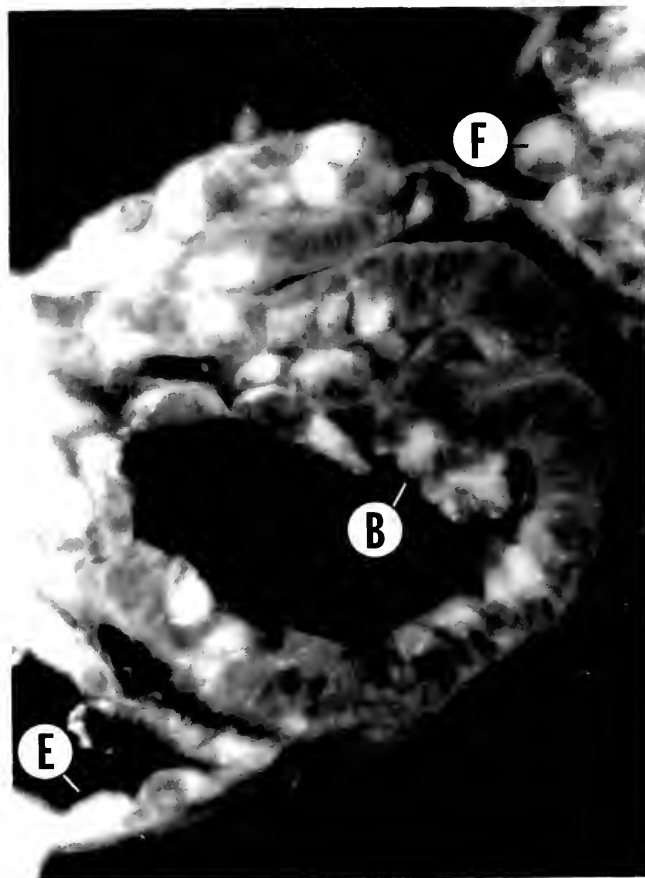


Fig. 14. Fluorescent Antibody Staining of Epidermal Cells, Imaginal Bud Tissues and Fatbody Tissue Infected with RMIV (250x).
(E - Epidermis; B - Imaginal bud; F - Fatbody)

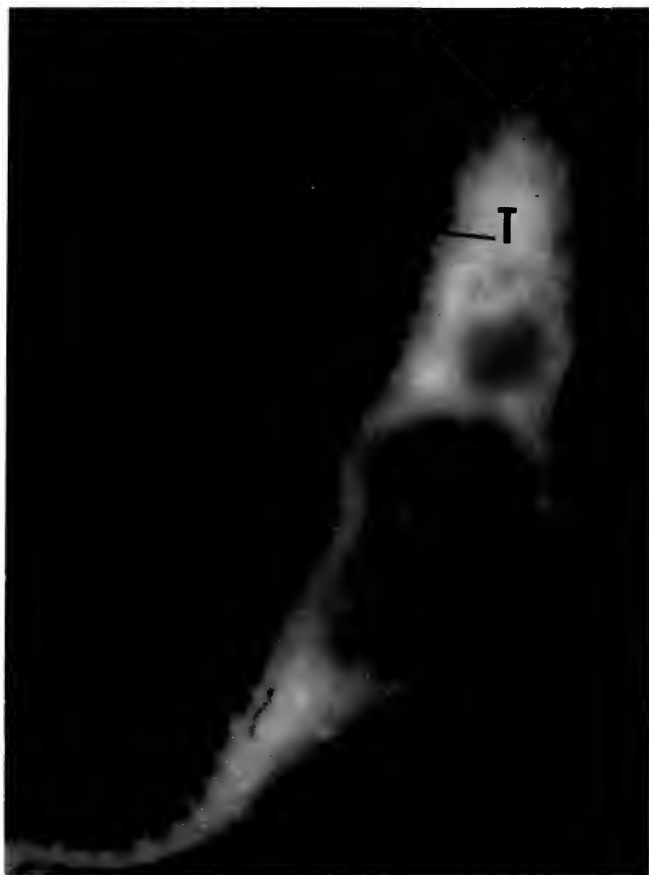


Fig. 15. Fluorescent Antibody Staining of RMIV Infected Tracheal Epithelium Cell (1000x).
(N - Nucleus; T - Tracheal taenidium)

size, and the foci of antigen were small and clearly defined. Both epidermis and fatbody were infected in third instar larvae, and occasionally a few cells of the imaginal buds had small, well-circumscribed foci of stained antigen. Again, foci occurred in the fatbody of each segment of the abdomen, and were now also common in the thorax. The number of infected cells per abdominal segment focus was fairly constant within, but varied between hosts. In some hosts only a few of the cells in each abdominal segment were infected; in others as many as 1/2 of all cells observed were infected. Epidermal cells were never observed to be infected except when they were adjacent to infected fatbody. Infected fatbody cells in third instar larvae were markedly enlarged and many were filled with the stained foci of viral antigen. Infections were massive in all infected fourth stadium larvae examined. In some larvae virtually every fatbody cell was infected, including those in the head capsule. Both epidermal cells not adjacent to infected fatbody, and tracheal epithelium cells were infected. Infection in the fatbody surrounding the testes was heavy (Figure 16), but no antigen was found within either the testes or the ovaries. No evidence of infection was seen in epithelium of foregut, midgut, or

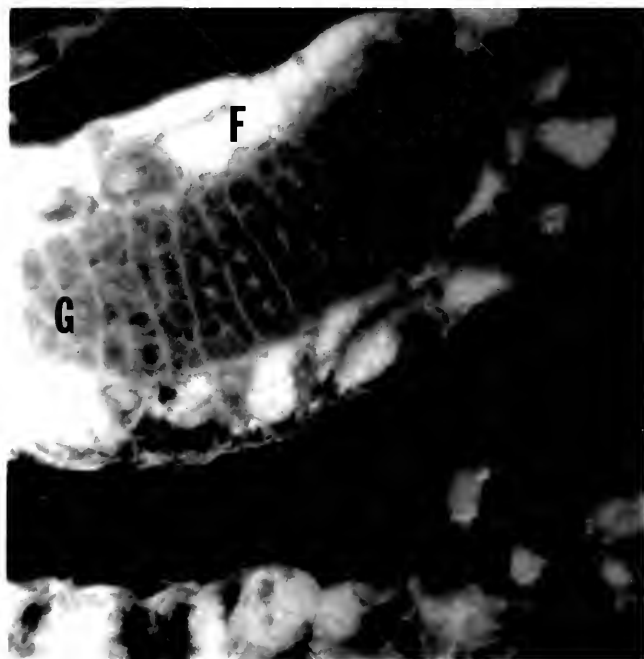


Fig. 16. Fluorescent Antibody Staining of Fatbody Surrounding Testis (250x).
(F - Fatbody; G - Testis)

hindgut or in nerve tissue. Infection in muscle tissue may have occurred, but probably could not have been seen because of the bright, silvery autofluorescence of that tissue. Some fluorescence was seen in a few oenocytes, but it appeared as small foci. There were several foci in each cell, and these probably represented phagocytized fragments of disrupted, infected cells.

The pattern of distribution of infected cells in the earliest infection observed suggested that infection was initiated in isolated cells throughout the body rather than at one location. This would be expected if virus penetrating the gut epithelium was distributed randomly by the hemolymph. If large numbers of infectious virus particles had penetrated a localized portion of midgut, a generalized infection, rather than a few isolated infected cells, would probably have resulted. It appears likely that a few infectious particles penetrate the gut, enter the hemolymph, are distributed randomly, and infect cells throughout the body. The virus replicates and accumulates in these cells which finally rupture. This releases the large number of infectious particles that initiate the generalized infection that rapidly kills the host.

Covert infections.--RMIV infections were not detected by fluorescent antibody staining among 100 advanced fourth instar larvae or among 100 unblooded female adults, killed and fixed 48 and 96 hr, respectively, after initiation of exposure to the virus. Also, infections were not detected in serial sections of 3 adult female mosquitoes that were known to have transmitted the virus transovarially. Only 1 of 100 pupae, killed and fixed 72 hr after the initiation of exposure, was infected, and this infection appeared abnormal. In sections of the infected pupa, infected fatbody cells were in groups of 3-10 cells per focus. These foci were scattered and infrequent in occurrence, rather than symmetrically located as in overtly infected larvae.

Failure to find evidence of infections among the larvae and unblooded females was surprising. Transovarially acquired infections occurred among 4.8 per cent of the progeny of the group of mosquitoes from which specimens examined were collected. This indicated that infections probably were present in about 6.5 per cent ($4.8\% \times 1.37$) of that group--yet, only 1 of 300 examined presented any evidence of infection when examined by fluorescent antibody staining. (As shown in the study entitled "Transovarial

Transmission of RMIV Through Isolated Females," per cent of infection among parents exposed as late instar larvae exceeds that observed among their progeny by a factor of about 1.37.) Adequate time passed between exposure and sampling for infections to have become detectable, since infections can be detected as early as 48 hr after initiation of exposure. This was shown in second instar larvae, which have much less fatbody than the specimens studied here. The method used has detected foci of infection as small as 1 cell. Therefore, it appears likely that infections by which transovarial transmission is effected are not massive and generalized as is characteristic for overt infections that result in death of the host.

An hypothetical explanation of the above observations follows. It incorporates the fact (Figure 2) that less transovarial transmission occurred through mosquitoes exposed during the sixth day of larval life than through those exposed during the fifth day of larval life. When late instar larvae feed on the cadavers of infected larvae, infectious virus particles enter the tissues of some of them. The rate at which infections develops in these larvae is proportional to the number of infectious particles acquired. The extent to which infections progress is

proportional both to the number of infectious particles that replicate and to time. Host changes associated with impending pupation stop or sharply reduce the rate of viral replication, halting the progression of the infection. When large numbers of infectious particles enter larvae early enough, infections may progress to such an extent that infected cells can still be found after pupation. When fewer infectious particles are acquired during exposure or when less time is available before pupation interrupts viral replication, infections do not progress to a point at which they can be detected by fluorescent antibody staining. However, the initial quantity of virus entering the host or that produced by subsequent replication will have been sufficient for infectious particles to have been distributed to reproductive tissues in some females. Receptiveness of reproductive tissues to virus invasion might be a factor working to assure infection of all oocytes in infected females. Also, virus might replicate, either in reproductive tissues or elsewhere, at a level not detectable by the methods used, with the result that all the oocytes become infected.

Number and Molecular Weights of
RMIV Structural Polypeptides

Proteins present in purified RMIV were resolved into 25 polypeptides by SDS-polyacrylamide gel disc electrophoresis. Table 6 is a list of proteins of known monomer number and molecular weight, which were electrophoresed at the same time as the viral polypeptides, to serve as markers for estimating the molecular weights of the RMIV polypeptides. The relative migration fractions (Rf's) of RMIV polypeptides in both 13 per cent and 10 per cent gels and their estimated molecular weights are shown in Table 7. Markers in the molecular weight range of the smaller viral polypeptides passed through 10 per cent gels with the dye front, as did the smaller viral polypeptides, and their Rf's could not be determined in 10 per cent gels. Therefore, molecular weights of small viral polypeptides were determined from a curve drawn with the Rf's of small markers from 13 per cent gels. The RF of thyroglobulin, molecular weight 335,000, was not included in drawing the curve. The larger markers and viral polypeptides penetrated the 13 per cent gels such a short distance that errors in measuring their migration distances were very large. Therefore, molecular weights of large viral polypeptides were determined from a curve drawn with the Rf's of large

Table 6

Proteins Used as Markers for the Estimation
of the Molecular Weights of RMIV
Structural Polypeptides

Protein	Monomer Molecular Weight	Log ₁₀ Monomer Molecular Weight	Relative Migration Fraction	
			13% Gels	10% Gels
Cytochrome C	12,400	4.0934	0.702	---
Myoglobin	17,500	4.2430	0.660	---
Apoferritin	24,000	4.3802	0.590	0.594
Chymotrypsinogen	25,000	4.3979	0.562	---
Aldolase	40,000	4.6021	0.312	0.486
Ovalbumin	45,000	4.6532	0.305	0.467
Human IgG (heavy chains)	50,000	4.6990	0.229	0.385
Bovine Serum Albumin	67,000	4.8261	0.179	0.301
Thyroglobulin	335,000	5.5250	0.019	0.053

Table 7

Relative Migration Fractions and Molecular
Weights of RMIV Structural Polypeptides

Poly- peptide	Relative Migration Fraction		Molecular Weight	
	13% Gel	10% Gel	13% Gel	10% Gel
1		0.044		331,000
2		0.068		290,000
3		0.080		271,000
4		0.097		246,000
5		0.112		224,000
6		0.146		181,000
7	0.076	0.155	80,700	174,000
8	0.093	0.186	77,200	146,000
9	0.098	0.191	75,800	141,000
10	0.123	0.214	72,700	124,000
11	0.132	0.252	69,200	100,000
12	0.164	0.306	64,100	73,000
13	0.216	0.369	<u>55,200</u>	<u>51,000</u>
14	0.221	0.379	54,500	48,800
15	0.225	0.408	53,900	29,600
16	0.275	0.471	47,100	22,200
17	0.309	0.519	42,900	20,900
18	0.319	0.529	41,800	17,400
19	0.345	0.563	39,000	16,300
20	0.358		38,000	
21	0.365		37,100	
22	0.414		32,600	
23	0.473		27,800	
24	0.483		27,300	
25	0.539		23,500	

markers from 10 per cent gels.

As can be seen in Table 7, the molecular weights of RMIV polypeptides determined in both 13 per cent and 10 per cent gels differed greatly. This difference was minimal (about 8%) for viral polypeptide 13. To determine the sum of the molecular weights, the molecular weights of polypeptides 1-13 from 10 per cent gels, rather than 13 per cent gels, were taken as probably more nearly accurate. Likewise, the molecular weights of polypeptides 14-25 from 13 per cent gels, rather than 10 per cent gels, were taken as probably more nearly accurate. The total of these molecular weights was 2,817,000. The molecular weight of the DNA in RMIV has been shown to be about 400×10^6 (Matta, 1970; Wagner et al., 1973). Assuming there is no redundancy in the DNA molecule, and that it does not code for 5S ribosomal RNA or transfer RNA, it should be able to code for about 20×10^6 daltons of protein. Thus, only about 14 per cent of the genome of RMIV would be able to code for its structural polypeptides. This figure is less than 1/2 of the 30 per cent of the genome of SIV and CIV required to code for their structural polypeptides (Kelly and Tinsley, 1972).

Separation of RMIV Structural Polypeptides
By Hydroxylapatite Chromatography

The structural polypeptides of RMIV could not be separated by hydroxylapatite chromatography at pH 6.4. Elution of the columns used was quantitative, but all fractions eluted as one peak.

SUMMARY

A system for the production of RMIV in A. taeniorhynchus larvae that yielded a maximum weekly production of 940 mg of purified virus was described. Several features of the production system accounted for its success. Larvae were exposed to an experimentally determined optimum quantity of inoculum (50 LEQ per 2500 larvae exposed). The larvae were exposed to inoculum during the second day of larval life, their age of greatest susceptibility. Methods were used to expose and rear larvae that caused essentially no mortality. This permitted standardization of procedures, and as a result, the virus production system is adaptable to mass production of the virus. Additionally, virus purification procedures were used that minimized waste of virus during purification. The production of virus from infected larvae was quantitated, with an average of 58.9 μg of purified virus per larva. This constituted 9.35 per cent of the host's dry weight. Attenuation of viral infectivity occurred throughout the purification procedure and could not be attributed to any single step in the

purification procedure.

Transovarial transmission of RMIV was found to increase as the age of the parent generation at per os exposure to virus increased, up to a point, but decreased if parents were exposed during the 24 hr immediately preceding pupation. Transovarial transmission studies with isolated females indicated that infected (transmitting) females produced fewer larvae (avg. = 63.3) than uninfected females (avg. = 94.2). Quantitation of this difference in fecundity provided a factor with which the per cent of covert infections among adults, exposed in the laboratory, was estimated from the per cent of infection among their progeny. With a means of estimating the per cent of covert infections available, total transmission was estimated when larvae of different ages were exposed to inoculum. This decreased as the age of larvae at exposure increased.

Males were shown to play no part in transovarial transmission of RMIV.

Preliminary studies of the autoradiographic detection of tritiated thymidine uptake in A. taeniorhynchus indicated that a 2 hr exposure of larvae (1 larva/ml) to a solution of 10 μ Ci/ml of ^3H -methyl thymidine (Sp. Act. 17 Ci/mM) was adequate to label most larval cells. An

autoradiographic study of developing RMIV infections showed that infected cells could be detected in fatbody, imaginal buds, epidermis and tracheal epithelium 84 hr after the initiation of exposure. This was the first attempt to study the development of RMIV infections by autoradiography, and the method displayed great potential.

A probable site of entry of RMIV into host tissue was detected by electronmicroscopy. For the first time, intact virus particles were seen between the peritrophic membrane and the midgut wall of second instar larvae that had been exposed to virus for 5 hr preceding fixation. Those seen were at the level of the foregut invagination into the midgut, and large numbers of virus particles were also seen in midgut epithelium. Although these particles were not packed in paracrystalline arrays, the number of particles present provided the first indication that RMIV may replicate in midgut cells.

An effective fluorescent antibody stain for RMIV was produced and used to study the development of overt infections with the virus. Viral antigen was detected in larvae as early as 48 hr after the initiation of exposure. Infection occurred in epidermis, fatbody, imaginal buds, and tracheal epithelium. However, covert infections

among known transovarial transmitters of RMIV could not be detected with fluorescent antibody staining. This observation plus the decrease in transovarial transmission through parents (exposed for 24 hr prior to pupation) suggested that virus replication in pupae and adults, and perhaps in late fourth stadium larvae, was arrested or retarded.

The number of structural polypeptides in RMIV was determined, and their molecular weights were estimated by polyacrylamide gel disc electrophoresis. It was calculated that a minimum of 14 per cent of the genome of RMIV could code for its structural polypeptides. This information is available for only 2 other iridescent viruses. When similar information becomes available for other iridescent viruses, it will be valuable in assessing the relationships among iridoviruses and used to facilitate identification.

BIBLIOGRAPHY

- Anderson, J. F. 1970. An iridescent virus infecting the mosquito Aedes stimulans. J. Invertebrate Pathol., 15, 219-224.
- Andrewes, C. H., and Horstmann, D. M. 1949. The susceptibility of viruses to ethyl ether. J. General Microbiol. 3, 290-297.
- Bellett, A. J. D. 1968. The iridescent virus group. Adv. Virus Res., 13, 225-246.
- Bellett, A. J. D., and Inman, R. B. 1967. Some properties of deoxyribonucleic acid preparations from Chilo, Sericesthis, and Tipula iridescent viruses. J. Mol. Biol., 25, 425-432.
- Bellett, A. J. D., and Mercer, E. H. 1964. The multiplication of Sericesthis iridescent virus in cell cultures from Antheraea eucalypti Scott. I. Qualitative experiments. Virology, 24, 645-653.
- Chamberlain, R. W. 1968. Arboviruses, the arthropod-borne animal viruses. Curr. Topics Microbiol. Immunol., 42, 38-58.
- Chapman, H. C., Clark, T. B., Anthony, D. W., and Glenn, F. E., Jr. 1971. An iridescent virus from larvae of Corethrella brakelevi (Diptera: Chaeboridae) in Louisiana. J. Invertebrate pathol., 18, 284-286.
- Chapman, H. C., Clark, T. B., Woodard, D. B., and Kellen, W. R., 1966. Additional mosquito hosts of the mosquito iridescent virus. J. Invertebrate Pathol., 8, 545-546.
- Clark, H. F., and Shepard, C. C. 1963. A dialysis technique for preparing fluorescent antibody. Virology, 20, 642-644.

- Clark, T. B. 1966. A method for the inoculation of mosquito larvae (viral and bacterial pathogens), *J. Invertebrate Pathol.*, 8, 425-426.
- Clark, T. B., Kellen, W. R., and Lum, P. T. 1965. A mosquito iridescent virus (MIV) from *Aedes taeniorhynchus* (Wiedemann). *J. Invertebrate Pathol.*, 7, 519-521.
- Cunningham, J. C., and Tinsley, T. W. 1968. A serological comparison of some iridescent non-occluded insect viruses. *J. General Virol.*, 3, 1-8.
- Faust, R. M., Dougherty, E. M., and Adams, J. R. 1968. Nucleic acid in the blue-green and orange mosquito iridescent viruses (MIV) isolated from larvae of *Aedes taeniorhynchus*. *J. Invertebrate Pathol.*, 10, 160.
- Glitz, D. G., Hills, G. J., and Rivers, C. F. 1968. A comparison of the *Tipula* and *Sericesthis* iridescent viruses. *J. General Virol.*, 3, 209-220.
- Goldman, M. 1968. "Fluorescent Antibody Methods." Academic Press, New York.
- Hall, D. W., and Anthony, D. W. 1971. Pathology of a mosquito iridescent virus (MIV) infecting *Aedes taeniorhynchus*. *J. Invertebrate Pathol.*, 18, 61-69.
- Hall, D. W., and Lowe, R. E. 1971. A new distribution record for the mosquito iridescent virus (MIV). *Mosquito News*, 31, 448-449.
- Hall, D. W., and Lowe, R. E. 1972. Physical and serological comparison of "R" and "T" strains of mosquito iridescent virus from *Aedes taeniorhynchus*. *J. Invertebrate Pathol.*, 19, 317-324.
- Hasan, S., Croizier, G., Vago, C., and Duthoit, J. J. 1970. Infection a virus irisant dans une population naturelle d'*Aedes detritus* Haliday en France. *Ann. Zool. Ecol. Anim.*, 2, 295-299.

- Holborow, E. J., and Johnson, G. D. 1967. Immunofluorescence, Chapt. 16, pp. 571-596 in "Handbook of Experimental Immunology." D. M. Weir, ed. Blackwell, Oxford, England.
- Kalmakoff, J., Moore, S., and Pottinger, R. P. 1972. An iridescent virus from the grass grub Costelytra zealandica: Serological study. J. Invertebrate Pathol., 20, 70-76.
- Kawamura, A., Jr. 1969. Editor, "Fluorescent Antibody Techniques and Their Applications," University Park Press, Baltimore.
- Kelly, D. C., and Tinsley, T. W. 1973. Ribonucleic acid polymerase activity associated with particles of iridescent virus types 2 and 6. J. Invertebrate Pathol., 22, 199-202.
- Klug, A., Franklin, R. E., and Humphreys-Owen, S. P. F. 1959. The crystal structure of Tipula iridescent virus as determined by Bragg reflection of visible light. Biochim. Biophys. Acta, 32, 203-219.
- Linley, J. R., and Nielsen, H. T. 1968a. Transmission of a mosquito iridescent virus in Aedes taeniorhynchus. I. Laboratory experiments. J. Invertebrate Pathol., 12, 7-16.
- Linley, J. R., and Nielsen, H. T. 1968b. Transmission of a mosquito iridescent virus in Aedes taeniorhynchus. II. Experiments related to transmission in nature. J. Invertebrate Pathol., 12, 17-24.
- Lowe, R. E., Hall, D. W., and Matta, J. F. 1970. Comparison of the mosquito iridescent viruses (MIV) with other insect iridescent viruses. Proc. 4th Int. Colloq. Insect Pathol., 1970, College Park, Maryland, pp. 163-170.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem., 193, 265-275.
- Maramorosch, K. 1968. Plant pathogenic viruses in insects. Curr. Topics Microbiol. Immunol., 42, 94-107.

- Maramorosch, K., and Jensen, D. D. 1963. Harmful and beneficial effects of plant viruses in insects. *Ann. Rev. Microbiol.*, 17, 495-530.
- Maizel, J. V., Jr. 1971. Polyacrylamide gel electrophoresis of viral proteins. Chapt. 5, pp. 180-244 in "Methods in Virology," Vol. 5, K. Maramorosch and H. Koprowski, eds., Academic Press, New York.
- Matta, J. F. 1970. The characteristics of a mosquito iridescent virus. II. Physicochemical characterization. *J. Invertebrate Pathol.*, 16, 157-164.
- Matta, J. F., and Lowe, R. E. 1969. A differential staining technique for a mosquito iridescent virus. *J. Invertebrate Pathol.*, 13, 457-458.
- Matta, J. F., and Lowe, R. E. 1970. The characterization of a mosquito iridescent virus (MIV). I. Biological characteristics, infectivity, and pathology. *J. Invertebrate Pathol.*, 16, 38-41.
- Mollenhauer, H. H. 1964. Plastic embedding mixtures for use in electronmicroscopy. *Stain Technol.*, 39, 111-114.
- Moss, B., and Rosenblum, E. N. 1972. Hydroxylapatite chromatography of protein-sodium dodecyl sulfate complexes. *J. Biol. Chem.*, 247, 5194-5198.
- Mrenova, M., and Albrecht, P. 1966. Stabilization of fluorescence in preparates treated by the fluorescent antibody technique. *Nature*, 212, 1256-1257.
- Nayar, J. K. 1967. The pupation rhythm in Aedes taeniorhynchus (Diptera: Culicidae). II. Ontogenetic timing, rate of development, and endogenous diurnal rhythm of pupation. *Ann. Ent. Soc. Am.*, 60, 946-971.
- Pease, A. G. E. 1953. "Histochemistry: Theoretical and Applied." Little, Brown and Co., Boston.
- Rogers, A. W. 1967. "Techniques of Autoradiography." Elsevier, Amsterdam.

- Service, M. W. 1968. The ecology of the immature stages of Aedes detritus (Diptera: Culicidae). J. Appl. Ecol., 5, 613-630.
- Smith, K. M. 1967. "Insect Virology." Academic Press, New York.
- Smith, K. M., and Williams, R. C. 1958. Insect viruses and their structure. Endeavor, 17, 12-21.
- Stoltz, D. B. 1971. The structure of icosahedral cytoplasmic deoxyriboviruses. J. Ultrastruct. Res., 37, 219-239.
- Stoltz, D. B., and Summers, M. D. 1971. Pathway of infection of mosquito iridescent virus. I. Preliminary observations on the fate of ingested virus. J. Virol., 8, 900-909.
- Thurston, J. M., and Jofte, D. L. 1963. Stains compatible with dipping radioautography. Stain Technol., 38, 231-235.
- Tinsley, T. W., and Harrap, K. A. 1972. "Moving frontiers in invertebrate virology," Monographs in Virology, Vol. 6, Krager, Basel, Switzerland.
- Tinsley, T. W., and Kelly, D. C. 1970. An interim nomenclature system for the iridescent group of insect viruses. J. Invertebrate Pathol., 16, 470-472.
- Tinsley, T. W., Robertson, J. S., Rivers, C. F., and Service, M. W. 1971. An iridescent virus of Aedes cantans in Great Britain. J. Invertebrate Pathol., 18, 427-428.
- Vago, C., Rioux, J. A., Duthoit, J. L., and Dedet, J. P. 1969. Infection spontanee a virus irisant dans une population d'Aedes detritus (Hal. 1833) des environs de Tunis. Ann. Parasit. Hum. et Comp., 44, 667-676.
- Venable, J. H., and Coggeshall, R. 1965. A simplified lead citrate stain for use in electron microscopy. J. Cell Biol., 25, 407-408.

- Wagner, G. W., Paschke, J. D., Campbell, W. R., and Webb, S. R. 1973. Biochemical and Biophysical properties of two strains of mosquito iridescent virus. *Virology*, 52, 72-80.
- Weiser, J. 1965. A new virus infection of mosquito larvae. *WHO Bull.*, 33, 586-588.
- Wildy, P. 1971. "Classification and Nomenclature of Viruses," Monographs in Virology, Vol. 3, Krager, Basel, Switzerland.
- Williams, R. C., and Smith, K. M. 1957. A crystallizable insect virus. *Nature*, 179, 119-120.
- Woodard, D. B., and Chapman, H. C. 1968. Laboratory studies with the mosquito iridescent virus (MIV). *J. Invertebrate Pathol.*, 11, 296-301.
- Wrigley, D. G. 1969. An electron microscope study of the structure of Sericesthis iridescent virus. *J. General Virol.*, 5, 123-134.
- Wrigley, D. G. 1970. An electron microscope study of the structure of Tipula iridescent virus. *J. General Virol.*, 6, 169-173.

BIOGRAPHICAL SKETCH

Stephen Carson Hembree was born September 20, 1938, in Murphy, Cherokee County, North Carolina. His parents, Noah Webster Hembree and Susie Ferguson Hembree, were descendants of pioneer farming families in western North Carolina. He graduated from high school in Murphy, North Carolina in 1956 and entered North Carolina State University at Raleigh, North Carolina, where he studied civil engineering for 2 years. He then entered the Army and served as an enlisted man for 3 years in the 101st Airborne Division. In 1961 he returned to North Carolina State University where he studied fishery biology and zoology and graduated with honors from the pre-medical curriculum in 1964. He entered graduate school at his alma mater in 1964 and completed a Master of Science degree in zoology, with a major in parasitology, in 1966. He re-entered the Army as a commissioned officer in 1966 and has since served at the 406th Medical Laboratory in Japan, at the Walter Reed Army Institute of Research in Washington, D. C., and with the U. S. Army Special Forces - Walter Reed Army Institute

of Research Field Epidemiology Research Team in South Vietnam. While in South Vietnam, he was awarded 2 Bronze Star Medals while conducting epidemiological research among the Montagnard tribes along the Cambodian and Laotian borders of South Vietnam. From September, 1970, until the present time he has worked toward the degree of Doctor of Philosophy at the University of Florida, Gainesville, Florida, under Army sponsorship.


Stephen Carson Hembree is married to the former Jane Van Horn and is the father of two sons, Michael and Sean.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



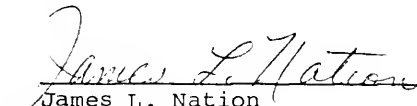
Franklin S. Blanton, Chairman
Professor of Entomology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



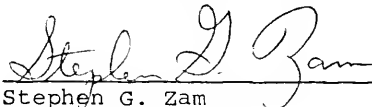
Ronald E. Lowe, Co-Chairman
Associate Professor of Entomology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



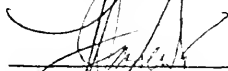
James L. Nation
Professor of Entomology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Stephen G. Zam
Associate Professor of Zoology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

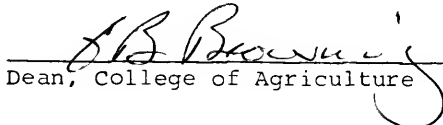


Francis W. Zettler

Associate Professor of Plant Pathology

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

March, 1974



Dean, College of Agriculture

Dean, Graduate School